

A factorial experiment for heritability estimation of the reproductive traits of the wild Persian sturgeon, *Acipenser persicus*

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Abstract

Persian sturgeon (*Acipenser persicus*) is one of the most valuable species of fish native in the Caspian Sea and Iran's waters. In order to evaluate the heritability of reproductive traits in wild Persian sturgeon (*A. persicus*) three males and three females' fish were caught in the southern area of Caspian Sea and then were crossed by using a 3×3 factorial with the aim of selecting the best parent to produce good larvae. Prior to the crossing, sperm density, motility, pH and osmolality, spermatocrit percentages, and the number of micropyles on the eggs' surface were determined. One hundred grams of eggs from each females were fertilized by 1mL of sperm from each males for a total of and 9 treatments combinations, (F1M1, F1M2, F1M3, F2M1, F2M2, F2M3, F3M1, F3M2 and F3M3) with 3 replicates each. Hatching percent was calculated at 5 h after fertilization and 405 eggs were measured for weighting and determining their diameter. Sperm concentration and number of micropyles had no significant effects on fertilization rate ($p>0.05$). Sperm density also had negative correlations with fertilization ($r=-0.603$, $p<0.01$) and hatching rates ($r=-0.175$, $p<0.01$). However, fertilization was positively correlated with osmolality ($r=0.511$) and number of micropyles ($r=0.574$). The correlation between osmolality and hatching rate was 0.288 and between eggs weight and its diameter was 0.698. Heritability estimates for weight and diameter of eggs were 0.043 ± 0.035 and 0.207 ± 0.103 , respectively. The results show treatment 9 (F3M3) had higher genetic values compared to the rest of the treatments. Also due to the large number of micropyle on sturgeon ova, in order to increase the percentage of fertilization rate, sperm density should be low but motility duration should be high.

Keywords: Persian sturgeon, Reproduction, Heritability, Genetic value

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Introduction

Sturgeons, the primitive fishes of the family Acipenseridae, have driven from the teleost's around 200 million years ago (Berg, 1948). They have been able to withstand widely fluctuating environmental conditions for centuries, while are currently found only in the northern hemisphere (Billard and Lecointre, 2001). Sturgeons are among the most valuable and luxury food fish producing the unique and exorbitant roe, caviar. While twenty-seven extant sturgeon species are known, most of them are at risk of extinction (Hallajian, 1998; Dapra *et al.*, 2009). Caspian Sea is a major natural habitat for the fish, hosting 6 species which incorporate 80-90 percent of the global sturgeon fishery (Khodorevskaya *et al.*, 1997). Persian sturgeon, *Acipenser persicus*, is a native fish to the southern coasts of the Caspian Sea comprising about 65% of annual catch of the sturgeons from the sea (Afraei-Bandei *et al.*, 2014). Due to the global climate changes and anthropogenic impacts- such as overfishing and pollution- ecological conditions of the Caspian Sea have adversely been changed, resulting in the alterations in natural life history of its living residents (Billard and Lecointre, 2001). The impacts are particularly destructive for sturgeons which generally have high age at maturation (up to 18 years for *Huso huso*) (Kohne-Shahri and Azeri-Takami, 1974). It is estimated that over 90 percent of the Caspian sturgeons will be extinct by 2050 (Ochokwu *et al.*, 2015). Thus, much attention has been drawn toward

their seed production and aquaculture for natural resources restoration.

Acipenseridae have a multi-layered ovum which, unlike the teleost fish, may have up to 57 sperm-penetrating routes, called micropyles (Hallajian, 1998). This exclusive structure makes fertilization of sturgeon's ova a critical stage in their life history, and much attention is paid in the hatcheries to maximize the hatching rate and to increase the survival rate of the produced larvae. Sperm quality also has a major role, being determined by such parameters as sperm density and motility (Tekin *et al.*, 2003). Active sperms in adequate quantities must be released at a proper time for a maximal fertilization (Gage *et al.*, 1995). Optimum fertilization is also dependent on the technique employed and the breeder's, chiefly male, size and age (Chechun *et al.*, 1994; Chereguini *et al.*, 1999).

