Ovary-Predominant wnt4 Expression During Gonadal Differentiation is not Conserved in the Rainbow Trout (Oncorhynchus mykiss)

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SUMMARY
The Wnt/β-catenin pathway is crucial for ovarian differentiation in mammals, and WNT4 is an important protein that regulates this process. While the role of Wnt4 in gonadal differentiation is relatively well characterized in mammals, little is known regarding its role in teleost fish. Therefore, we investigated the potential activity of wnt4 in gonadal differentiation in rainbow trout (Oncorhynchus mykiss), focusing on the teleost and salmonid gene duplications. Phylogenetic and synteny analyses demonstrated that teleost fish possess two wnt4 genes, wnt4a and wnt4b, as a consequence of the teleost-specific whole-genome duplication (3R). In rainbow trout, we also identified an additional wnt4 gene, which is a wnt4a paralog that likely resulted from the salmonid-specific whole-genome duplication (4R). These two Wnt4a proteins (Wnt4a1 and Wnt4a2) share a high identity (>80%) with other vertebrate Wnt4 proteins, whereas Wnt4b is clearly more divergent (60% identity). During embryogenesis and adulthood, the wnt4a1/2 transcripts were expressed in various tissues, including the ovaries and testes. In contrast, wnt4b expression was restricted to the nervous system, suggesting a sub- or a neo-functionalization of this divergent paralog. During early gonadal differentiation in both males and females, the wnt4a1/2 transcripts were detected in the somatic cells surrounding the germ cells, with a slight sexual dimorphism in favor of males. These results demonstrate that, unlike mammals, rainbow trout do not display an ovary-predominant wnt4 expression profile during early gonadal differentiation.

INTRODUCTION
Teleost fish are the largest group of vertebrates, and exhibit an extraordinary diversity of sex determination and sex differentiation systems, including gonochorism (with either genetic or temperature-dependent sex determination) and natural hermaphroditism (Devlin and Nagahama, 2002). In medaka (Oryzias latipes), the major sex-determining gene, dmt1Y, has been characterized (Matsuda et al., 2002; Nanda et al., 2002), but dmt1Y is not the universal master sex-determining gene in fish (Volff et al., 2003). Furthermore, the molecular pathways triggering male and female gonadal differentiation have not been completely elucidated. It is well established, however, that steroids play a crucial role in sex differentiation in fish, and it has been demonstrated that estrogens and the...
enzyme Cyp19a1a are needed for triggering and maintaining ovarian differentiation (Guiguen et al., 2010). The rainbow trout, Onchorhynchus mykiss, is a teleost fish from the salmonid family with a rather strict genotypic sex determination and a male heterogamy (XX/XY). The histological differentiation of rainbow trout gonads occurs at approximately 60–65 days post-fertilization (dpf) with the formation of the ovarian lamellae. By investigating earlier stages of development using genetically all-male and all-female populations (Guiguen et al., 1999), some sexually dimorphic gene expression patterns, such as cyp19a1a and foxl2 in the ovary, have been characterized in histologically undifferentiated gonads (Baron et al., 2004, 2005a; Vizziano et al., 2007; Cavileer et al., 2009). This period of molecular sexual dimorphism that precedes histological gonadal differentiation has been termed “early molecular sex differentiation” (Vizziano et al., 2007).

Over the past decade, it has been clearly established that Wnt4 (wingless-type MMTV integration site family, member 4) is a key factor in ovarian differentiation in mammals. In mice, Wnt4 is expressed very early in undifferentiated male and female gonads; however, this expression becomes female specific at the onset of sex determination (11.5 days post coitum). The lack of Wnt4 expression in female mice results in the masculinization of XX gonads (Vainio et al., 1999), whereas the overexpression of Wnt4 disrupts the testicular vasculature and testosterone synthesis (Jordan et al., 2003). This essential role of WNT4 has also been demonstrated in humans: the duplication of a portion of the chromosome containing the WNT4 locus induces partial male-to-female sex reversal in XY patients (Jordan et al., 2001). The predominant expression of Wnt4 in the differentiating ovary has been examined in various mammalian species, including the goat (Capra aegagrus hircus) (Pailhoux et al., 2002), the tammar wallaby (Macropus eugenii) (Yu et al., 2006; Pask et al., 2010), and humans (Peltoketo et al., 2004; Jaakselainen et al., 2010). Although the role of Wnt4 in ovarian differentiation is well described in mammals, few studies have explored its potential role in non-mammalian vertebrates, where its specific role in ovarian differentiation may not be as clear.

In order to investigate if Wnt4 plays a role in gonadal differentiation in rainbow trout, we first analyzed Wnt4 gene duplications over evolution. Based on this analysis, the Wnt4 gene appeared very early in the metazoan lineage and was duplicated after the teleost-specific whole-genome duplication (3R). This 3R duplication resulted in two paralogs, wnt4a and wnt4b, in most teleost fish. A third wnt4 gene, wnt4a2, however, was found in rainbow trout; this was likely the result of the additional whole-genome duplication of the salmonid family (4R). With regards to gonadal sex differentiation, two wnt4 genes (wnt4a1 and wnt4a2) are expressed in differentiating rainbow trout gonads, with a slight dimorphic expression in favor of males. In contrast with Wnt4 expression in mammals, wnt4a1 and wnt4a2 are not predominantly expressed in the differentiating ovary during early sex differentiation.

