

## Research Article

### Protein Profiling of Gonads of Males and Sex Reversed Males in *Nemacheilus angorae*

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**Abstract:** In the present study a proteomics approach has been taken to analyze differential protein expression between mature male and sex reversed male of *Nemacheilus angorae* gonads. In regard to the fruitful studies of sex reversal in mammalian species and the fact that some major sex determination molecules are conserved among vertebrates, *Nemacheilus angorae* (Angorae loach) seems to be a good model system in studying molecules involved in sex differentiation. *N. angorae* is a teleost fish exhibiting a spontaneous sex reversal (male to female) pattern. The gonads of adult individuals were dissected and used for histological investigation and protein analysis. Proteins were next analyzed using two-dimensional gel electrophoresis and the distinguished spots have been compared in two experimental samples. Among them, 23 differentially expressed proteins spots were identified by MALDI-TOF/TOF analysis. Two spots in sex reversed testis with high score showed significant similarity to Vasa (assembling of the pole plasm and the pronuclear region of the oocyte) and Proline 4-hydroxylase proteins. Vasa are involved in germ cell development both in invertebrates and vertebrates. This data could be considered as starting base for subsequent studies to identify proteins involved in sex reversal and differentiation at different stages of gonadal maturation in fish.

**Keywords:** *Nemacheilus angorae*, protein profile, sex reversal

## INTRODUCTION

Fishes are an attractive group of organisms to study sex determination from the evolutionary point of view, because members of this class exemplify a complete range of various types of sex differentiation from hermaphroditism to gonochorism and from environmental to genetic sex determination (Baroiller, 1999; Devlin and Nagahama, 2002). Reproduction in teleost fish is a complex process that begins with the formation of the primordial, sexually undifferentiated gonads early during ontogenesis and continues through anatomical and cytological sexual differentiation, puberty and finally sexual maturation and spawning. Reproductive modes in fish are varied and include processes such as gonochorism (ovaries and testes occurring in separate individuals), hermaphroditism, and parthenogenesis (Nakamura *et al.*, 1998). Fishes have both major gonosomal type of sex determination. For example, *Tilapia mossambicus* and *Tilapia niloticus* have homogametic (XX) females and heterogametic (XY) males, whereas *Tilapia hornorum* and *Tilapia aureus* have heterogametic (ZW) females and homogametic (ZZ) males (Chen, 1969).

Among gonochoristic species, the primary sex determination of the gonads can be genetically predetermined by Mendelian inheritance (Genotypic Sex Determination, GSD) or be directed by environmental factors (Environmental Sex Determination, ESD). Environmental cues influencing sex determination include variety of factors such as social interactions, pollution, pH, and temperature, among others (Francis, 1992).

Plasticity in sex determination is also reported in some gonochoristic fish species (Francis, 1992; Crews, 1996). Temperature-Dependent Sex Determination (TSD) has been shown to be operative in most teleosts. It has been shown that cold temperature usually produces female biased sex in some fishes such as goldfish and tilapias (Baroiller and Dcotta, 2001). Temperature, pH, steroid hormones and social cues are some exogenous factors that influence sex ratios in fish. Steroid hormones are the natural inducers of gonadal sex differentiation in fishes. Indeed, the genetically prescribed sex can easily be overridden with exogenous steroids if they are applied at the appropriate time and dosage during early development (Hunter and Donaldson, 1983). In general, estrogens induce feminization and androgens induce masculinization.

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During the critical period of differentiation, treatments with exogenous sex hormones often induce sex reversal (Yamamoto, 1969).

Although androgens seem to initiate sex reversal in some species, results are less convincing in others (Shapiro, 1988). Exogenous androgens also have been used extensively in aquaculture to induce sex reversal and increase growth in gonochoristic species such as tilapia and catfish (MacIntosh *et al.*, 1985; Gannam and Lovell, 1991). The effect of temperature on sex determination in southern flounder was shown. High and low temperature induced phenotypic sex reversal in juvenile southern flounder, producing a higher proportion of males. Raising southern flounder at the midrange temperature held sex ratios close to 1:1 (Luckenbach *et al.*, 2003).

Recently, a first candidate gene for the male sex-determining in a fish species was cloned in the medaka *Oryzias latipes*. The male sex-determining candidate gene of medaka is a duplicated version of the autosomally located *dmrt1a* gene. It is designated *dmrt1bY* (Matsuda *et al.*, 2002).

