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ARTICLE *in* MOLECULAR REPRODUCTION AND DEVELOPMENT · AUGUST 2012

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RESEARCH ARTICLE

Molecular Reproduction & Development 79:504–516 (2012)

Expression and Phylogeny of Candidate Genes For Sex Differentiation in a Primitive Fish Species, the Siberian Sturgeon, *Acipenser baerii*

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SUMMARY

The molecular mechanisms underlying testis differentiation in basal actinopterygian fish remains poorly understood. The sex differentiation period was investigated in the Siberian sturgeon, *Acipenser baerii*, by expression profiling of Sertoli cell transcription factors (*dmrt1*, *sox9*) that control testis differentiation in vertebrates; Leydig cell factors (*cyp17a1*, *star*) affecting androgen production; the androgen receptor (*ar*); a growth factor controlling testis development (*igf1*); and a gene coding for a gonadotropin hormone (*lh*). Two genes were characterised for the first time in the Siberian sturgeon (*dmrt1*, *cyp17a1*), while the others came from public databases. Sturgeon gonad development is very slow, with a late sexual differentiation time during their juvenile stage, and are still immature at 3 years of age. Immature fish showed a sex-dimorphic pattern; all the genes studied displayed a higher expression level in male gonads. We took advantage of the presence of juvenile fish with pre- and post-differentiated gonads (16 and 18 months old) to characterise them at the molecular level. The post-differentiated fish displayed a sex dimorphism of gene expression in their gonads for all genes studied, with the exception of *sox9*. The trends in undifferentiated fish lead us to propose that sturgeons undergoing male differentiation express high levels of Sertoli cell factors (*dmrt1*, *sox9*) and of genes involved in the production and receptivity of androgens (*cyp17a1*, *star* and *ar*) together with *lh*. Expression profiles and phylogenetic studies suggest that these genes are potential regulators of testis development in the Siberian sturgeon.

Mol. Reprod. Dev. 79: 504–516, 2012. © *2012 Wiley Periodicals, Inc.*

Received 26 December 2011; Accepted 11 May 2012

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Grant sponsor: Programa de Desarrollo Tecnológico del Uruguay; Grant number: 71-13

Published online 22 May 2012 in Wiley Online Library (wileyonlinelibrary.com).
DOI 10.1002/mrd.22053

INTRODUCTION

The morphological differentiation of vertebrate gonads is preceded by molecular changes that are not completely understood in different taxa. A master gene that determines

Abbreviations: *Amh*, anti-Müllerian hormone; *Ar*, androgen receptor; *Cyp17a1*, cytochrome P450, family 17, subfamily a, polypeptide 1; *Dmrt1*, doublesex and Mab-3 related transcription factor 1; *Igf1*, insulin like growth factor I; *Lh*, luteinising hormone; *Sox9*, SRY-box containing gene 9; *Sry*, sex-determining region of chromosome Y; *Star*, steroidogenic acute regulatory protein.

gender has been already identified in some vertebrates. Generally, this master gene encodes a transcription factor, and it is lineage- or even species-specific: SRY (sex-determining region of the Y chromosome) in placental mammals (Sinclair et al., 1990; Koopman et al., 1991), *DMY*/*dmrt1bY* (*Y-specific DM-domain gene required for male development*) in medaka (Matsuda et al., 2002; Nanda et al., 2002), *Dmrt1* (*doublesex and Mab-3 related transcription factor 1*) in chicken (Smith et al., 2009), and *Dm-W* (*W-linked paralogue of dmrt1*) in the African clawed frog (Yoshimoto et al., 2008). Recently, it has been shown that a Y-linked duplicated copy of the anti-Müllerian hormone (*amhy*) plays a critical role in the sex determination of the Patagonian pejerrey (*Odontesthes hatcheri*) (Hattori et al., 2012). On the other hand, many unsuccessful efforts have been undertaken to discover the gender-determining gene in sturgeons (Wuertz et al., 2006; McCormick et al., 2008; Keyvanshokook and Gharaei, 2010). Studies involving different vertebrates revealed that even if the sex-determining gene seems not to be conserved, the downstream sex differentiation cascade includes common genes that interact to induce gonad differentiation (Brennan and Capel, 2004; Piferrer and Guiguen, 2008; Herpin and Schartl, 2011). *Sox9* (*SRY-box containing gene 9*), *Dmrt1*, *Amh* (*anti-Müllerian hormone*), *Nr5a1* (*nuclear receptor subfamily5, group a, member 1*), *Nr0b1* (*nuclear receptor subfamily 0, group b, member 1*) and *Wt1* (*Wilm's tumour suppressor-1 gene*) have been described as conserved genes involved in the masculine pathway (Bowles and Koopman, 2001; Brennan and Capel, 2004; Piferrer and Guiguen, 2008).

Since the major sex-determining factor is still unknown for most fish, it is not possible to sex the fish at early stages of development, before the period of morphological gender differentiation. To circumvent this problem, genetically all-male and all-female populations were obtained for some species, allowing the study of the changes in gonadal gene expression before their gender was expressed at a morphological level. The information on factors involved in testis differentiation came from species in which monosex populations and genetic resources are available. The over-expression of certain genes before morphological gonad sex differentiation was described in some teleost species (Guiguen et al., 1999; Marchand et al., 2000; Wang et al., 2004; Baron et al., 2005; Nagahama, 2005; Vizziano et al., 2007; Ijiri et al., 2008; Lareyre et al., 2008; Shibata et al., 2010; Yano et al., 2011). These studies showed that testis differentiation was preceded by a clear over-expression of Sertoli cell transcription factors (i.e. *dmrt1*, *sox9*, *nr0b1* or *dax1*, and *tbx1* (or *T-box transcription factor gene family*)) (Marchand et al., 2000; Vizziano et al., 2007; Yano et al., 2011), and transforming growth factors such as *amh* and *gsdf* (*gonadal soma-derived growth factor*) (Baron et al., 2005; Vizziano et al., 2007; Lareyre et al., 2008; Shibata et al., 2010). *dmrt1* expression was previously studied in sturgeon gonads, and shown to be sex dimorphic in Lake sturgeon (Hale et al., 2010) but not in the Shovelnose sturgeon (Amberg et al., 2010). Leydig cell differentiation occurs for some species before testis differentiation, with an over-expression and/or activation of enzymes and factors controlling steroid synthesis (Vizziano et al., 2007; Blasco et al., 2012). In contrast, the steroid synthesis capacity appears after testis differentiation in tilapia (Ijiri et al., 2008). The role of steroids in gonad differentiation is unknown in primitive fish like sturgeons. Finally, another interesting candidate involved in gonadal development is the *igf1* (*insulin-like growth factor I*) (Le Gac et al., 1996), which is up-regulated during late testis differentiation in trouts (Baron et al., 2005).