RESULTS

Sequence and Phylogenetic Analyses

Three different wnt4 cDNAs were cloned from rainbow trout. These cDNAs were designated wnt4a1 [GenBank: JF815553], wnt4a2 [GenBank: JF815554], and wnt4b [GenBank: JF815555] based on the phylogenetic analyses presented below. The wnt4a1, wnt4a2, and wnt4b sequences were 2,012 bp, 1,337 bp, and 2,298 bp in length, respectively, with an open reading frame encoding putative 352-, 352- and 351-amino acid proteins, respectively. All of these deduced protein sequences displayed significant similarity with vertebrate WNT4 proteins. The amino acid sequences of the rainbow trout Wnt4a1 and Wnt4a2 proteins had high identity with other teleost fish Wnt4a proteins (approximately 90%), and were highly conserved throughout vertebrate evolution (over 80% identical). In contrast, whereas the amino acid sequence of the rainbow trout Wnt4b protein shared a high identity with other teleost fish Wnt4b proteins (90%), it was clearly divergent when compared with Wnt4a sequences from other teleost fish or other vertebrates (~60% identical; Table 1).

The phylogenetic reconstruction based on publicly available WNT4 protein sequences and on the predicted rainbow trout Wnt4 proteins confirmed that all of the rainbow trout proteins belonged to the WNT4 subgroup (Fig. 1). WNT4 has been identified in various metazoan species, including sea anemones (Nematostella vectensis), annelids (Platyneris dumerilii), and nematodes (Caenorhabditis elegans). The reconstruction also showed that teleost fish have at least two wnt4 genes, wnt4a and wnt4b, while other vertebrates and an amphioxus (Branchiostoma floridana), possess a single copy of the gene. Interestingly, the teleost-specific Wnt4b sequences form a clade that branches separately from the clade that includes all vertebrate WNT4 and teleost fish Wnt4a sequences. The rainbow trout Wnt4a1 and Wnt4a2 sequences both branch together within the Wnt4a clade (Fig. 1). Our phylogenetic analysis also showed that the partial sequence of the black porgy wnt4 locus, which has been recently associated with ovarian development (Wu and Chang, 2009), is included in the Wnt4a clade (data not shown).

A synteny analysis (Fig. 2) showed that the teleost fish wnt4a genes were closely related to the epha8 and cdc42 genes, similar to WNT4 in chicken, mouse (Mus musculus), and humans (Homo sapiens). Other genes, including lepre1...
and cldn19, were also associated with Wnt4 on the same chromosome/scaffold in teleost fish and chicken, but not in mammals. Interestingly, the teleost fish wnt4b gene was always adjacent to cdc42-like, a paralog of the cdc42 gene. The gene leper-like2, a paralog of lepre1, was also present in the same chromosomal region in medaka, tetraodon (Tetraodon nigroviridis), and fugu (Takifugu rubripes). Therefore, the wnt4a and wnt4b genes appeared as a result of the teleost fish whole-genome duplication.

### Table 1. Percentage of Identity Between WNT4 Full Length Proteins

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The deduced amino-acid sequences of rainbow trout Wnt4a1, Wnt4a2, and Wnt4b reveal high conservation with, respectively, the teleost fishes Wnt4a and Wnt4b amino-acid sequences. Wnt4a1 and Wnt4b amino acid sequences are highly conserved throughout vertebrate evolution, whereas Wnt4b differs from WNT4 sequences of other vertebrates.

### Figure 1
Rainbow trout possess multiple wnt4 genes. Teleost fish carry two wnt4 genes, with the exception of the salmonid rainbow trout, which possess a third wnt4 gene that is highly related to the wnt4a phylogenetic group. The phylogenetic tree reconstruction was calculated using the FigenX automated phylogenomic annotation pipeline. The bootstrap values obtained using neighbor-joining, maximum parsimony, and maximum likelihood methods are provided for each node or replaced by an asterisk in the absence of the node for a given phylogenetic method.

### Figure 2
Synteny conservation of Wnt4 genes throughout vertebrate evolution. The wnt4a and wnt4b genes appeared as a result of whole-genome duplication in teleost fish (3R). The synteny was constructed using Synteny Database and Genomicus. Color figure can be viewed in the online issue which is available at wileyonlinelibrary.com.