The members of the Sox gene family have been highly conserved across evolution and have been found in a wide variety of species such as human, mice, marsupials, birds, turtles, *Xenopus*, alligator, lizards, *Drosophila* (Rui *et al.*, 2003) and fishes (Fukada *et al.*, 1995; Ito *et al.*, 1995). In teleost fish, rainbow trout *sox9* is also expressed in the testis. Two *sox9* genes, *sox9a* and *sox9b*, were isolated in zebrafish and they are expressed in the chondrogenic tissues. In the zebrafish gonad, *sox9a* is expressed in the testis while *sox9b* is expressed in the ovary (Chiang *et al.*, 2001). These results suggested that the chondrogenic expression of Sox9 is conserved among vertebrates, whereas the expression of Sox9 in the gonad seems to be somewhat diversified in fish species (Chiang *et al.*, 2001).

*Nemacheilus angorae* a teleosts fish first identified by Steindachner in 1897, shows differentiated gonochorism strategy and demonstrating kind of sex reversal pattern which seems to be under social control. *N. angorae* is a cyprinid fish distributed in some freshwaters of Middle East countries including Iran and seems to be a good model system in studying molecules involved in sexual differentiation. The reproductive season in *N. angorae* spans through May to July during which the fish shows sex ratio of 1:1. The sex ratio fluctuates up to 3:1 during winter. To keep the sex ratio balance in population some of the male individuals shift to females. The diversion of sex is demonstrated by appearance of the ovules in sex-reversed male testes. The frequency of sex reversed males increased in winter. Moreover, the numbers of ovules during winter are more frequent than the number seen in other seasons. To investigate the molecular components of sex reversal in *N. angorae*, the protein profile of gonads

of male and sex reversed male individuals were compared during winter and showed apparent differences among these two individuals which are analyzed by mass spectrometry.

## MATERIALS AND METHODS

**Fish sampling:** The *Nemacheilus angorae* samples were hunted from Jajrood river located in 34 Km north of Tehran (Iran). Fishing was performed with screen placed diagonally in the river-bed. The fishes were arrived at screen with river flow. Fish samples were collected over three time intervals from May 2005 to February 2006. Since ovules in gonadal tissues of sex reversed male fishes are more abundant after their reproduction, December samples were chosen for 2DE analysis.

**Gonad histology:** The gonads of male fishes were dissected out and divided into two groups. One half a gonad of a fish was considered for histological technique and the other half for protein analysis (samples were kept in -70°C). A part of the testes which was reserved for microscopic studies, processed by conventional microtome and histological procedures. Half of the testis were cut and preserved in Bouin's fixative and subsequently were processed histologically to observe the process of sex change throughout the gonadal lobe. Transverse sections (5-7 2 µm) were stained with Harris haematoxylin and counterstained with eosin. The slides of cross-sectioned gonads investigated with light microscopy to show de novo appearance of ovules in sex reversed males' gonads.

**Protein extracts preparation:** The procedure for protein extraction was based on Damerval *et al.* (1986) and Kamo *et al.* (1995), with some modifications. Samples were ground in liquid nitrogen and suspended in 10% w/v TCA in acetone at -20°C for 45 min., followed by centrifugation for 15 min at 14000 g. The pellets were resuspended in acetone and Phenyl Methyl Sulfonyl Fluoride (PMSF) and then incubated at -20°C for 45 min., followed by centrifugation again at 4°C for 15 min. After that, the pellets were solubilized in lysis buffer (6 M urea, 2 M thiourea; 15 mM 2 ME, 2% [w/v] NP-40, 0.5% ampholin and pH [3-10]) and the protein concentration was determined by the Bradford assay, using BSA as the standard (Bradford, 1976).

**2D Electrophoresis (2-DE):** In order for first-dimensional Is Electric Focusing (IEF), total protein extract (60 µg) was loaded onto 18 cm IPG gel strips (pH 3 to 10 linear gradient and Bio-Rad, USA) during strip rehydration overnight. According to the manufacturer's protocol, samples were combined with rehydration buffer (8 M urea, 2% [w/v] CHAPS, 1%

[v/v] of 50 mM Dithiothreitol [DTT], 0.2% [v/v] of BioLyte Ampholyte and trace amounts of bromophenol blue) to reach to a final volume of 315  $\mu$ L.

IEF was then performed using a rapid voltage ramping method, up to 40 KVh at 20°C. Following IEF, strips were incubated for 20 min. at room temperature with continuous shaking in equilibration buffer (6 M urea, 20% [v/v] glycerol and 2% [w/v] SDS in 0.05 M Tris-HCl, pH 8.8 and a trace of bromophenol blue) containing 1% [w/v] DTT. Then, the strips were washed briefly in water and incubated for 20 min. in equilibration buffer with 2.5% [w/v] iodoacetamide. Separation of proteins in the second dimension by SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE) was achieved using 12% continuous gels. Electrophoresis was carried out at 30 mA for 30 min., followed by 55 mA for 5 h at 4°C. Silver staining protocol was used to visualize spots in analytic gels (Shevchenko *et al.*, 1996). Silver-stained gels were stored in a solution of 1% acetic acid at 4°C until analyzed.