Understanding the sources of diversity of the commercially-important traits and predicting the selection response is essential for designing the selective breeding programs. Heritability is a tool for the selection of the traits with suitable genetic attributes, leading to the production of the individuals with genetic values higher than their parental averages (Cameron, 1997). However, there are very few studies on the heritability of fish egg characteristics despite, for instance, the importance of egg size on the quality of larvae (Bascinar and Okumus, 2004). Effects of semen pH, dilution rate, ionic compositions and osmolality on sperm motility and the

impact of sperm concentration and pH on fertilization and hatching rates have been studied in some Caspian sturgeons (Nazari *et al.*, 2005; Dadras *et al.*, 2011; Shaluei *et al.*, 2009), and in bony fish, the effect of sperm on the reproduction of fish such as rainbow trout (*Oncorhynchus mykiss* Walbaum 1972) (Shamspour and Khara, 2016), brown trout (*Salmo trutta*) (Rahbar and Khara, 2015) and *Rutilus frisii* (Fallah Shamsi and Khara, 2015) referred. Nevertheless, the heritability of reproductive characteristics of sturgeons has never been investigated in Iran.

Owing to the importance of the estimation of fertilization and hatching rates and heritability prediction of the egg characteristics in artificial propagation of sturgeons, this study was designed as a factorial experiment using wild Persian sturgeon breeders caught from Iranian coasts of the Caspian Sea. The aim of the study was to discover a baseline for the selection of breeders with superior genetic merits producing eggs with maximal hatching rates and more viable larvae suitable for establishment of future adult cohorts.

Materials and methods

Fish

A number of 6 *A. persicus* breeders (3 males and 3 females) caught by gillnets in the southern Caspian Sea in March 2016 were transported to Dr. Shahid Beheshti Sturgeon Hatchery Center, Rasht, Iran in canvas fish tanks containing the sea water supplied by oxygen. Male and female fish were kept separately in the breeders ponds.

Biometric measurements

Total and fork lengths, and the body weight and circumference of the fish were measured. Samples of the fish eggs were obtained from the oviduct and their diameter was determined for the estimation of polar index according to Dettlaff *et al.*, (1993). The fish were aged by analyzing their pectoral fin spines (Parafkandeh- Haghighi, 2008).

Hormonal induction

The fish were injected subcutaneously with an analogue of the luteinized hormone releasing hormone (LHRH-A2) to promote egg and sperm maturation and ovulation. The injection was performed in two steps in females (10 and 90% of the total dose, respectively over a 10-12 h period), while the males got a single injection at the time of the female's second shot. The dose of the hormone differed based on the water temperature and was adjusted through its dilution by physiological serum (Mohammadi *et al.*, 2015).

Sperm and egg evaluation

Prior to the fertilization, 10 mL of the sperms of each of the males were sampled and the sperm osmolality was quantified as milli-osmol L⁻¹ (mosmol L⁻¹) by a digital osmometer (Nr.9610003- Rebling, Germany). Proportion of the motile sperms was also determined under a Nikon light microscope. Sperm concentration was estimated after a 10% dilution by using a hemocytometer slide under a microscope at 40x magnification (Cosson, 2008; Kopeika and Kopeika

2008). The pH of the semen was measured by a digital pH-meter, while spermatocrit was determined by micro hematocrit capillary tubes after centrifugation at 5000 min⁻¹ for 5 min (Williot *et al.*, 2002). The ova were also examined to estimate their number per gram, weight and diameter, and the number of micropyles (Hallajian, 2010).

Artificial fertilization, incubation and heritability estimation

For fertilization, 300 g ova were taken from each female and transferred to three separate buckets (100g each).

Artificial fertilization was taken place by the conventional semi-dry method based on a 3×3 factorial design in which a female was fertilized by three males. Thus, there were 9 treatments, triplicates per each treatment (Table 1). One milliliter of the sperms was added to fertilize the ova of each bucket after mixing them gently for 5 min. Egg cohesion was prevented by the addition of sterile clay to the mixture and washing for 40 min with tap water. The fertilized eggs were transferred to the Yushchenko incubators with a water flow of 0.3-0.5 L sec⁻¹.

Table 1: Array of the treatments showing the crosses between different male (M) and female (F) *Acipenser persicus*.