### Tissue Expression of the wnt4 Genes in Rainbow Trout Embryos and Adults

In order to characterize the expression pattern of the wnt4 genes during early development, whole mount in situ hybridizations were performed on 18-day-post-fertilization (dpf) rainbow trout embryos (Fig. 3). Because of the high identity between the wnt4a1 and wnt4a2 sequences, we were not able to investigate the localization of wnt4a1 and wnt4a2 without observing cross hybridization (data not shown). Consequently, in situ hybridizations were performed using a single probe generated from a partial wnt4a1 cDNA, and these hybridizations are referred to as “wnt4a1/2.” During rainbow trout embryogenesis, wnt4a1/2 transcripts were detected in the neural tube, floor plate, otic vesicle, and faintly in the skin. By contrast, wnt4b expression was weaker and restricted to the floor plate and the epithelium of the head. The expression patterns of wnt4a1, wnt4a2, and wnt4b were then quantified in various tissues from adult rainbow trout using real-time RT-PCR (Fig. 4). For each gene, the relative expression was calculated as the percentage of the highest expression level recorded, but overall, wnt4a2 and wnt4b expression was weaker than wnt4a1 (data not shown). Interestingly, the wnt4a1 and wnt4a2 transcripts were predominately...
expressed in the gonads. In addition, \textit{wnt4a1} was also expressed in the gills, brain, spleen, and skin, while \textit{wnt4a2} was expressed in the gills, pituitary, skin, and brain. By contrast, \textit{wnt4b} expression was restricted to the brain and pituitary.

**Gonadal Expression of the Rainbow Trout \textit{wnt4a1} and \textit{wnt4a2} Genes During Early Sex Differentiation and Gametogenesis**

To study the sex-specific expression of the \textit{wnt4} genes during the period that precedes histological gonadal differentiation, we used genetically all-male and all-female rainbow trout populations (Guiguen et al., 1999). As no expression of \textit{wnt4b} was observed in differentiating rainbow trout gonads (data not shown), only \textit{wnt4a1} and \textit{wnt4a2} expression are reported. \textit{wnt4a1} expression was detected in female and male gonads during early molecular differentiation (Fig. 5A). Its expression increased between 35 and 63 dpf in both male and female gonads, with a slight overexpression in males. The mean of the male-to-female \textit{wnt4a1} expression ratio (M/F) was 1.428 ± 0.343 during early molecular sex differentiation, and this ratio was significantly greater than 1 (\textit{t}-test; \( P < 0.01 \)). \textit{wnt4a1} expression decreased during gametogenesis in both male and female gonads (Fig. 5B), and no expression of \textit{wnt4a1} was observed in mature rainbow trout oocytes. \textit{wnt4a2} was also detected very early during gonadal differentiation (Fig. 5A); however, its expression was weaker than \textit{wnt4a1} (data not shown). Similar to \textit{wnt4a1}, the mean of the male-to-female \textit{wnt4a2} expression ratio (M/F) was significantly >1 during early molecular sex differentiation (1.392 ± 0.360; \textit{t}-test; \( P < 0.01 \)).

**Figure 3.** The expression pattern of \textit{wnt4a} and \textit{wnt4b} differs in rainbow trout embryos. The \textit{wnt4a} transcript is expressed in multiple tissues, including the neural tube (nt), floor plate (fp), and skin during rainbow trout embryogenesis, whereas weak \textit{wnt4b} expression is restricted to the floor plate and the epithelium of the head. The localization of the transcripts in 18-dpf rainbow trout embryos was performed using whole mount in situ hybridization with a probe that cross-hybridized with \textit{wnt4a1} and \textit{wnt4a2}, or with a specific probe for \textit{wnt4b}. Scale bar: 50 μm; fp, floor plate; n, notochord; nt, neural tube; ov, otic vesicle. Color figure can be viewed in the online issue which is available at wileyonlinelibrary.com.

**Figure 4.** \textit{wnt4a1/2} transcripts are detected in multiple tissues, whereas \textit{wnt4b} expression is restricted to the nervous system. Real-time PCR analysis was conducted using total RNA from the following tissues isolated from adult rainbow trout: brain (Br), pituitary (Pi), gills (Gi), heart (He), stomach (St), liver (Li), intestine (In), muscle (Mu), skin (Sk), spleen (Sp), head kidney (HK), trunk kidney (TK), late-vitellogenesis ovary (Ov l.v.), and stage II testis (Te II). For each tissue, three separate RT reactions were carried out using distinct RNA samples originating from three different fish. The RT reactions were pooled and used to run real-time PCR in triplicate. After normalization with the 18S reference gene, the relative expression was calculated as the percentage of the highest expression level recorded for each gene. Significant differences in expression (Wilcoxon test; \( P < 0.05 \)) are indicated with a different letter (\( a > b > c > \) other tissues).
In males, the highest expression was observed in stage-I testes, followed by a decrease in the more advanced stages of spermatogenesis. wnt4a2 expression remained very low in the ovaries during oogenesis, and no expression of wnt4a2 was detected in mature oocytes (Fig. 5B). The expression of wnt4a1/2 was detected in both male and female gonads using in situ hybridization as early as hatching (33 dpf; Fig. 6). At this stage, the gonads are characterized by the presence of three categories of cells, which can be distinguished by their shape, size, or location. Germ cells are easily identified by their large size, round shape, and location in the center of the gonads. The germ cells are surrounded by small, peri-germinal somatic cells. The germ cells and peri-germinal somatic cells are surrounded by epithelial cells, which delineate the gonads. At 33 dpf and 47 dpf, wnt4a1/2 transcripts were detected in some somatic cells located close to the germ cells in both male and female gonads, and at low levels in the germ cells. Then, at 68 dpf, as histological differentiation of the gonads occurs with the formation of the ovarian lamellae (Baron et al., 2004; Vizziano et al., 2007), wnt4a1/2 expression was exclusively found in the peri-germinal somatic cells located within the ovarian lamellae in the ovaries or the distal portion of the testes (Fig. 6).