Contrasting with information in teleost fish, the molecular mechanisms underlying testis differentiation in basal actinopterygian fish such as sturgeons remains poorly understood (McCormick et al., 2008; Amberg et al., 2010; Keyvanshokook and Gharaei, 2010). No monosex populations are available for this class of fish, sex-linked genetic markers are not known (Wuertz et al., 2006), and neither their complete genome sequences nor gonad transcriptomes are publicly available to study their sexual development. In order to explore the possible regulators involved in testis differentiation in *Acipenser baerii*, we selected two Sertoli cell factors (*dmrt1*, *sox9*); two Leydig cell factors, *cyp17a1* (*cytochrome P450, family 17, subfamily a, polypeptide 1*) and *star* (*steroidogenic acute regulatory protein*); and another three markers, *ar* (*androgen receptor*), *lh* (*luteinising hormone*), and *igf1*, which have been reported to be over-expressed in the differentiating gonads of trouts (Baron et al., 2005). Here, we compare recently differentiated gonads to gonads in the period preceding their morphological differentiation. A phylogenetic analysis of the selected genes was made in order to confirm their relationship with already characterised vertebrate sexual regulators.

RESULTS

Gonad Development

In the rearing conditions used, the fish were sexually differentiated but still immature at 3 years old. Juvenile fish of 16 and 18 months were analysed by microscopy. The histological analysis of juvenile gonads showed 12 fish with undifferentiated gonads at both the morphological and histological levels; 5 fish already differentiated into females, with ovaries containing early pre-vitellogenic oocytes; and 4 fish already differentiated into males, with testes in which the future tubules are recognisable. The undifferentiated gonads were characterised by an absence of features identifying them as testes or ovaries; the somatic tissue had no special arrangement indicating the sex of the gonad, and contained only gonias (gc) (Fig. 1, 'Undifferentiated'). The differentiated ovaries showed future lamellae with plenty of oogonia (oo) and a few pre-vitellogenic oocytes (Prev-OV) (Fig. 1, 'Differentiated ovary I') or contained an ovarian lamellae filled with pre-vitellogenic oocytes and groups of oogonia (oo) (Fig. 1, 'Differentiated ovary II'). The differentiated testes showed the somatic cells (sc) surrounding the spermatogonia (spg) and organised to form the future tubules (Fig. 1, 'Differentiated testis').

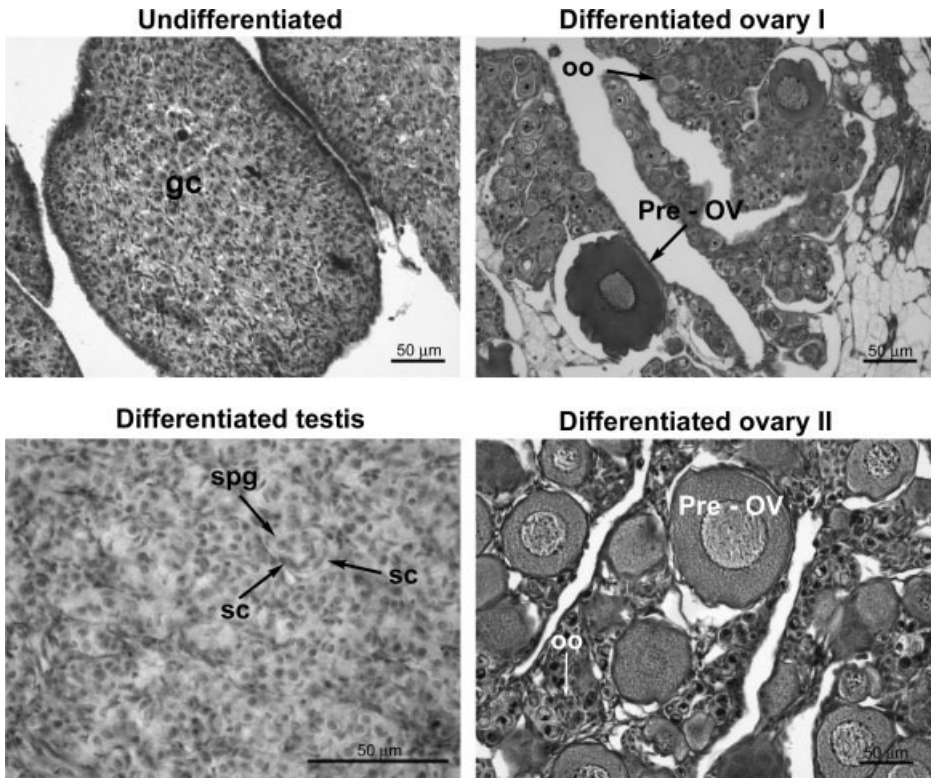

Figure 1. Histological analyses of Siberian sturgeon juvenile gonads. Germ cells (gc), oogonias (oo), pre-vitellogenic oocytes (Pre-ov), somatic cells (sc), spermatogonias (spg). Scale bar = 50 µm.

Phylogenetic Characterisation of Gonadal *Dmrt1, Cyp17a1, Star, Igf1, ar, lh,* and *Sox9*

Phylogenetic studies of Siberian sturgeon Dmrt1 showed that it is unambiguously orthologous to all the Dmrt1 proteins found in other chordate species (data not shown). It does not harbour any long branches, as in tetrapods or teleost species (Fig. 2A). Cyp17a1 is also in a basal phylogenetic position from tetrapods and teleost fish, although the branch leading to sturgeons is supported by a very weak bootstrap value. Again, the branch of sturgeons is short compared to that of mammals (Fig. 2B). This feature is even stronger for the Star protein (Fig. 2C). Igf1 is found in all chordate species, and the phylogenetic position of this gene for the Siberian sturgeon follows the species classification (Fig. 2D). Similarly, the phylogeny of the Ar follows the species classification. The branch length of the Ar in sturgeons is similar to that of the Ar in other tetrapods, the Ar-a of teleost fish, and even three chondrichthyan species (Fig. 2E). The acipenser Lh orthologs cluster with the tetrapod species instead of the fish species, although the bootstrap values are weak in the common branch of sturgeons and tetrapods and in the branch leading to the actinopterygians. This could be a consequence of a long-branch attraction to the very differentiated Lh homologs found in the metatherian species used here (Fig. 2F). The sturgeon Sox9 branches at the base of the actinopterygians, although its bootstrap value is also weak (Fig. 2G).

Expression Patterns in Tissues From Immature Fish

A quantitative expression study through qPCR in immature gonads of Siberian sturgeons showed that *dmrt1*, *sox9*, *cyp17a1*, *star*, *ar*, *igf1*, and *lh* are sex-dimorphic in gonads, with higher expression in testes (Fig. 3). *dmrt1* and *cyp17a1* are slightly expressed in brain, muscle, liver, gills and kidney. In contrast, *sox9*, *ar*, and *lh* have a higher expression in the tissues studied, with a particularly high expression in male muscle and brain when compared to females. *igf1* showed higher expression in the liver of males and females when compared to the other tissues studied, while the expression of *star* was particularly high in kidneys.