Treatment	1	2	3	4	5	6	7	8	9
Cross	F1M1	F1M2	F1M3	F2M1	F2M2	F2M3	F3M1	F3M2	F3M3

Fertilization rate (FR) was estimated after five hours. For this, samples of eggs were taken randomly by an aquarium net and fixed in 10% formalin. Fertilization rate was calculated by using the following formula (Bromage and Cumalantunga, 1988):

$$FR = (\text{normal fertilized eggs} / \text{total examined ova}) \times 100$$

The eggs were hatched after 6 days (144 degree days) at 18.2 ± 1.34 °C. Hatching rate (HR) was determined by the following formula (Hanjavanit *et al.*, 2008):

$$HR = (\text{number of hatched larvae} / \text{number of fertilized eggs}) \times 100$$

For heritability estimation, 405 eggs were weighted by a digital balance and their diameter was measured by a light microscope equipped with a monitor

and measuring device (Digital Sight-Nikon).

Water temperature, oxygen and pH were assessed daily by precise instruments. The water had a temperature of 18.2±1.34 °C (mean±standard deviation), average dissolved oxygen was 8.1± 1.09 mg L⁻¹, and water pH was 7.5±0.41.

Statistical analysis

Analysis of variance for egg weight and diameter was performed using the R software (Developed by Core, 2011) under a linear mixed model (LMM) with Lme4 package (Bates *et al.*, 2011) and SAS, 9.2 programs. Paternal and maternal heritability and their interaction were estimated in Excel environment using outputs for variance components from following model:

$$y_{ijk} = \mu + s_i + d_j + (s \times d)_{ij} + e_{ijk}$$

(Model 1)

Where y_{ijk} is an individual observation, μ is the overall mean, s_i is the random effect of sire i , d_j is the random effect of dam j , $(s \times d)_{ij}$ is the random interaction effect of the cross between the j th dam and i th sire and e_{ijk} is the random residual. Variance components were estimated for effect of sires (σ^2_s), of dams (σ^2_D), and the interaction between sires and dams (σ^2_{SD}) and accordingly the heritability was estimated as:

$$h^2_s = 4\sigma^2_s / \sigma^2_p \quad h^2_D = 4\sigma^2_D / \sigma^2_p$$

(Model 2)

Where σ^2_p is phenotypic variance (Becker, 1984).

Fertilization and hatching rates were compared by a one-way analysis of variance (ANOVA) followed by a Tukey's test at $p < 0.05$. Pearson's correlation coefficient analysis was employed for uncovering the relationships between the examined reproductive traits. The latter statistical analyses were performed by SPSS, 20 software packages.

Results

The male and female fish had an average weight of 13.3 ± 1.5 and 24.7 ± 7.5 kg, respectively. Average total and fork lengths, and body circumference were 179.3 ± 17.5 , 160.7 ± 15 and 31.7 ± 1.7 cm in the females and 145 ± 4.5 , 132 ± 5.1 and 23.5 ± 1.3 cm in the males, respectively. The males and females had mean ages of 14 ± 0.5 and 17 ± 1.5 years, respectively. Tables 2 and 3 show the

values of the estimated sperm and egg parameters of the studied fish.

Table 4 compares fertilization and hatching rates of the breeders. Maximum fertilization and hatching rates, obtained from crossing 3 males and one female fish, were 92.97 ± 0.5 and 73.29 ± 13.89 %, respectively.

Statistical analysis revealed that there were no significant differences between fertilization rate and the sperm concentration, motility and osmolality ($p > 0.05$). Sperm concentration negatively correlated with fertilization rate ($r = -0.60$, $p < 0.01$) and hatching percent ($r = -0.18$, $p < 0.01$). On the other hand, there were positive correlations between the number of micropyles and fertilization rate ($r = 0.57$, $p < 0.01$), and between osmolality and fertilization rate ($r = 0.51$, $p < 0.01$) and hatching rate ($r = 0.29$, $p < 0.01$). Sperm pH had a very weak correlation with fertilization rate ($r = 0.015$, $p < 0.01$) and a negative correlation with hatching rate ($r = -0.28$, $p < 0.01$).