**Effects of Sex Inversion Steroid Treatments on Gonadal wnt4a1 Expression**

The expression of wnt4a1 was examined in the gonads of genetically all-male rainbow trout treated with 17a-ethinylestradiol (EE2; Fig. 7A) and genetically all-female rainbow trout treated with the androgen 11β-hydro-
xyandrostenedione (11βOHΔ4) or with an aromatase inhibitor (ATD; Fig. 7B). Between 53 and 116 dpf, the expression of wnt4a1 slowly decreased in the gonads of both the control male and female populations. The masculinization of females with an inhibitor of estrogen synthesis (ATD) had no effect on gonadal wnt4a1 expression. In both males feminized with EE2 and in females masculinized with 11βOHΔ4, however, wnt4a1 expression was strongly downregulated 7 days after the initiation of treatment, and gonadal wnt4a1 expression remained low in both of these treated groups (EE2 and 11βOHΔ4). The treatments had no effect on the already low wnt4a2 expression (data not shown).

DISCUSSION

Although Wnt4 is involved in many important developmental processes in different fish species (Ungar et al., 1995; Liu et al., 2000; Yokoi et al., 2003; Matsui et al., 2005; Wu and Chang, 2009; Inohaya et al., 2010), including reproduction and sex inversion (Wu and Chang, 2009), no detailed evolutionary gene history has been carried out on teleost fish wnt4. Therefore, we analyzed Wnt4 evolution in metazoans, with a particular focus on the teleost fish whole-genome duplications. This analysis was then used to characterize the expression patterns of the wnt4 genes during gonadal differentiation in rainbow trout.

Wnt4, like almost all members of the Wnt family, has been found in various metazoan phyla. In addition to chordates, Wnt4 has been found in protostomes, including various lophotrochozoans (Prud’homme et al., 2002; Cho et al., 2010), such as mollusks and the annelid P. dumerilii, and in various ecdysozoans, such as nematodes and arthropods (with the exception of insects, which have lost Wnt4) (Bolognesi et al., 2008; Janssen et al., 2010). Wnt4 has also been found in the sister group to bilaterian metazoans, specifically in the cnidarian N. vectensis (Kusserow et al., 2005). Therefore, the Wnt4 subgroup likely originates before the divergence of cnidarians and bilaterians. It is possible that the Wnt4 subgroup appeared earlier, as other Wnt ligands have been found in early animal phyla, such as placozoans (Srivastava et al., 2008; Richards and Degnan, 2009), poriferans (Adamska et al., 2007; Lapebie et al., 2009), and ctenophores (Pang et al., 2010), suggesting that the diversification of the Wnt family occurred very early in metazoan evolution. Our analysis shows that most teleost fish have two wnt4 genes, wnt4a and wnt4b, whereas a single Wnt4 gene is found in other vertebrates and in non-vertebrate species. It is now well established that teleost fish underwent a whole-genome duplication 320 million years ago (Meyer and Van de Peer, 2005), and we confirmed that these two wnt4 genes are paralogous copies derived from this whole-genome duplication using synteny analysis. This paralogous relationship is not supported by our phylogenetic analysis, however, as all teleost fish Wnt4b sequences clearly branched outside of the clade formed by all other vertebrate WNT4 sequences. This inconsistency can be explained by a long-branch attraction artifact (Delsuc et al., 2005) resulting from the important divergence of these Wnt4b sequences. Such a high rate of molecular evolution appears to be quite frequent for many of the genes resulting from genome duplications in fish, and these duplicated genes are often associated with functional shifts via sub- or neo-functionalization (Steinke et al., 2006).
In addition to the first duplication of \textit{wnt4} in teleost fish, we also found a second duplication in rainbow trout that resulted in two copies of \textit{wnt4} that we designated \textit{wnt4a1} and \textit{wnt4a2}. As only one \textit{wnt4} gene is found in other fish, such as zebrafish, medaka, and tetraodon, this additional copy likely originated from salmonid-specific whole-genome duplication (Allendorf and Thorgaard, 1984). While we have not been able to find another salmonid sequence homologous to \textit{Wnt4a} in public databases, the sequencing of the rainbow trout and Atlantic salmon (\textit{Salmo salar}) genomes is ongoing. These sequences will allow us to determine if other salmonids possess this additional \textit{wnt4a} gene, and if this is the result of salmonid-specific whole-genome duplication.

