Mixed milt fertilization of endangered Caspian brown trout *Salmo trutta caspius* influences effective population size of breeders

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Abstract

The maintenance of the endangered Caspian brown trout *Salmo trutta caspius* populations in Iran depends on its artificial breeding. There is no information on how current hatchery breeding protocol influences effective population size of breeders (N_e), which is a critical parameter to maintain genetic diversity in future generations. The current hatchery protocol (CHP) was comparatively evaluated with a balanced hatchery protocol (BHP), where mixtures of equal amounts of gametes per male and female breeder were used to balance parental contributions to progeny. To evaluate putative differences in viability between families, individual crosses were performed and fertilized ova of different families were mixed to constitute balanced family pools. 1440 alevins were totally sampled in the crosses performed from the 11 breeders. An exclusion-based parentage approach using three polymorphic microsatellite markers unambiguously assigned more than 93% of progeny to a single pair of parents. Significantly different contributions of breeders to progeny were observed in CHP ($p<0.05$). The primary constraint on N_e in BHP was the unbalanced contribution of males, which seemed a consequence of sperm competition in mixed fertilization caused by differences in sperm quality. Sperm motility duration was positively correlated with the number of sired progeny by each male. The results illustrate the limitations of the BHP in minimizing the loss of genetic diversity observed in CHP. A protocol based on mixture of equal number of fertilized ova from individual male × female crosses emerged as the best alternative for conservation of Caspian brown trout.

Keywords: Caspian brown trout, *Salmo trutta caspius*, Mixed milt fertilization, Effective population size, Sperm competition, Microsatellite.

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Introduction

The endemic Caspian brown trout, *S. trutta caspius* Kessler, 1877 is a member of brown trout Danubian lineage which is found mainly in the southwest part of the Caspian Sea, Iranian waters (Vera *et al.*, 2011). Caspian brown trout populations in Iran have experienced such a drastic decline in recent decades as a result of overfishing, river pollution and destruction of natural spawning areas that have been considered as critically endangered according to IUCN criteria (Coad, 2000). Therefore, artificial breeding has been attempted in Iranian hatcheries and huge investments by the government have been made for recovering wild populations through the release of fingerlings produced in hatcheries (Jalali and Amiri, 2009).

Artificial breeding of adults and the subsequent release of juveniles to the wild are commonly performed in conservation programs to avoid the short-term extinction probability of endangered species. Many salmonids are propagated through mixed milt fertilization in hatchery conservation programs (Campton, 2004). Large variation in contribution of breeders to progeny is expected with such fertilization systems that would result in lower effective population sizes (N_e) than the census size of breeders (N_c) (Koljonen *et al.*, 2002; Machado-Schiaffino *et al.*, 2007). Low N_e increases the inbreeding coefficient and reduces genetic diversity of hatchery-produced progeny (Campton, 2004; Porta *et al.*, 2006). As a consequence, these stocks are not representative of the original population, and their release into the wild is not the best option to recover or maintain genetic diversity in natural populations for their conservation (Bohlin *et al.*, 2002; Heggenes *et al.*, 2002).

In order to maintain genetic diversity within hatchery populations, it is necessary to have access to reliable parentage information, which permits to know the different contributions of breeders to progeny (Evans *et al.*, 2004; Jerry *et al.*, 2004). Microsatellite markers are hyper-variable DNA markers with great discriminating power for parentage analysis when progeny from mixed fertilizations are reared together (Chistiakov *et al.*, 2006; Martinez and Fernndez, 2008). In the last decade several studies have demonstrated the ability of microsatellites to establish parentage in some species including Japanese flounder (*Paralichthys olivaceus*) (Hara and Sekino, 2003), Gilthead sea bream (*Sparus aurata*) (Brown *et al.*, 2005), Senegal sole (*Solea senegalensis*) (Castro *et al.*, 2006), Barramundi (*Lates calcarifer*) (Frost *et al.*, 2006) and Steelhead trout (*Oncorhynchus mykiss*) (Araki *et al.*, 2007).