**Gel image and data analysis:** All gels were scanned by scanner and analyzed with Melanie III and Image Master 2D Elite software followed by an additional visual analysis. Gels from male and sex reversed male gonads were analyzed and averaged separately and then compared with each other. The PI were determined using a linear 3-10 distribution, and MWs determinations were based on a Broad Range Protein Molecular Weight Marker (Promega), using a logarithmic curve. The spots visualized on the gels shown were marked with Adobe Photoshop over one representative pooled gel from each sample. Upon the completion of the procedure, the gels were compared to one another and the differentially expressed protein spots were visually checked in the individual pattern of each sample. In-gel digestion and MALDI TOF-TOF MS were performed by the Protein and Proteomics Centre in Singapore.

## RESULTS

**Sex change:** Macroscopic and histological examination of the *N. angorae* male gonads showed sex changes in some male samples. In functional males the process of sex reversal begins in July (after reproduction season), so a small number of ovules appear in the testis (Fig. 1). These cells are usually found embedded within the testicular tissue. As the process of sex reversal proceeds, the oocytes increase in number (December is the month with most number of oocytes) and it shows the completion of the transformation of a functional male into a transitional individual. During these changes, neither functional female nor complete degeneration of the male tissues were observed. Thus,

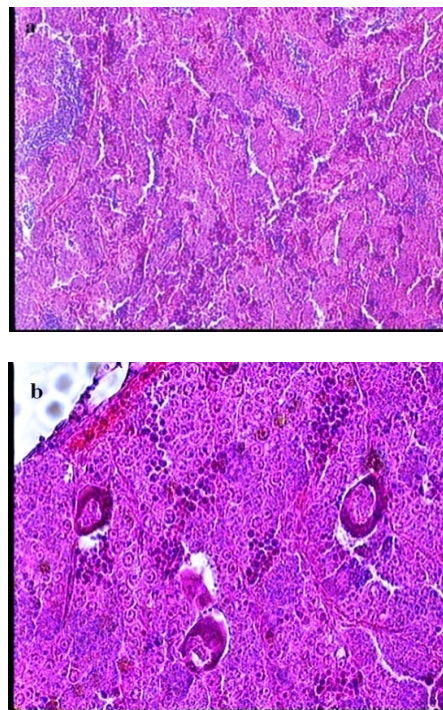


Fig. 1: Section of testis of *N. angorae*: (a) functional male, (b) Transitional individual or sex reversed male, Stained with haematoxylin and eosin

this sex reversal may only occur to keep the 1:1 sex ratio in the population (Fig. 1).

**Proteome comparison of reproductive systems in male and sex reversed male in *N. Angorae*:** In an attempt to provide a comparative analysis of the global proteome expression pattern of reproductive systems tissues from male and sex reversed male were isolated, 2-DE gels of respective protein samples of two gonads were prepared and resolved proteins were visualized by silver staining. Each sample was individually analyzed three times. The gels were analyzed and averaged, producing a unique protein profile (Fig. 2).

In order to identify protein patterns on the average gels of the testis, 23 unique spots were distinguished in testis and 10 proteins' spots of interests were cut out from the gel and identified by MALDI-TOF-TOF analysis. The spectrums obtained were compared with the databases found in Mascot Search software that has the NCBIInr, Swissprot, OWL, dbEST and MSDB protein databases Identification of differentially expressed proteins by MALDI TOF-TOF: Upon comparison of protein expression patterns of male and transitional male, a set of 10 spots with different expression patterns were excised. Identified proteins are listed in Table 1. Score above 78 for proteins indicate significant ( $p < 0.05$ ) difference. According to the table, spot numbered 8 was expressed only in males. Two of the remaining spots, numbered 4, 7 seem to be