Gene Expression in Gonads of Juvenile Fish

Sertoli and Leydig cell markers were studied in already-differentiated gonads of juvenile fish. Among the Sertoli cell markers, *dmrt1* mRNA expression exhibited a clear sexual dimorphism, being higher (>200-fold) in males than in females when gonads were just-differentiated

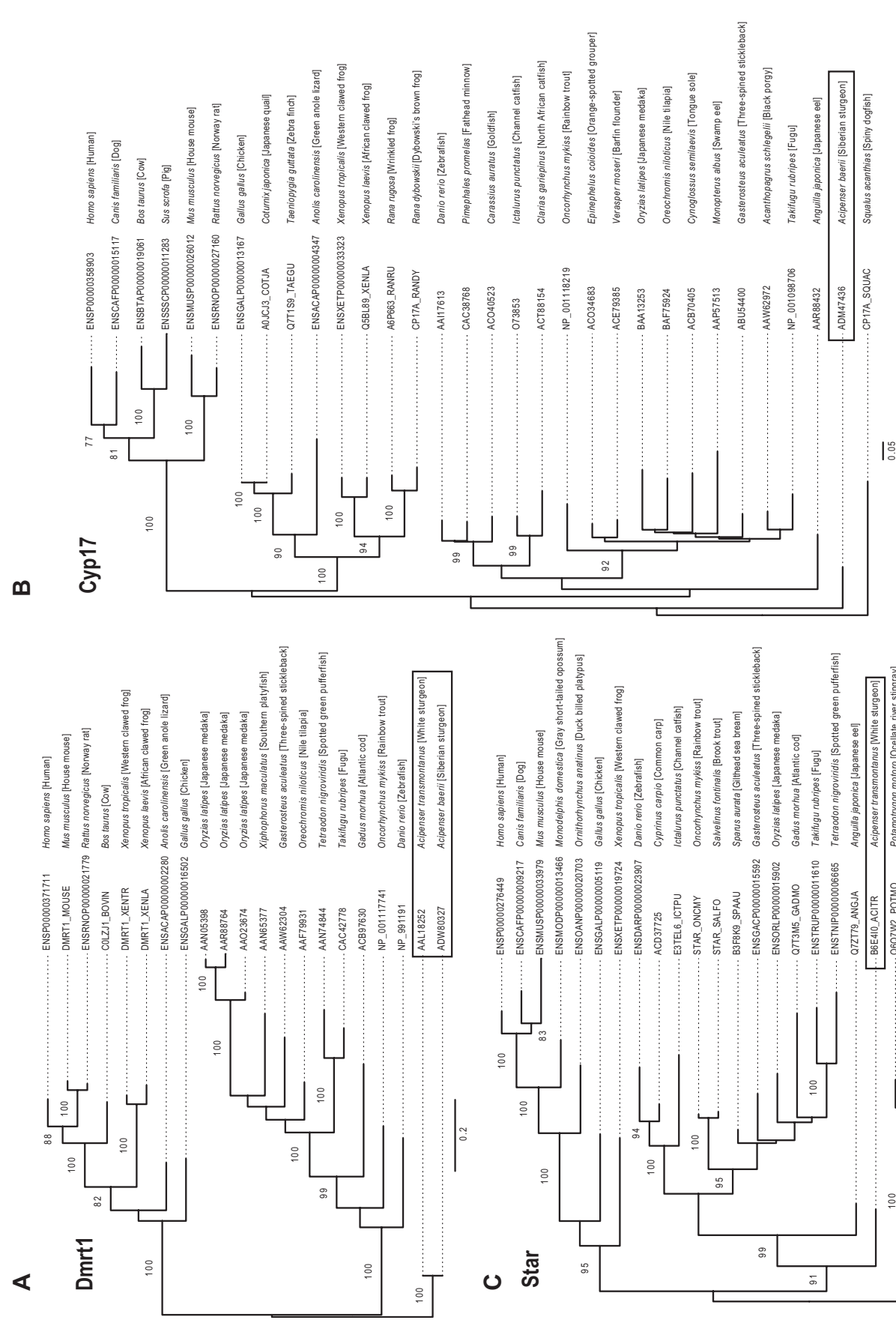

Figure 2. Phylogenetic analyses, using Maximum Likelihood, of factors involved in testis differentiation: (**A**) Dmrt1, (**B**) Cyp17a1, (**C**) Star, (**D**) Igf1, (**E**) Ar, (**F**) GtHb2/Lh and (**G**) Sox9. Sturgeon fish are in square boxes. Protein access numbers are given with species' names. Bootstrap values of 1,000 iterations, shown as a percent, were generated using PHYML.