The embryonic expression pattern of \textit{wnt4} is well conserved throughout vertebrate evolution. In rainbow trout, \textit{wnt4a} is expressed in the neural tube and floor plate, whereas \textit{wnt4b} expression is restricted to the floor plate. These results are in agreement with what has been previously described in other teleost fish, including zebrafish (Ungar et al., 1995; Matsui et al., 2005) and medaka (Yokoi et al., 2003; Inohaya et al., 2010). In addition, frog (\textit{Xenopus laevis}) and mouse \textit{Wnt4} are also expressed in the neural tube and the floor plate (McGrew et al., 1992; Parr et al., 1993). In non-vertebrate species, such as the annelid \textit{Helobdella robusta} (Cho et al., 2010) and the cephalochordate \textit{B. iloride} (Schubert et al., 2000), \textit{Wnt4} has also been linked with neural development. The restricted expression of teleost \textit{wnt4b} in the floor plate suggests a neo- or sub-functionalization of this gene, which is also supported by its inconsistent position in our phylogenetic analysis. This is also in agreement with recent work in medaka, demonstrating that the absence of \textit{wnt4b} expression in the floor plate results in defective segmentation of the vertebral column, and that this phenotype is not exacerbated by the knockdown of \textit{wnt4a} (Inohaya et al., 2010). Therefore, this essential role of \textit{wnt4b} in the segmentation pattern of the vertebral column is not redundant with \textit{wnt4a} functions (Inohaya et al., 2010). Altogether, this strongly corroborates our hypothesis of a sub- or neo-functionalization of \textit{wnt4b}, specifically a role during embryonic development that is restricted to the floor plate. The expression of \textit{wnt4b} and \textit{wnt4a} were also clearly different in adult rainbow trout tissue, with the expression of \textit{wnt4b} detected in the brain and pituitary whereas the expression of \textit{wnt4a} (1 and 2) was mainly detected in the gonads. To date, only one study has reported the gonadal expression of a \textit{wnt4} gene in a teleost species (black porgy; Wu and Chang, 2009). Unfortunately, the black porgy \textit{wnt4} gene has not been characterized regarding potential gene duplications. According to our analysis, this \textit{wnt4} gene is clearly a \textit{wnt4a} paralog (data not shown), and this supports the hypothesis that only \textit{wnt4a} genes are expressed in the gonads.

Based on these results, we restricted our analysis of \textit{wnt4} expression sex differentiation to the \textit{wnt4a} genes. We found that \textit{wnt4a1} and \textit{wnt4a2} were both expressed in differentiating male and female gonads in the rainbow trout. For both genes, the mean expression ratio in male and females gonads (M/F) was significantly >1, indicating that \textit{wnt4a1} and \textit{wnt4a2} were slightly, but significantly, expressed more (approximately 40% increased expression) in male gonads during early molecular sex differentiation. The fact that \textit{wnt4a1} and \textit{wnt4a2} have different expression profiles during gonadal differentiation.
and gametogenesis suggests that they play different roles during early and late gonadal development. It should also be noted that expression of \textit{wnt4a2} is extremely low, regardless of the stage of gonadal development. The difference in the expression of \textit{wnt4a1} and \textit{wnt4a2} can be explained by the differential evolution of their promoter. As the sequence of the rainbow trout genome is not yet available, we did not study the promoter of the \textit{wnt4} genes. Interestingly, in the protandrous hermaphrodite black porgy, which first differentiates as male, \textit{wnt4} expression has been detected during sex differentiation, and its expression remains unchanged during early ovarian development (Wu and Chang, 2009). The rainbow trout \textit{wnt4} expression profile differs from the profiles seen in other species, which exhibit either ovary-predominant \textit{Wnt4} expression (Vainio et al., 1999; Pailhoux et al., 2002; Oreal et al., 2002; Yu et al., 2006; Smith et al., 2008; Jaaskelainen et al., 2010; Tripathi and Raman, 2010) or non-dimorphic \textit{Wnt4} expression (Oshima et al., 2005; Shoemaker et al., 2007). In mice, steroidogenesis occurs earlier in male embryonic gonads than in female embryonic gonads, and the high expression of \textit{Wnt4} in the future ovary has been associated with the inhibition of steroidogenic cell recruitment and steroidogenic gene expression (Jeays-Ward et al., 2003; Jordan et al., 2003). In rainbow trout, steroidogenesis begins earlier in female embryonic gonads than in males embryonic gonads (Vizziano et al., 2007). Therefore, in contrast to mouse gonadal development, the slight overexpression of \textit{wnt4a1/2} in rainbow trout male gonads could be involved in the delay of steroidogenesis in males.

Regardless of sexual dimorphism, the cellular localization of \textit{Wnt4} in the differentiating gonad seems to be well conserved across species. In rainbow trout, the \textit{Wnt4} transcript was detected in peri-germinal somatic cells located inside the differentiating male and female gonads. A similar expression pattern in somatic cells has been observed in mice at the transcriptional level (Vainio et al., 1999; Yao et al., 2004) and at the protein level in the tammar wallaby (Yu et al., 2006). \textit{WNT4} expression has also been reported in somatic cells of the “inner part” of differentiating embryonic chicken gonads (Oreal et al., 2002), and in somatic cells located in the cortical region of the ovary and in the putative testicular sex cords of turtle gonads (Shoemaker et al., 2007). This localization in differentiating gonads is consistent with expression in pre-granulosa cells in the females. Following sex differentiation, \textit{Wnt4} expression has been detected in the granulosa cells of small follicles in human fetuses at both the transcriptional and protein level (Jaaskelainen et al., 2010), and in mice (Hsiew et al., 2002) and chickens (Oreal et al., 2002) at the transcriptional level. This is also in agreement with what has been reported in the black porgy, where \textit{wnt4} (\textit{wnt4a} expression was detected at both the transcriptional and protein level in the follicle cells surrounding oogonia and early meiotic oocytes, and in the granulosa cells surrounding more developed oocytes (Wu and Chang, 2009).