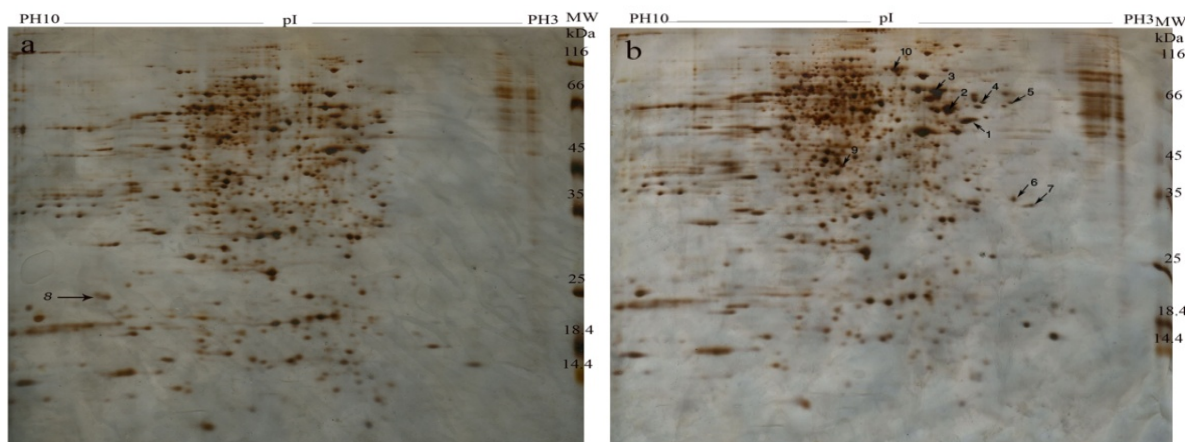


Fig. 2: Representative gels of protein spots expressed in gonad of: a, male; and b, transitional male of *Nemacheilus angorae*. silver stained 2-DE pattern of whole-cell protein extracts

Table 1: Protein identified by peptide mass spectrometry

Spot	Protein name	MASCOT score	Mr (Da) <sup>1</sup>	Seq Cov (%) <sup>2</sup>	PI <sup>3</sup>
1	proline4-hydroxylase	79	56762	7	4.71
2	General secretion path way protein D	62	79705	11	5.65
3	Cycloartenol synthase	61	87256	9	6.10
4	DNAK protein	50	68152	10	4.84
5	Transcarboxylase 12S subunit	53	66113	9	5.09
6	Vasa protein	123	50148	13	5.33
7	Histidinol dehydrogenase	53	45752	10	5.36
8	TnpC protein	54	22665	14	8.72
9	Unnamed protein product	57	55628	16	6.49
10	Ubiquity specific protease 37 Isoform 3	54	111185	6	5.79

1: Molecular mass; 2: Sequence coverage; 3: Isoelectric pH

expressed solely in sex reversed males. Others showed expression in gonads of males and sex reversed males. The most significantly expressed one among the latter is vasa protein (spot number 6).

## DISCUSSION AND CONCLUSION

Among 23 differentially expressed proteins in male and sex changed male of *Nemacheilus angorae*, appearing as distinguished spots in 2D gels, 10 spots were identified by mass spectrometry. The most significant ones with higher MASCOT score were procollagn-prolin, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase) and VASA protein (most similar to *Monopterus albus* protein). Seven proteins were identified with known function but no significant score. Moreover, one protein was found that has not been described yet. In light of our own finding and related studies concerning VASA protein, the importance and possible functions of this protein were discussed with focus on possible roles in reproduction and sex reversal.

Inferring from Fig. 2b, the sixth spot seems to be expressed in both male and sex reversed male gonads. Mass analysis indicated that this spot represents vasa protein similar to VASA protein found in *Monopterus albus* and *Carassius auratus gibelio*. There was not any

report concerning the expression or the function of vasa in *Nemacheilus angorae*. However, the potentially essential role of VASA protein in other teleost such as goldfish (*Carassius auratus gibelio*) (Chen *et al.*, 2005; Xu *et al.*, 2005), zebrafish (*Danio rerio*) (Xiang *et al.*, 2004), rice field eel (*Monopterus albus*) (Lu *et al.*, 2005; Ding *et al.*, 2007) and European sea bass (*Dicentrarchus labrax*) (Mercedes *et al.*, 2011) was investigated. The vasa gene was originally identified in *Drosophila* and has since been found in other invertebrates and vertebrates. The presence of these vasa homologs has revealed a highly conserved role for VASA Protein among different organisms (Erez, 2000). Work in a variety of animals suggests that a group of conserved molecular determinants function in this germ line maintenance and function. The most universal of these genes are vasa and vasa-like DEAD box RNA helicase genes (Gustafson and Wessel, 2010). The VASA protein can be detected in the germ line cells of *Drosophila* throughout their development; in early embryos it is specifically localized to polar granules, which are located where the germ cells are specified. *Drosophila* embryos that inherit mutant maternal vasa RNA and protein fail to form germ cells and females carrying null mutations in vasa display a range of defects in oogenesis (Liang *et al.*, 1994; Erez, 2000). Following the isolation of the *Drosophila* vasa gene, vasa-like DEAD-box RNA helicase genes that are

expressed in germ cells were identified in many species, including mouse, rat, frog, zebrafish, medaka (*Oryzias latipes*), trout, planarian, chick, ascidian, nematode, silkworm, human and the flour beetle (Erez, 2000). In continue, some studies concerning expression and function of vasa gene during gonadal development in some teleost will be reported.