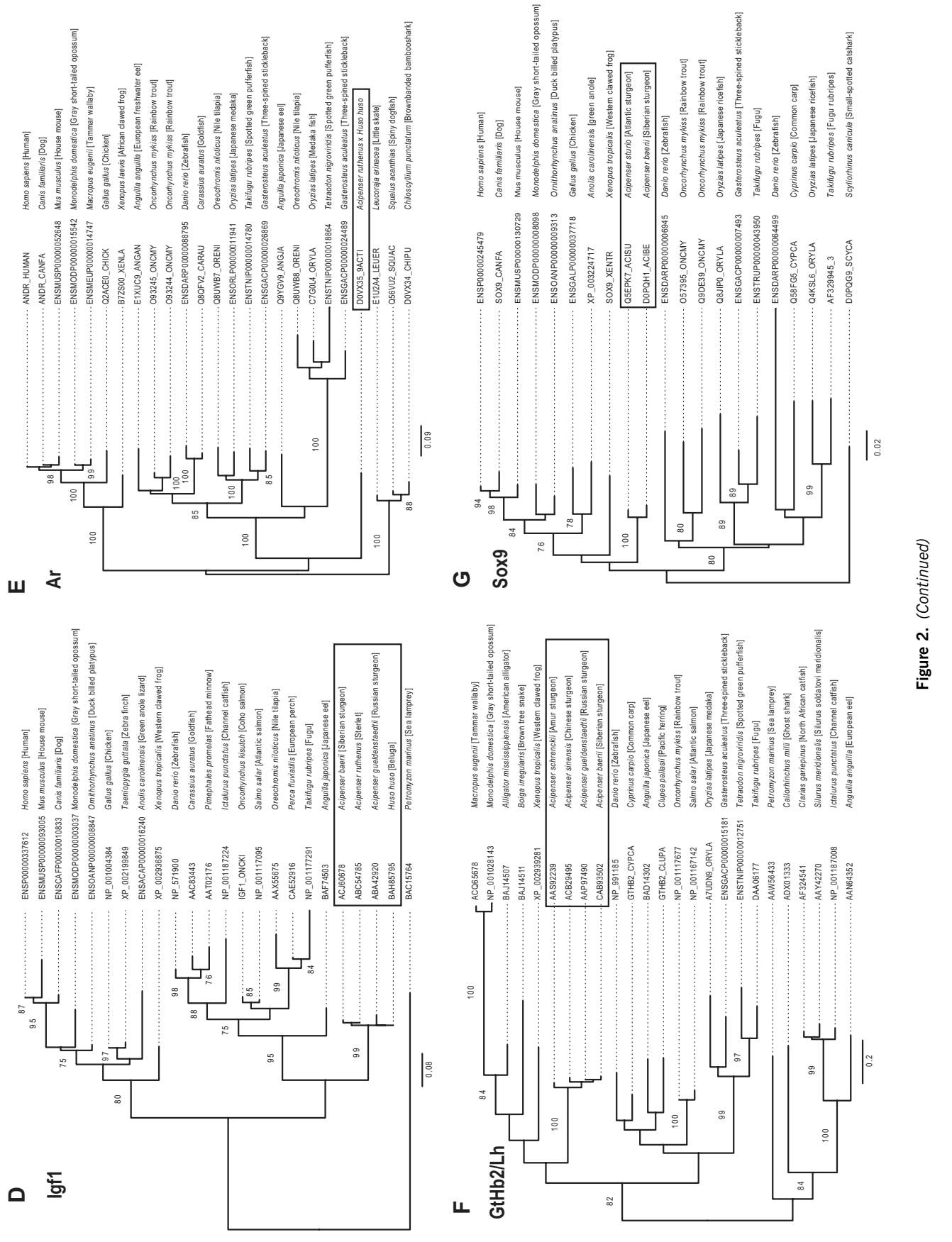

Figure 2. *(Continued)*

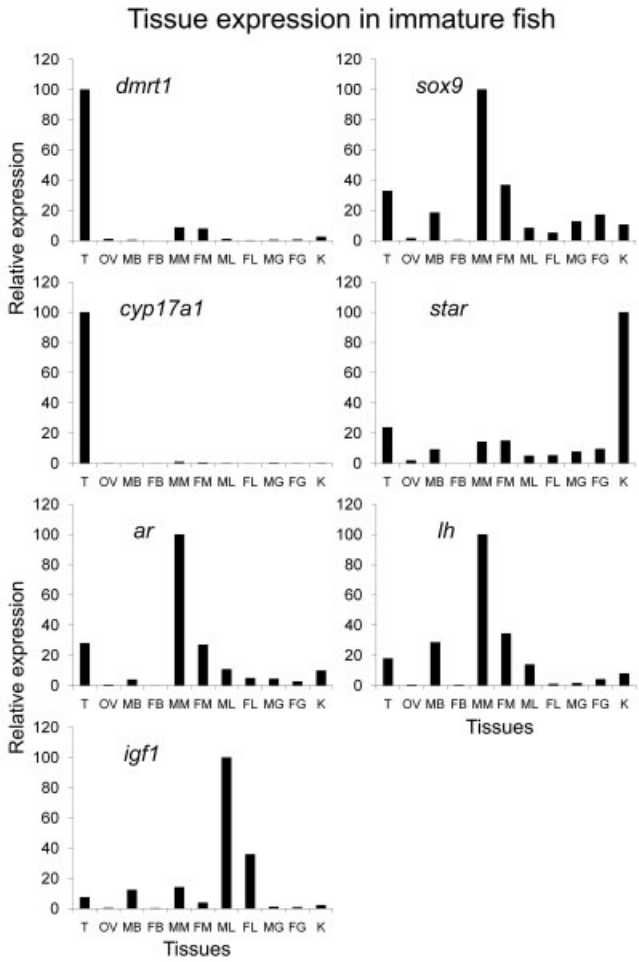

Figure 3. Tissue expression of *dmrt1*, *sox9*, *cyp17a1*, *star*, *ar*, *lh*, and *igf1* transcripts in immature Siberian sturgeons. qPCR was performed on testes (T), ovaries (OV), male brain (MB), female brain (FB), male muscle (MM), female muscle (FM), male liver (ML), female liver (FL), male gills (MG), female gills (FG), and kidney (K). Relative quantification was carried out by normalisation of the values relative to those of the housekeeping gene β-actin.

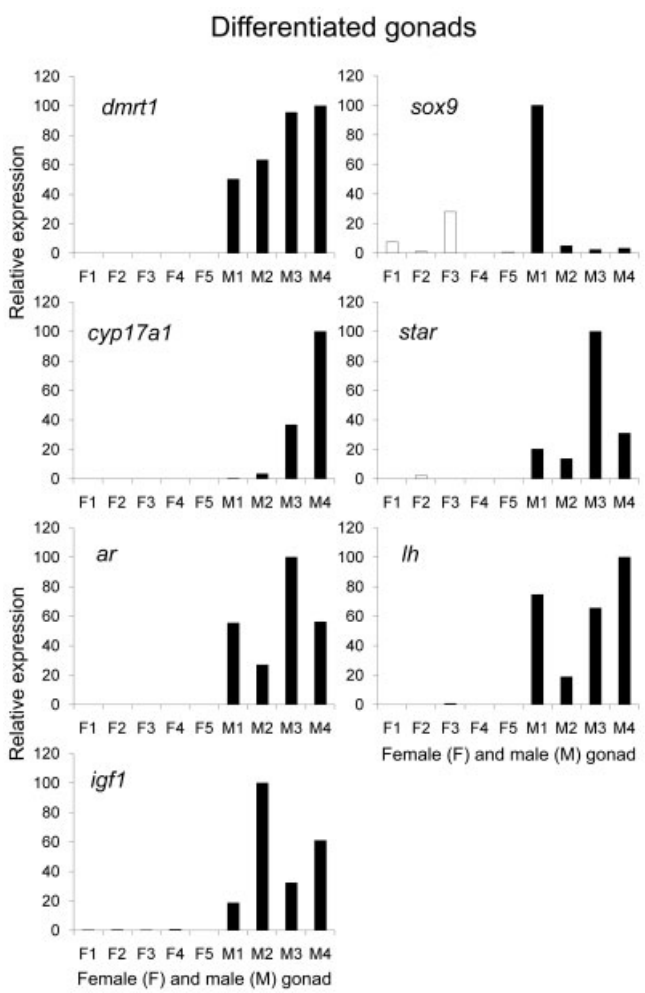

Figure 4. Expression levels of *dmrt1*, *sox9*, *cyp17a1*, *star*, *ar*, *lh*, and *igf1* transcripts was analysed through qPCR on gonad samples from already-differentiated female (F1 to F6) and already-differentiated male (M1 to M4), 16-month-old fish. Relative quantification was carried out by normalisation of the values relative to those of the housekeeping gene β-actin. The expression level was significantly higher in male than in female gonads for *dmrt1* ($P < 0.001$), *cyp17a1* ($P < 0.001$), *star* ($P < 0.001$), *ar* ($P < 0.001$), *lh* ($P < 0.001$), and *igf1* ($P < 0.0001$). We found no difference in *sox9* expression between male and female gonads.