In this protandrous hermaphroditic fish species, male-to-female sex inversion induced by treatment with estrogens or an aromatase inhibitor strongly upregulates \textit{wnt4a} expression in the gonads (Wu and Chang, 2009). Furthermore, during the natural sex inversion process, ovarian \textit{Wnt4} expression is high when the ovarian tissue begins to develop. In rainbow trout, both feminizing (EE2) and masculinizing (11βOH\(\Delta\)4) steroid treatments resulted in the rapid and sustained inhibition of \textit{wnt4a1} expression, whereas masculinization induced by an aromatase inhibitor (ATD) had no effect. While this seems to be in disagreement with the results obtained in the black porgy, it should be noted that gonadal differentiation and development are very different in these two species: black porgy are functional males for the first 2 years of life, after which they differentiate into females (Wu and Chang, 2009), whereas rainbow trout are gonochoristic. Therefore, our treatments were initiated early in development, when gonadal differentiation is still labile, and mRNA expression was measured shortly after the initiation of treatment. It has also been previously shown that in rainbow trout, these sex inversion steroid treatments do not always reflect what is observed during natural gonadal differentiation, and that the masculinization induced by the inhibition of estrogen synthesis (e.g., ATD treatment) is more physiological than the masculinization induced by androgen treatment (Vizziano-Cantonnet et al., 2008; Vizziano et al., 2008). Therefore, the steroid-induced downregulation of \textit{wnt4a1} may be more a consequence of the profound disruption of the endocrine environment of the differentiating gonads.

One intriguing question that still remains to be answered is why \textit{Wnt4} is predominantly expressed during early ovarian differentiation in some species (e.g., mammals, the garden lizard and chicken) but not in others (e.g., turtle, frog, and rainbow trout). Its early role during ovarian differentiation is often described as an anti-testis factor that antagonizes the action of testis-specific genes such as \textit{Fgf9} (Kim et al., 2006). One hypothesis is that this proposed role of \textit{Wnt4} would not be necessary during ovarian differentiation in species that do not rely on the sex-specific expression of specific genes, such as \textit{Fgf9}. This hypothesis accounts for the non-dimorphic expression of \textit{Wnt4} observed in the frog, \textit{R. rugosa}, as the expression of \textit{Fgf9} does not appear to be sexually dimorphic during gonadal differentiation in this species (Yamamura et al., 2005). The fact that the \textit{fgf9} gene is no longer present in teleost genomes (Itoh and Konishi, 2007) may also support this hypothesis, and explain why the \textit{wnt4a} genes are not predominantly expressed in the differentiating ovaries in the rainbow trout. Such an early ovary-predominant expression in mammals does not, however, mean that \textit{Wnt4} is required exclusively for female gonadal development, and in that regards, it has been shown that male \textit{Wnt4} knockout mice present with defects in the differentiation of Sertoli cells (Jeays-Ward et al., 2004). Thus, although \textit{Wnt4} is well known for its pivotal role in ovarian differentiation in mammals, it is probably also required for proper testicular development. Therefore, \textit{wnt4} may also play a role in both male and female sex differentiation in the rainbow trout. Most of the sexually dimorphic genes identified in differentiating rainbow trout gonads, such as \textit{cyp19a1a} and \textit{foxl2} in the ovary (Baron et al., 2004, 2005a; Vizziano et al., 2007;
Cavileer et al., 2009), were already implicated in sex differentiation in other vertebrate species, including mammals (Baron et al., 2005b). This suggests that despite some differences in the process of sex determination and differentiation, some molecular pathways are conserved throughout vertebrates. In mammals, another gene, Follistatin, has been implicated in early ovarian differentiation. Follistatin is a target gene of the Wnt/β-Catenin pathway (de Groot et al., 2000; Willert et al., 2002), and acts downstream of Wnt4 and the Wnt pathway during ovarian differentiation (Yao et al., 2004; Chassot et al., 2008; Liu et al., 2009).

Interestingly, follistatin has been implicated in ovarian developmental processes, including folliculogenesis and oocyte maturation, in teleost fish (Pang and Ge, 1999; Wu et al., 2000; Wang and Ge, 2004), including rainbow trout (Lankford and Weber, 2010). Moreover, in rainbow trout, follistatin gene was found to be over-expressed in differentiating female gonads (Baron et al., 2005a; Vizziano et al., 2007). These data suggest an implication of wnt4 in ovarian differentiation and development in rainbow trout. Yet the role of wnt4 in ovarian and testicular development still remains unclear with regards to the evolution of vertebrate gonadal differentiation, and functional analyses in non-mammalian species are needed to better understand its potential role in these processes.