Maximum, minimum and average egg weights were 31, 18 and 22.7 ± 3.01 mg, while the values for egg diameter were 4.18, 3.11 and 3.57 ± 0.19 mm, respectively. Results of the estimation of such parameters as genetic variance and heritability, and the heritable values of the examined traits in the *A. persicus* eggs are presented in Tables 5 and 6. Accordingly, as can be seen in Table 5, the paternal and maternal heritability and their interaction in the Persian sturgeon's eggs with mean weight of 22.70 ± 3.01 mg were 0.439 ± 0.035 , 0.225 ± 0.128 and 0.134 ± 0.093 , respectively. For the eggs with mean

diameter of 3.57 ± 0.19 mm, these values were 0.207 ± 0.103 , 0.807 ± 0.572 and 0.507 ± 0.384 , respectively. There was a significantly positive correlation between egg weight and diameter ($r=0.698$, $p<0.01$) (Fig. 1).

Table 2: Values (mean $\pm$ SD) of the reproductive parameters of the examined female Persian sturgeon, *Acipenser persicus*.

No.	The number per gram (pcs)	The average weight of the ova(g)	The average diameter of the ova(mm)	The average number micropyle	GV%
1	54	18.57 ± 0.93	3.2 ± 0.160	6 ± 1.18	8.6
2	52	19.25 ± 1.01	3.5 ± 0.166	8 ± 1.26	8.8
3	49	20.4 ± 0.48	3.5 ± 0.124	9 ± 1.34	8.9

Table 3: Values of the reproductive parameters of the examined male Persian sturgeon, *Acipenser persicus*.

No.	pH	Time motility(s)	osmolality (mOsm L ⁻¹)	Motility%	Quality mobility	spermatocrit percent	density (mm ³)
1	9.35	195	154	50	middle	11	3.356×10^9
2	9.67	110	146	60	middle	10	2.550×10^9
3	9.4	260	193	80	Good	8	1.051×10^9

Table 4: Fertilization and hatching rates obtained from different crosses between the Persian sturgeon, *Acipenser persicus* breeders ($\pm$ SD).

Mating		Fertilization rate	Average fertilization rate	Hatching rate	Average Hatching rate
Male and female					
Male 1	Female 1	82	85.87 ± 6.7	42.57	68.02 ± 22.2
	Female 2	92		83.6	
	Female 3	78.6		77.9	
Male 2	Female 1	91.2	88.67 ± 4.5	45.68	59.54 ± 22.6
	Female 2	91.4		47.33	
	Female 3	83.4		85.63	
Male 3	Female 1	93.2	92.97 ± 0.5	59.6	73.29 ± 13.9
	Female 2	92.3		72.9	
	Female 3	93.4		87.39	

Table 5: Estimated variance components for sire (σ^2_s), dam (σ^2_d), sire by dam interaction (σ^2_{sd}), phenotypic variance (σ^2_p) and corresponding heritability ($\pm$ SD) for weight and diameter of eggs in Persian sturgeon.

Trait	σ^2_s	σ^2_d	σ^2_{sd}	σ^2_p	h^2_s	h^2_d	h^2_{sd}
weight	0.1011	0.5225	1.2472	9.2684	0.043 ± 0.035	0.225 ± 0.128	0.134 ± 0.093
diameter	0.00867	0.03377	0.02119	0.04175	0.207 ± 0.103	0.807 ± 0.572	0.507 ± 0.384

Table 6: Estimated genetic values of parents in Persian sturgeon.

Trait	Female 1	Female 2	Female 3	Male 1	Male 2	Male 3
weight	-0.414	-0.374	0.787	-0.139	-0.148	0.287
diameter	-0.064	-0.033	0.098	0	0	0

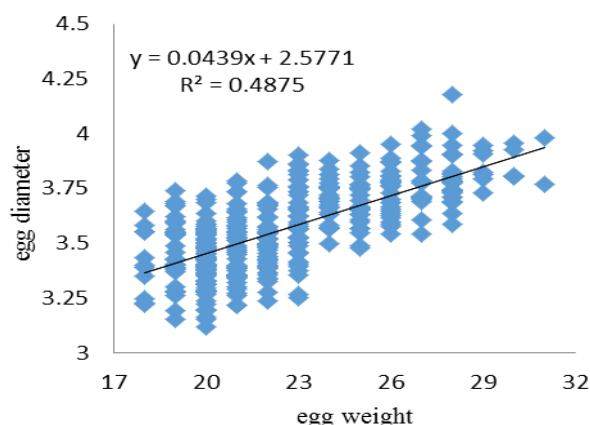


Figure 1: Correlation between egg weight and diameter in Persian sturgeon eggs.