($P < 0.001$); in contrast, *sox9* was not dimorphic (Fig. 4). The Leydig cell markers assayed, *cyp17a1* and *star*, showed a clear sexual dimorphism, being higher in males than in females at this stage ($P < 0.001$) (Fig. 4). *ar* was also up-regulated in males ($P < 0.001$); the same trend was observed for *lh* ($P < 0.001$) and *igf1* ($P < 0.0001$), although these two genes were poorly expressed at this stage.

Gene Expression in Gonads of Undifferentiated Fish

Interestingly, within the group of undifferentiated, 16-month-old fish, individual number 4 (see Fig. 5) over-expressed *dmrt1*, *sox9*, *cyp17a1*, *star*, *ar*, and *lh* in its gonads compared to a much lower expression of these genes in other fish from the same group (Fig. 5, fish number 1, 2, 3, 5, 6 and 7). *igf1* did not share the same behaviour as the other dimorphic genes at 16 months (Fig. 5). In a second group of undifferentiated fish at 18 months, two individuals showed an over-expression of *dmrt1*, *sox9*, *cyp17a1* and *ar* (Fig. 6, fish number 1 and 4), while the others had low expression levels of these genes (Fig. 6, animals 2, 3 and 5). The other genes that were tested (*star*, *lh* and *igf1*) did not show the same expression pattern.

DISCUSSION

This is the first characterisation of the molecular changes that occur in sturgeons during the gonad gender

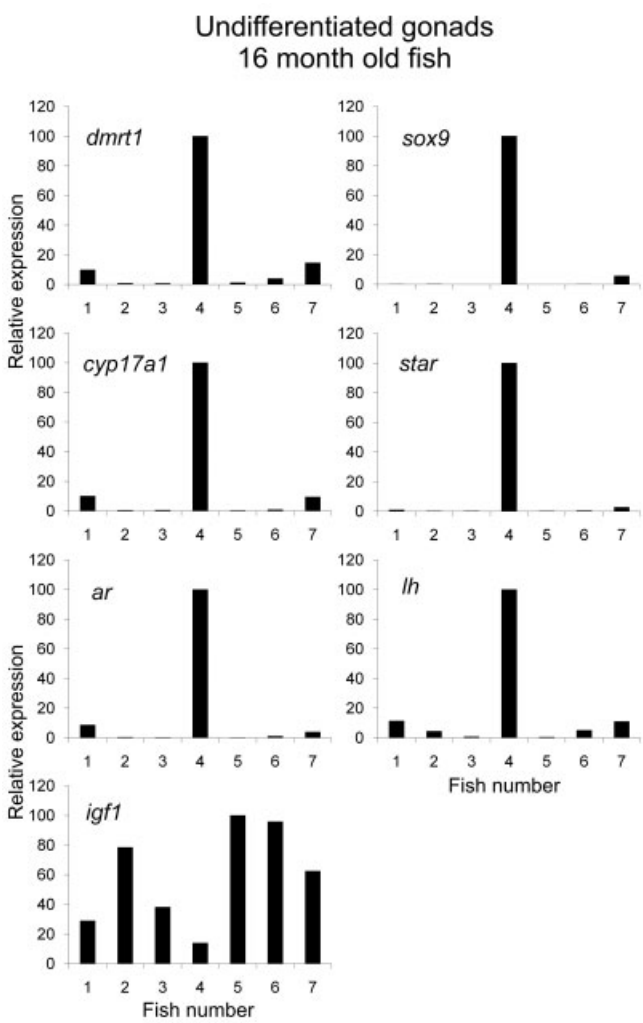

Figure 5. Expression level of *dmrt1*, *sox9*, *cyp17a1*, *star*, *ar*, *lh*, and *igf1* transcripts was analysed through qPCR on gonad samples from undifferentiated, 16-month-old fish. Each number (1–7) corresponds to the gonad of an individual fish. Relative quantification was carried out by normalisation of the values relative to those of the housekeeping gene β-actin.

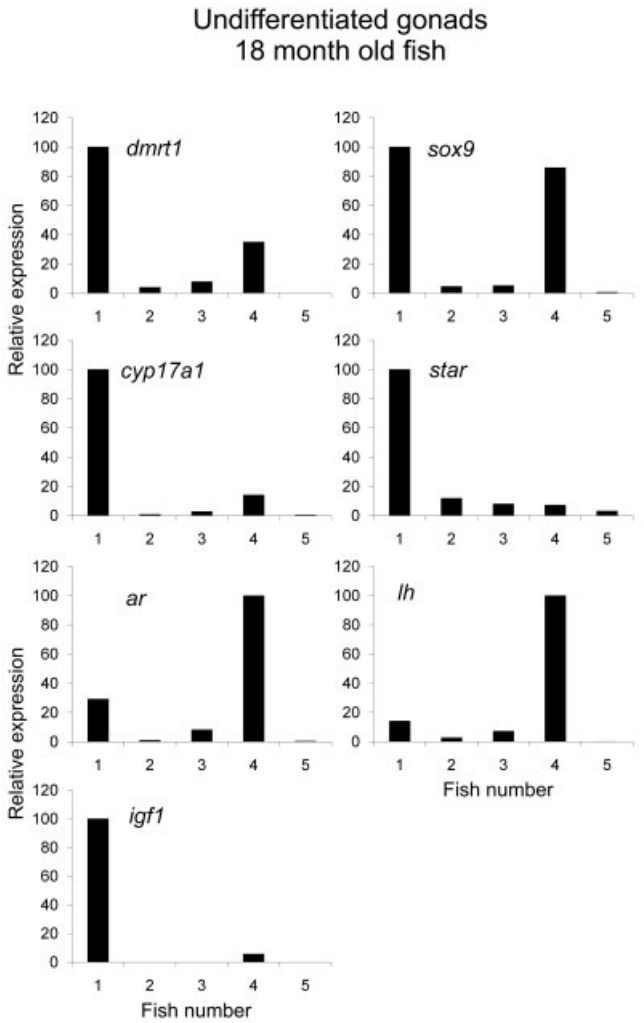

Figure 6. Expression level of *dmrt1*, *sox9*, *cyp17a1*, *star*, *ar*, *lh*, and *igf1* transcripts was analysed through qPCR on gonad samples of undifferentiated, 18-month-old fish. Each number (1–5) corresponds to the gonad of an individual fish. Relative quantification was carried out by normalisation of the values relative to those of the housekeeping gene β-actin.

differentiation. We observed a clear sexual dimorphism in the genes selected (*dmrt1*, *sox9*, *cyp17a1*, *star*, *ar*, *igf1* and *lh*), with their over-expression in testes when compared to ovaries of immature fish (3 years old). The dimorphism was also present in the juvenile fish with differentiated gonads, except for *sox9*. Prior to the morphological sex differentiation, the pattern of expression observed in Sertoli cell factors (*dmrt1*, *sox9*), androgen production and receptivity factors (*cyp17a1*, *star*, *ar*), and *lh* suggests that these individuals were probably undergoing male differentiation. The lack of genetic all-male and all-female populations or sex genetic markers prevents a definitive conclusion about the involvement of the factors studied during gonad differentiation. Even though the trends observed suggest they take part in the process of testes differentiation, more studies must be carried out in earlier stages of gonad development to validate the observed trends.