In this study, we report the characterization of three wnt4 genes in a teleost fish (rainbow trout). These genes likely resulted from specific whole-genome duplications. Two of these genes showed greater conservation with other vertebrates, and were expressed in early differentiating gonads, with a slight dimorphism in favor of males. These results show that, unlike mammals, rainbow trout do not display an ovary-predominant Wnt4 expression profile during early gonadal differentiation.

**MATERIALS AND METHODS**

**cDNA Cloning of wnt4 Genes**

Partial rainbow trout wnt4a1, wnt4a2, and wnt4b cDNA were amplified by PCR using degenerated primers, obtained by sequence alignment of zebrafish, medaka, and tetraodon wnt4a and wnt4b using ClustalW. The complete coding sequences were then obtained by 5′ and 3′ RACE-PCR, using cDNA from differentiating gonads for wnt4a1 and wnt4a2, and from pituitary for wnt4b. All these PCR products were subcloned in pGEMTeasy (Promega, Madison, WI) according to manufacturer recommendations. Accession numbers are indicated in Table 2.

**Sequences Analysis**

Phylogenetic tree reconstruction of WNT4 protein sequences was carried out with the FIGENIX genomic annotation platform (Gouret et al., 2005), based on neighbor-joining, maximum parsimony, and maximum likelihood methods. Bootstrap values are indicated for the nodes that are present in all three phylogenetic reconstruction methods, and the absence of a node for a given phylogenetic method is denoted by an asterisk. Accession numbers of the protein sequences used in this work are indicated in Table 2. Synteny analysis was performed using the Synteny Database (Catchen et al., 2009) and Genomicus genome browser (Muffato et al., 2010).

**TABLE 2. Accession Number of Full Length Wnt4 Proteins According to Species Used in This Study**

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<th>Species</th>
<th>Name</th>
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Animals and Sampling

Research involving animal experimentation conformed to the principles for the use and care of laboratory animals, in compliance with French and European regulations on animal welfare. Genetically all-male (XY) and all-female (XX) rainbow trout larvae were obtained from the Institut National de la Recherche Agronomique experimental facilities (Drennec, France) as previously described (Guiguen et al., 1999). In order to determine the tissue distribution of wnt4 genes, gills, brain, heart, stomach, liver, pituitary, intestine, muscle, skin, spleen, head kidney, trunk kidney, and late vitellogenesis ovary were sampled in three adult females. This tissue collection was complemented by samples of stage-II testis that were also collected in three different males. To analyze expression profile of wnt4a1 and wnt4a2 during early gonad differentiation, from 50 to 100 gonads depending on the age of the fish, were sampled and pooled at 35, 36, 39, 42, 43, 44, 45, 46, 49, 52, 56, and 63 days post fertilization (dpf) in genetically all-male (XY) and all-female (XX). Ovaries were sampled at the following stages: late vitellogenesis, post-ovulatory ovaries along with mature oocytes, and testis were sampled at different key stages of male reproductive cycle: testis stage I, with slowly dividing type A spermatogonia; testis stage II, with type A and actively-dividing type B spermatogonia; testis stage III, maturing testes containing meiotic spermatocytes; testis stage VI, with post-miotic spermatids; and testis stage VIII, spawning testes with fully developed spermatozoa. All these gonadal samples were collected from three individuals. All biological samples were immediately frozen in liquid nitrogen and stored at −80°C until RNA extraction. For in situ hybridization of 18 dpf rainbow trout, embryos were mechanically dechorionated prior to fixation. Rainbow trout trunks were dissected at stages 33, 47, and 68 dpf and were mechanically dechorionated prior to fixation. After fixation overnight in 4% paraformaldehyde at 4°C, samples were dehydrated in 100% methanol, and stored at −20°C.

Sex Inversion Steroid Treatment of Male and Female Rainbow Trout

The masculinization (induced by inhibition of estrogen synthesis or by androgens) or feminization (induced by estrogen) of rainbow trout was carried out as previously described (Baron et al., 2007; Vizziano-Cantonnet et al., 2008; Vizziano et al., 2008). Briefly, at the onset of the first feeding (55 days post-fertilization), genetic all-female rainbow trout larvae were fed daily with a commercial diet supplemented with 10 mg/kg 11b-hydroxyandrostenedione (11bOH14; Sigma, St. Louis, MO) or 50 mg/kg 1,4,6-androstatriene-3,17-dione (ATD). In contrast, genetic all-male rainbow trout were fed a commercial diet supplemented with 20 mg/kg of 17α-ethinylestradiol (EE2, Sigma). The fish were treated for 2 months starting from the first feeding (approximately 50–55 dpf). Control, all-female and all-male genetic populations were fed with the same commercial diet, without steroid supplementation. For each treatment, the gonads from 20 to 100 animals were sampled and pooled in duplicates for real-time PCR analysis at the following stages of development post fertilization: 53, 60, 69, 83, and 116 dpf. All samples were immediately frozen in liquid nitrogen and stored at −80°C until RNA extraction.