Discussion

Egg and sperm quality is a major concern in artificial fish propagation, and its evaluation is crucial for fertilization and hatching success. Low quality sperms and eggs are regarded as the main obstacles of aquaculture development worldwide (Alavi *et al.*, 2004; Ochokwu *et al.*, 2015). Gametes with good quality will guarantee the production of normal eggs and embryos and strong healthy larvae. In this regard, sperm concentration plays a major role (Krol *et al.*, 2006; Coban *et al.*, 2011). In various fish species, concentration of sperm is dependent on the periodic sperm discharge, and the male age and body weight (Ingermann *et al.*, 2002). However, the existence of several micropyles on the sturgeon's eggs surface, whose number is counted to be 8-9 in *A. persicus* (Hallajian, 1998), may cause polyspermy (Azeri-Takami, 2009) and consequently, lower the fertilization and hatching rates. This was confirmed by the results of this study in which the eggs fertilized by higher concentration of sperms from male 1 ($3.36 \times 10^9 \text{ mm}^3$) had the least fertilization rate ($85.87 \pm 6.7\%$), while

those fertilized by less-dense spermatozoa of male 3 ($1.05 \times 10^9 \text{ mm}^3$) had the maximum fertilization ($92.97 \pm 0.5\%$) and hatching ($73.29 \pm 13.89\%$) rates (Table 4). As female 3 with highest number of micropyles (9) was only crossed with male 3 which had minimum sperm concentration, their cross was among the three producing. Shown that F3M3 treatments had the highest fertilization rate among other treatments. Dadras *et al.* (2011) by studying on the reproductive traits of 11 male Persian sturgeons observed that sperm concentration inversely correlated with fertilization and hatching rates. Nazari *et al.* (2005) investigated the crosses between 16 male and 16 female Persian sturgeons and figured out that even when the eggs of each female were fertilized by the sperms of a single male, increased sperm concentration significantly enhanced polyspermy rate ($p < 0.5$). Nevertheless, Ottesen *et al.* (2009) reported that while sperm concentration has a significant correlation with the fertilization success, cannot consistently be an indicator of sperm fertilizing capability.

In salmonid fish, sperm motility is controlled by potassium ion, while in sturgeons; osmolality is also a key element affecting the sperm activity (Cosson, 2004; Shaluei *et al.*, 2009). Higher and lower osmolality prevent sperm motility in carps and marine fish, respectively (Alavi and Cosson, 2006). Shaluei *et al.* (2009) in their study on the spermatozoa of Ship sturgeon found that the osmolalities of 68-94 mosmol L⁻¹ hindered the sperm motility to some extent, while the sperms were motionless at osmotic pressures of higher than 133 mosmol L⁻¹. This is in contrast with the results of the present study, as the male 3 with highest sperm osmolality promoted maximum fertilization and hatching rates (Tables 3 and 4). The differences in fertilization rates observed in different studies are due to the fact that such factors as the female genetic background, feeding and body size (Brooks *et al.*, 1997), and egg size and number of micropyles (Hallajian, 1998) are also important for a successful fertilization. This can be seen in table 4 where the cross between male3 and female3 has led to the maximum fertilization and hatching rates.

An animal model that includes a random effect for the additive genetic effect of each individual and incorporates a complete set of additive genetic relationships among all the individuals, allows an unbiased estimation of variance components, even for the data involving selection and non-random mating (Sorensen and Kennedy, 1986). Heritability is among the main genetic indices in animal

selective breeding, indicating the level of genetic improvement expected or gained following the selection of a trait. It has already been estimated for egg size and volume in a number of fish and shrimp species (Gall and Gross, 1978; Gall and Huang, 1988; Huang and Gall, 1990; Su *et al.*, 1997; Macbeth *et al.*, 2007; Tan *et al.*, 2017). Larger eggs produce larger larvae with greater yolk reserve and this can eventually be beneficial for aquaculture development (Bascinar and Okumus, 2004).