Among the studied genes, transcription factor *dmrt1* is the only one conserved in invertebrates and vertebrates as a major factor involved in male fate (Volff et al., 2003; Haag and Doty, 2005). Dmrt1 belong to the *dmrt*/*mab3* multigenic family, and has several paralogs (Hong et al., 2007). At the functional level, it has been shown that *Dmrt1* is essential for maintaining mammalian testes determination and gonad sex after foetal choice between male and female in mouse (Matson et al., 2011). The deletion of *Dmrt1* in Sertoli cells induces feminisation, and reprograms differentiated Sertoli cells into granulose-type cells in mouse (Matson et al.,

2011). *Dmrt1* has also been proposed as the gene required for male development in birds, as two doses of Z-linked *dmrt1* in ZZ males are needed for gonad masculinisation (Smith et al., 2009). The knockdown of *Dmrt1* using RNA interference resulted in partial sex reversal of males and gonad feminisation (Smith et al., 2009). In amphibians, a *dmrt1* gene linked to the W chromosome is crucial for primary ovary formation in *Xenopus laevis*, which uses a ZZ–ZW sex determination system (Yoshimoto et al., 2008). The results observed in birds and *X. laevis* suggest that there are two alternative forms of sex determination in ZZ/ZW animals: the dosage of a Z-linked gene or the expression of a female sex-determining gene in the W chromosome.

In teleost fish, *dmrt1* appears as an important factor related to testis differentiation (Piferrer and Guiguen, 2008). This assumption is based on its pattern of expression during the very early gonad development in teleosts (Vizziano et al., 2007; Ijiri et al., 2008) and on its behaviour when masculinising and feminising treatments are used. *dmrt1* is up-regulated during the early phases of gonad transdifferentiation induced by masculinising treatments (Raghuveer et al., 2005; Vizziano et al., 2008), and down-regulated during the feminisation induced by estrogens (Raghuveer et al., 2005; Vizziano et al., 2008). In primitive fish such as sturgeons, *dmrt1* has been described in two species (*Acipenser transmontanus* and *A. baerii*, present work) and roots distinctively and unambiguously with respect to all the *Dmrt1* genes found in other chordate species. *dmrt1* in sturgeons harbours a very short branch when compared to tetrapods or teleost fish, suggesting that this gene retains its original function. The sexual dimorphism of *dmrt1* during early and advanced stages of gonad development in the Siberian sturgeon agrees with trends observed in teleosts (Marchand et al., 2000; Piferrer and Guiguen, 2008) and other sturgeons (Hale et al., 2010), and sustains the idea that it is an essential factor needed to maintain the gonad sex, as was described recently in mammals (Matson et al., 2011). It must be reported that *dmrt1* seems not to be sex dimorphic in all the sturgeon species studied (Amberg et al., 2010). In fish with XX/XY sex determination, *dmrt1* is expressed in both male and female gonads. It seems that for this kind of sex determinism, *dmrt1* is not a good candidate to be the master gene involved in testis determination. Yet, a double dosage of *Dmrt1* in ZZ males could be critical for testis determination in organisms with ZZ/ZW, such as birds and more recently, the Siberian sturgeon (Fopp-Bayat, 2010). Whether the double dosage of *dmrt1* in male ZZ Siberian sturgeons is critical for testis determination remains to be elucidated. The phylogenetic analysis together with the expression pattern observed for *dmrt1* suggests that the gene already acquired its function as a critical regulator of testis differentiation in this primitive fish.

Sox9 was another Sertolian factor selected as a possible candidate involved in testis differentiation in Siberian sturgeons. The *Sox9* genes belong to the *SoxE* subdivision among another large, multigenic family (Schepers et al., 2002). Within the *SoxE* group, *Sox8* and *Sox9* are involved in male sex determination in mammals (Bowles et al., 2000; Chaboissier et al., 2004; Koopman, 2005). At a functional level, *Sox9* has a crucial role in Sertoli-cell differentiation; it is one of the earliest genes to be up-regulated in pre-Sertoli cells following the expression of *Sry*, and it has a key role in sex differentiation in mammals (Bowles and Koopman, 2001; Brennan and Capel, 2004). Yet, *Sox9* is dispensable for testis differentiation after sex determination (Barrionuevo et al., 2009), leaving its involvement in fish testis differentiation unclear. In a previous work on sturgeons, no evidence was observed for *sox9* duplication (Hett et al., 2005), as is the case in teleost fish (Cresko et al., 2003; Zhou et al., 2003), and no sex-specific differences were observed. The phylogenetic analyses of *Sox9* evolution revealed a basal position for sturgeon *sox9* (*Acipenser sturio*, Hett et al., 2005, *A. baerii* present work). In medaka and pejerrey (*Odontestes bonariensis*), *sox9* has been proposed as being involved in the formation of testis structures, but not in testis differentiation (Nakamoto et al., 2005; Blasco et al., 2010). In trout, *sox9* paralogs (*sox9a1*, *sox9a2*) showed an incomplete sexually dimorphic expression (Vizziano et al., 2007), suggesting that they are not essential for testis differentiation. The pattern of *sox9* expression reported in the present work suggests a potential involvement of *sox9* in late testis differentiation in the Siberian sturgeon. Its role in early events of gonad differentiation remains to be studied in undifferentiated gonads of younger fish (i.e. 3- to 6-months old).