RNA Extraction and Real-Time PCR Analysis

Total RNA was extracted using TRIzol reagent (Invitrogen) as previously described (Govoroun et al., 2001), and reverse transcription (RT) was performed as previously described for the early molecular differentiation kinetics (Vizziano et al., 2007). For the gametogenesis kinetics, total RNA was treated with RQ1 DNase (Promega) according to manufacturer recommendations. RT reactions were carried out using 1 μg of RNA samples. Control reactions were performed without reverse transcriptase. RT was carried out with M-MLV reverse transcriptase (Promega) as recommended by the manufacturer. Real-time PCR was performed using the Step One Plus system (Applied Biosystems, Foster City, USA). cDNA templates and negative RT control reactions were diluted to 1/25, and 4 μl were used for real-time PCR, using Fast SYBR Green Master Mix (Applied Biosystem) and 600 nm of each primer. The following primers were used: wnt4a1, Fw: 5′-CCACCGAGTCAGTCGAA-3′ and Rv: 5′-TACCCCTCCTCACAATCAACA-3′; wnt4a2, Fw: 5′-CCAT- GGAGTCAGTCCGGAG-3′ and Rv: 5′-TACTCCTCCTCC- TTACGTCATA-3′; and wnt4b, Fw: 5′-TGGTCTGGGG- TGCAATGATA-3′ and Rv: 5′-GGGAATAGCCTCTC- GTCCA-3′. Relative expression was normalized with 18S gene, and was calculated as the percentage of the highest expression level recorded for each gene. All statistical analyses were performed using R, version 2.9.2. For each of the time points during early sex differentiation, the ratio of gene expression in males and females (M/F) was calculated, and a t-test was conducted to determine if the M/F expression ratio was significantly >1 (P < 0.01).

In situ Hybridization

A 1 kb region of the wnt4a1 and wnt4b cDNA was PCR amplified using primers specific for wnt4a1 (Fw: 5′-TTGT- CCACACTGGACACATC-3′; Rv: 5′-TCCCGTGCCAGTG- TACATT-3′) or wnt4b (Fw: 5′-TACCTGTCATGTGAT- GCAA-3′; Rv: 5′-CAGCGCCGTGGATTGGACA-3′) and cloned into the pGEMTeasy vector (Promega). These plasmids were used as DNA templates for the production of digoxigenin-labeled anti-sense RNA probes. In situ hybridizations (ISH) were performed using an Intavis Insitu Pro AG robotic workstation. After rehydration in graded methanol/PBS baths, embryos or trunks were digested with 25 μg/ml proteinase K for 30 min at room temperature. The digestion was stopped with 2 mg/ml glycine in PBS-T, and the samples were fixed in 4% paraformaldehyde/PBS for 20 min and rinsed in PBST. Pre-hybridization (2 hr, 65°C) and hybridization (16 hr, 65°C) were carried out in 50% formamide, 5× SSC, 0.1% Tween 20, 0.005% heparin, and 100 μg/ml tRNA. For hybridization, the digoxigenin-labeled RNA probes were diluted in hybridization buffer

to a final concentration of 1 ng/μl. The washing steps (2× SSC, three times for 40 min; 0.2× SSC, three times for 40 min; and PBST, three times for 40 min) were performed at 55°C. The samples were then blocked for 1 hr at room temperature in PBST containing 2% inactivated sheep serum. The digoxigenin signal was detected using an anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche Diagnostics, Mannheim, Germany) and a NBT/BCIP detection system (Roche Diagnostics), as recommended by the manufacturer. After labeling, the samples were fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin using a Citadel 1000 tissue processor (Shandon, Pittsburgh, PA) and a HistoEmbedder (TBS88; Medite, Burgdorf, Germany). Serial, 5-μm thick cross-sections were cut for each sample. After deparaffinization and rehydration of the sections, the slides were mounted with Mowiol 4-88 medium (Calbiochem, Meudon, France).

ACKNOWLEDGMENTS

The authors are grateful to the experimental facility staff of the INRA Scribe laboratory for taking care of fish rearing. The authors thank Dr. Jean-Jacques Lareyre for supplying stage I to stage VIII testis RNA, and Dr. Pierre Pontarotti and Olivier Chabrol for the helpful advices on phylogeny analysis. B.N. gratefully acknowledges the funding received for her PhD from the Brittany province (Region Bretagne) and the INRA Institute (PHASE department). The research leading to these results has received funding from the European Community’s Seventh Framework Program. The authors thank Dr. Jean-Jacques Lareyre for supplying stage I to stage VIII testis RNA, and Dr. Pierre Pontarotti and Olivier Chabrol for the helpful advices on phylogeny analysis. B.N. gratefully acknowledges the funding received for her PhD from the Brittany province (Region Bretagne) and the INRA Institute (PHASE department). The research leading to these results has received funding from the European Community’s Seventh Framework Program (FP7/2007-2013) under grant agreement no. 222719-LIFECYCLE.

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