Two isoforms of vasa mRNA and protein are present in a teleost fish, tilapia. Expression of two VASA isoforms is dependent upon germ cell differentiation and sex (Kobayashi *et al.*, 2002).

Vasa are highly expressed in germ cell lines and differentiated gonads. Joseph and Ian (2004) used vasa as a marker for germ cell migration and sex determination in the little skate. Use of vasa as a marker of normal germ cell migration and gonadal development may provide insight into the normal developmental processes and gonadal differentiation (Joseph and Ian, 2004).

Xu *et al.* (2005) cloned and characterized CagVasa, a Vasa homolog from the gibel carp, a fish that reproduces either bisexually or gynogenetically. In bisexually reproducing gibel carp, vasa are maternally expressed and its zygotic expression is restricted to gonads. By in situ hybridization on testicular sections, vasa expression seems to be low in spermatogonia, high in primary spermatocytes, reduced in secondary spermatocytes and disappears in spermatids and sperm. In contrast, vasa expression persists throughout oogenesis, displaying low-high-low levels from oogonia over vitellogenic oocytes to maturing oocytes (Xu *et al.*, 2005).

Using in situ hybridization with DIG antisense RNA probe, Chen *et al.* (2005) detected the expression and distributing of goldfish (*Carassius auratus*) DEAD-box family gene vasa during oogenesis and spermatogenesis. The results showed similarity to Xu *et al.* (2005) experiments, that is during oogenesis the positive signals of vasa RNA were detected in the cytoplasm of oocytes at all stages; however, vasa gene may play an important role only at the early stages of goldfish spermatogenesis (Chen *et al.*, 2005).

In zebrafish, differential expression of vasa mRNA in different stages of oogenesis suggest that vasa gene may play an important role during oogenesis (Xiang *et al.*, 2004). During sex reversal of the rice field eel (*Monopterus albus*), expression of vasa gene can also be observed in degenerated oocytes. Some vasa mRNA positive cells detected in the membrane of III, IV stage ovary and these may be the Primordial Germ Cells (PGC) which migrate to the membrane of the ovary and will differentiate to spermatogonia. These PGCs will migrate to the seminiferous tubule and develop into spermatids during sex reversal. The expression of vasa mRNA in the mature testis is restricted to the spermatogonia and spermatocytes (Lu *et al.*, 2005).

By using degenerate PCR and RACE, Ding *et al.* (2007) also cloned the vasa gene of the rice field eel (*Monopterus albus*) and named it ma-vas (*Monopterus albus vas*). Ma-vas was exclusively expressed in the gonads of the female, intersex members and males. During gonadal natural sex reversal, ma-vas is expressed in oocytes at all stages of oogenesis, in degenerating oocytes of ovotestis and in spermatogonia and spermatocytes at early stages (Ding *et al.*, 2007).

As described before, mass analysis indicated that the sixth spot in Fig. 2 is the most similar to VASA protein found in *monopterus albus*. According to findings in other fishes, vasa expressed in gametogenesis both in oogenesis and spermatogenesis, during oogenesis vasa expression detected at all stages, however; this expression may detected only in the early stage of spermatogenesis (Xiang *et al.*, 2004; Chen *et al.*, 2005; Xu *et al.*, 2005). Expression of vasa in transitional male gonad was reported and represented in Fig. 2. This data showed that vasa exclusively expressed in the intersex and male gonads, as Ding *et al.* (2007) and Lu *et al.* (2005) reported in their studies on natural sex reversal rice field eel (*monopterus albus*). In the gonads of intersex individuals of *Nemacheilus angorae* new oocytes have been appeared (Fig. 1) therefore, this individual not only showed spermatogenesis but also demonstrated oogenesis in the testes. In addition, real-time PCR results in catfish, *Clarias gariepinus*, has been shown that expression of vasa was seen throughout the development from embryonic stage to adult. However, the expression was more in ovary than in testis during gonadal development (Raghuveer and Senthilkumaran, 2010). Consequently, vasa expression in *Nemacheilus angorae* may increase in the gonads of sex changed males.

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