Additive genetic diversity has led to the differing heritability values for a single trait. The quality of the egg, in terms of fertility and embryo development, is determined by the time at which the egg is stripped from the female relative to time of ovulation and the temperature of the holding water. Male fertility is also an essentially unknown factor. Heritability estimates according to Table 5 for egg weight and diameter were 0.04 and 0.20, respectively. Gall (1975) estimated the heritability of rainbow trout, *O. mykiss*, egg size, number and volume to be 0.20±0.05, 0.19±0.06 and 0.20±0.05, respectively. He also found that egg size had positive correlations with egg number ($r=0.09\pm0.13$) and growth rate ($r=0.47\pm0.13$) and negative correlation with egg volume (-0.55 ± 0.12). Gall and Huang (1988) obtained heritability variance components for the rainbow trout egg volume, number and size to be 0.3, 0.32 and 0.28, respectively. Gall and Neira (2004) estimated the heritability of rainbow trout green eggs weight and number as 0.39 and 0.42, respectively, while the heritability of

eyed-eggs weight and numbers were 0.32 and 0.33, respectively. They also found a strong correlation between the green eggs weigh and number ($r=0.79$). Su *et al.*, (1997, 2002) using restricted maximum-likelihood (REML) method estimated the heritability of rainbow trout egg size (0.6) and volume (0.52) and their correlation (0.25) as well as the correlation between the fish body weight and egg size (0.29), between the egg number and volume (0.67), and between egg size and hatching rate (0.35). Kinnison *et al.*, (2001) determined the heritability of Chinook salmon, *O. tshawytscha*, egg size (0.50-0.78) and number (0.00-0.76) in two regions of New Zealand. They also noticed a significantly negative correlation between egg size and number ($r=-0.75$). Arcos *et al.* (2004) reported that the white-leg shrimp, *Litopenaeus vannamei*, egg number and diameter had heritability values of 0.17 ± 0.24 and 0.07 ± 0.23 , respectively, and genetic correlation of 0.16. These heritability and correlation estimates in the shrimp, *L. vannamei* were 0.12 ± 0.08 , 0.01 ± 0.04 and -0.77 ± 1.14 , respectively in the study of Tan *et al.*, (2017).

Similar to the results of the previous studies (Gall and Gross, 1978; Gall and Huang, 1988), our results show that the cross between male and female fish with higher body measures (e.g. weight and length) can have superior genetic components and heritability rates. So that, the treatment 9 (F3M3) produced by male3 and female 3 had higher genetic values compared to the rest of the crosses, Average heritability of the

egg weight and size suggests that selection of these traits can trigger the genetic improvement. The heritability of the reproductive traits estimated in this study was in the range of 0-0.76. This range of the heritability values indicates that there is an additive genetic diversity in the examined traits, so that it is expected that their selection can eventually be mirrored in the suitable body weight (Su *et al.*, 1997). Heritability of useful traits together with the variables such as growth and survival rates is important in the development of commercial aquaculture through genetic and environmental manipulation. But normally reproductive traits normally are not considered to be important components of breed improvement programs for sturgeon because of the high fecundity of the species. However, it is important to monitor reproductive performance so the breeder can identify any changes in reproductive productivity that may occur during selection and breed improvement. The results reported here indicate that the Iranian sturgeon population under study contains a high level of additive genetic variation for all traits related to female reproduction.

The estimates of genetic parameters provided by this study indicate that genetic improvement of growth and reproduction would be possible with few antagonist complications. However, they must be viewed as initial estimates from a unique population and replicate estimates are needed. In addition, information is urgently needed concerning the validity of a volume

measure of egg size, volume measure of egg number, effect of egg size on hatchability, reproductive performance of males and the maternal influence of the egg on fingerling growth and survival. The findings from such studies could greatly modify the conclusions one can make from the present experiments. This will, eventually, minimize the expenses of sturgeon larviculture by lessening the proportion of weak and slow-growing larvae in the culture system.

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