cyp17a1, *star*, and *ar* are related to steroid production and receptivity. *cyp17a1* encodes for the 17-hydroxylase that converts progestins into androgens; *star* encodes for a protein that controls steroid synthesis, and *ar* encodes for androgen receptors. Androgens are the main mediators of vertebrate masculinisation after the testes are differentiated (Borg, 1994; Brennan and Capel, 2004). In teleost fish, however, it is controversial whether androgens participate in the gender differentiation of testes or start acting only after the onset of spermatogenesis. In this group, the more potent androgens are those that have an oxygen at carbon eleven (11-oxygenated androgens) (Borg, 1994), and the presence of these steroids is usually related to masculine development. Yet, females of several teleost fish species also produce high levels of 11-oxygenated androgens (Lokman et al., 2002), as does the basal actinopterygian Siberian sturgeon (Cuisset et al., 1995; Williot, 2011). Thus, the enzyme involved in 11-oxygenation (11β-hydroxylase) seems not to be a good choice to study the masculine pathway, at least in the Siberian sturgeon. In fish in which the production of 11-oxygenated androgen synthesis was observed only in male gonads, there is some evidence that androgens precede testis differentiation. This is the case of the pejerrey, *O. bonariensis*, in which a production of 11-oxygenated androgens was observed during the early phases of gonad development prior to testis differentiation (Hattori et al., 2009; Blasco et al., 2012). Moreover, an early over expression of the gene encoding for the 11β-hydroxylase (*cyp11b*, cytochrome P450, family 11, subfamily b, polypeptide) was observed in all male genetic trout populations 20 days before testis differentiation (Vizziano et al.,

2007). In contrast, there was no expression at the gene and protein levels for steroid synthesis enzymes before testis differentiation in tilapias (Ijiri et al., 2008). This shows that androgens are not universally involved in testis differentiation in fish.

The androgen content (testosterone and 11-ketotestosterone) in young sturgeons discriminates between genders fairly well as soon as differentiation has occurred—that is, stage II, as is the case for *A. transmontanus* (Feist et al., 2004) and *Huso huso* (Mola et al., 2011), but not *Acipenser gueldenstaedtii*, which did not exhibit any sex-related differences (Barannikova et al., 2000). This occurs at a similar stage as the stage in Siberian sturgeon development, during which we observed an over-expression of *star* and *cyp17a1*, indicating that, in recently differentiated sturgeons, androgens are produced and detectable. The expression of *star* and *cyp17a1* in undifferentiated fish opens the question about the role of androgens prior to gonad differentiation in Siberian sturgeons. Besides the potential to produce steroids, we found that the expression of the *ar* gene was clearly dimorphic in recently differentiated and immature testes when compared to ovaries in the Siberian sturgeon. The over-expression of *ar* in the same undifferentiated fish that show high expression of *dmrt1* and *sox9* suggests there is a higher sensitivity for androgens in male gonads before their morphological differentiation. Studies focused on early developmental stages in order to identify steroid synthesis as well as the androgen effect on gonad development will help understand if androgens are involved in testis differentiation in sturgeons. The phylogenetic analysis of *cyp17a1*, *star*, and *ar* indicates a probable conservation of gene function.

Another interesting feature we found was the male over-expression of *igf1* in recently differentiated Siberian sturgeons. The expression of *igf1* has been shown to occur in germ and somatic cells of rainbow trout, tilapia and seabass (Le Gac et al., 1996; Reinecke et al., 1997; Berishvili et al., 2006; Viñas and Piferrer, 2008), suggesting an autocrine or paracrine function. *igf1* is also expressed in somatic cells when gonads are still undifferentiated, suggesting a crucial importance to further gonad development and differentiation (Reinecke, 2010). A study using knockdown of the type-1 Igf receptor by morpholino oligonucleotides in zebrafish embryos demonstrated a high impact on gonad development, leading to mismigration and apoptosis of primordial germ cells (Schlueter et al., 2007). The pattern of *igf1* expression in undifferentiated gonads of Siberian sturgeons was not similar to the pattern observed for the other male markers (*dmrt1*, *sox9*, *ar*, *lh*, *cyp17a1*). Moreover, the trends observed prior to gonad differentiation in the Siberian sturgeon did not suggest any dimorphic expression for *igf1*. This factor probably controls the first steps of testis development after differentiation, as it has been shown for some teleost species. The phylogenetics showed that *Igf1* was found in all chordate species, and suggests that the gene's function should not have changed.

lh has been found to be expressed in trout and is over-expressed specifically in ovaries during the first oocyte meiosis (Baron et al., 2005). Such a result was interpreted as a possible anti-apoptotic factor at that time. In the Siberian sturgeon, *lh* expression was higher in testes than in ovaries, and the pattern observed in undifferentiated fish suggests that it could be a masculine marker.

In conclusion, Sertoli cell transcription factors (*dmrt1* and *sox9*), Leydig cell functional markers as (*cyp17a1*, *star*), as well as *ar*, *igf1*, and *lh*, seem to have retained an ancestral function in sexual development, specifically testis differentiation. Some of these genes seem to be involved in the process of testis differentiation (*dmrt1*, *ar*, *lh*, *cyp17a1*, *star*, *sox9*) while others are active at initial stages of gametogenesis (*igf1*). Along these lines, individual Siberian sturgeons over-expressing *dmrt1*, *sox9*, *cyp17*, *star*, *ar*, and *lh* are potential males in advanced molecular differentiation.

MATERIALS AND METHODS

Animals and Sampling

Research involving animal experimentation conformed to the principles for the use and care of laboratory animals, and was in compliance with Uruguayan regulations on animal welfare (Comisión Honoraria de Experimentación Animal).

Siberian sturgeon juveniles (*A. baerii*) of different ages were obtained from a fish farm, (Esturiones del Río Negro), where they were reared at environment temperature. Fish were transported in aerated water tanks of 1 m^3. The acclimatisation time depended on the difference between the temperature of the water in the tank and the water in the experimental facilities, taking 1 hr for each degree of difference. Fish were maintained for 2 months in the Instituto de Investigaciones Pesqueras (Facultad de Veterinaria, Universidad de la República Oriental del Uruguay, Uruguay) at environment temperature before sampling. They were fed ad libitum with 38% protein, following the diet developed by the enterprise Esturiones del Río Negro, with a feeding rate of 3% of their biomass. Different batches were used. A first group of 16-month-old fish measured 37 ± 5 cm and weighed 110 ± 46 g. A second group of 16-month-old fish measured 70 ± 7 cm in length and weighed $1{,}228 \pm 165$ g. A third group of 18-month-old specimens measured 40 ± 2 cm and weighed 150 ± 37 g. Each group of fish was maintained in the same tank. The late differentiation of the gonads at 16 and 18 months with respect to previous reports (Williot, 2011) can be explained by the slow growth observed in the fish used. This difference did not affect the histological identification of the developmental stage of each specimen selected to compare undifferentiated to differentiated fish.

Three-year-old fish showed a well differentiated but immature gonad. For molecular analysis and cloning, the testes and ovaries of 3-year-old fish were sampled. To validate the pattern of expression of genes studied, gonads, brain, muscle, liver and gills were collected.

For the comparison between already differentiated and undifferentiated fish, the gonads of all the fish in the different groups were evaluated at a microscopic level. One gonad was fixed in Bouin for histology and the contralateral one

was frozen in liquid nitrogen and kept at −80°C for RNA extraction. Among the fish, we found individuals with undifferentiated gonads and other with gonads that were already differentiated into testes or ovaries. For qPCR, we studied seven undifferentiated gonads from 16-month-old fish, and five undifferentiated gonads from 18-month-old fish. In addition, five ovaries and four testes of 16-month-old fish were studied.

RNA Extraction and Reverse Transcription

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to manufacturer instructions, and quality was assessed by gel electrophoresis. cDNA synthesis was carried out on 3 μg of total RNA. RNA was denatured in the presence of random hexamers (0.5 μg) for 5 min at 70°C, and then chilled on ice. Reverse transcription (RT) was performed at 37°C for 1 hr using M-MLV reverse transcriptase (Promega, Madison, WI) as described by the manufacturer.

cDNA Characterisation and Real-Time PCR

No sequences of genes relevant to *A. baerii* gonadal development are available in public databases. Hence, there is a need to start characterising those genes that are expressed during early gonad development. Phylogenetic position determines significant genetic divergence compared to other fish, and makes the selection of primers with homology quite difficult (Berbejillo et al., 2010). The primers we used were designed based on highly conserved regions from fish and tetrapod orthologues, inferred from sequence alignments using the ClustalW multiple sequence alignment algorithms provided in the BioEdit software (Hall, 1999). The gene list and primer selection is shown in Table 1.

The partial cDNA sequences encoding for *dmrt1* (HQ110106), *ar* (HQ110107), and *cyp17a1* (HQ026486) were characterised and sent to GenBank. Primers for qPCR were designed based on the gene sequences characterised here (*dmrt1*, *cyp17a1*) and on sequences available in Gen Bank (Table 1).

Real-time RT-PCR was carried out using an Applied Biosystems instrument (ABI 7500). Reactions were performed in 20-μl volumes with 300 nM of each primer, 5 μl of a 1:10 dilution of the RT reaction, and the Kapa Sybr qPCR master mix, according to the manufacturer's instructions. The incubation steps used were: Holding stage (95°C for 5 min), Cycling stage (95°C for 15 sec, 60°C 1 min), and Melt Curve Stage (95°C 15 sec, 60°C 1 min, 95°C 30 sec, 60°C, 15 sec) followed by 40 PCR cycles.

The validity of the qPCR was confirmed through the analysis of the melting curves, checking amplified fragments in an agarose gel, and through sequencing. The Systemizing Quotient (SQ) values were calculated as described in Baron et al. (2005). The housekeeping gene used for data normalisation was β-actin. The relative expression was calculated as a percentage of the highest expression level recorded for each gene.

TABLE 1. Nucleotide Sequence of Real-Time PCR Primers and GenBank Accession Number for All Target Genes

Gene	Symbol gene name	GenBank accession number	Forward sequence	Reverse sequence	Size (bp)
androgen receptor	*ar*	DQ388357.1	TGAAGAAGATGAAGGGAGCAGAAGAT	TCTCCCCCAGTTCATTCAAGC	227
luteinising hormone	*lh*	AJ251656.1	CTGCAGAGAAGGAGGAATGT	GCGAAGATCCTTATAGGTGCA	152
cytochrome P450, family 17, subfamily a, polypeptide 1	*cyp17a1*	HQ026486, ADM47436.1	TCACACACTCCAGTATTGGTG	CCATTCCTTTTCATCTGATG	141
doublesex and mab-3-related transcription factor 1	*dmrt1*	HQ110106, ADW80327	GGCCCAGGTAGCACTGAGGA	GTTGTGGCTGGACAAACGGC	390 kb
insulin-like growth factor I	*igf1*	FJ428828.1	AGCTGAGCTTGTGGACAC	AAGCAGCACTCATTCACGAT	126 kb
sry-box containing gene 9	*sox9*	EU241882.1	AGCAGCAAAAACAAGCCT CA	AGCTCCGCGTTGTGAAGAT	113 kb
steroidogenic acute regulatory protein	*star*	FJ205610.1	CAGAAGTCAATCAGCATCCT	TCAGCACCTTGTCTCCATTG	67 kb
β-actin	*β-actin*	FJ205611.1	TATCCTGACCCTGAAGTACCC	CTCATCGTACTCCTGCTTGCT	

Gene symbols and gene names are depicted according to the gene zebrafish nomenclature (www.zfin.org).

Histology

For histological analyses, the gonads were fixed for 24 hr in Bouin's fixative, and stored in 70% ethanol. They were then dehydrated, embedded in paraffin, cut to 5-μm thick sections, and stained with Regaud hematoxiline, orange G, and aniline blue or Mayer haematoxylin and eosin (Gabe, 1968).

Database Search and Alignments

Homologous genes of interest were searched for using BlastP in the Ensembl database (www.ensembl.org/), UniProt (www.uniprot.org/), and the non-redundant (nr) database at NCBI (www.ncbi.nlm.nih.gov/). Retrieved homologous sequences were screened semi-automatically to remove redundant sequences and to select representative species in groups of interest. Too-short or poorly annotated sequences were discarded manually from protein sequence alignments generated with Muscle v3.8.31 (Edgar, 2004) (www.drive5.com/muscle). Total protein sequence alignments as well as large phylogenies are available upon request. Phylogenetic analyses were first generated using BIONJ (Gascuel, 1997), implemented in Seaview Version 4.2.12 (Gouy et al., 2010) (pbil.univ-lyon1.fr/software/seaview.html). Simplified trees were generated with a selection of well-annotated protein sequences of representative species from key taxonomic groups. In this sense, *A. baerii* sequences did not always meet these criteria, so we used an orthologous sequence for another sturgeon species instead. Maximum Likelihood phylogenetic trees were generated using PhyML (www.atgc-montpellier.fr/phyml/) (Guindon et al., 2010) using JTT as a substitution model and with robustness calculated by 1,000 bootstrap replicates.

Statistical Analysis

We carried out the comparative expression analysis of each gene for males and females. Both groups of data (male and female) were compared to find that the variances for most genes (*dmrt1*, *ar*, *lh*, *cyp17*, *igf1*) were not equal, and thus the results were analysed using the Mann–Whitney test. For *star*, the variances were equal and the results were analysed using the Student's *t*-test.

ACKNOWLEDGMENTS

We thank Dr. Walter Norbis (Facultad de Ciencias, Universidad de la República Oriental del Uruguay, Uruguay) for his help in statistics; Dr. Daniel Carnevia, Dr. Alejandro Perreta and Dr. Maite Letamendía (Instituto de Investigaciones Pesqueras, Facultad de Veterinaria, Universidad de la República Oriental del Uruguay, Uruguay) for their help in the maintenance and sampling of fish, and Lic. Andrés Alberro for technical assistance. We also thank 'Esturiones del Río Negro' (Uruguay) for kindly providing the biological samples used in this work.

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