In mixed milt fertilization, broodstock contribution to progeny seems more variable in male than in female breeders (Bekkevold *et al.*, 2002; Brown *et al.*, 2005). According to Campton (2004) and Frost *et al.* (2006), this observation may be caused by sperm competition, which occurs when sperm from two or more males compete for fertilization of eggs. Implications of sperm competition for mating systems are just beginning to be understood (Gage *et al.*, 2004). The success of hatchery induced sperm competition may depend on differences in sperm quality traits such as milt volume,

sperm motility (motility duration and percentage motility), velocity, sperm density and spermatocrit (Kaspar *et al.*, 2007; Wedekind *et al.*, 2007). There are controversial reports on how the differences in these traits affect the outcome of sperm competition (Gage *et al.*, 2004, in Atlantic salmon *Salmo salar*; Vermeirssen *et al.*, 2004, in Atlantic halibut; Casselman *et al.*, 2006, in Walleye (*Sander vitreus*); Stoltz and Neff, 2006, in Bluegill (*Lepomis macrochirus*)).

CHP of Caspian brown trout consists of mixed fertilization of stripped ova from 2-4 females with the milt of 2-4 males in each breeding cross. Unequal parental contributions of Caspian brown trout to progeny, especially from males, in mixed milt fertilization was reported by Sourinejad *et al.* (2010). The results showed that in CHP, differential contribution of breeder individuals to progeny reduced N_e/N_b to 0.53, and genetic diversity parameters were also reduced in progeny compared to their parents.

The goal of hatchery breeding programs should be to minimize the genetic change between the broodstock and the progeny produced. To achieve this goal, breeding protocols should maximize N_e by increasing the genetic contribution of broodstock to progeny.

The objective of the present study was to assess if an alternative breeding protocol based on equal amounts of gametes from breeders (BHP) would produce balanced parental contributions and reduce the genetic diversity change between breeders and the progeny. The results were analyzed for proposing a new hatchery breeding protocol if needed to conserve more genetic diversity of the endangered Caspian brown trout for the first time. Furthermore, the relationship between sperm parameters and the contribution of males at each trial was investigated.

Materials and methods
Fertilization scheme and sampling
Collection of specimens was limited by scarcity of Caspian brown trout in the rivers during the spawning season. Ova and sperm for the experiments were stripped from 11 breeders caught in the wild in the 2009 spawning season from Tonekabon River, northern Iran and held in captivity at Kelardasht hatchery center (36°29' 50.66 N, 51°08' 044.52 E), where the Iranian Caspian brown trout restoration program is being implemented. The samples for our experiment included 5 females (numbers 1, 2, 3, 4 and 5) and 6 males (numbers 1, 2, 3, 4, 5 and 6). It is worth mentioning that only about 30 wild male breeders were caught during the 2009 spawning season for the restoration program.

In the CHP, a single hatchery trial was performed using the gametes of four females (1, 2, 3 and 4) and two males (1 and 2). In the BHP, two independent trials were performed (1 and 2). In trial 1, equal milt volumes (300 µl) of four males (1, 2, 3 and 4) were mixed and added to the mixture of equal ova numbers (n=150) of four females (1, 2, 3 and 5). Trial 2 was performed like trial 1, but males 3 and 4 were substituted with two different ones (5 and 6). Two trials were also performed in BMF. In the first trial, two males (3 and 4) and two females (1 and 5) were

individually crossed and the fertilized ova mixed in equal proportions. In the second trial, males 5 and 6 and females 1, 3 and 5 were used following the same scheme to evaluate if differences in viability between families may distort parental contributions. All trials were carried out in two replicates and the mean was considered for statistical analyses (Table 1). Caudal fin clips were taken from all the breeders and preserved in absolute ethanol for subsequent genotype analyses. Fertilized eggs from the different trials were incubated until day 90 post-fertilization when the yolk sac of

hatched alevins was completely absorbed. Fertilization success and hatching rate of trials were determined. In each trial, a random sample of alevins equal to the number of possible parental contributions multiplied by 15 was collected to obtain at least a minimum representation of alevins in each trial. Therefore, 240 alevins from the CHP, 960 alevins from the BHP (480 alevins from each trial) and 240 alevins from the BMF (120 alevins from each trial) were collected and preserved in absolute ethanol for further DNA analyses.

Table 1: Overall scheme of the different crosses performed from 11 Caspian brown trout breeders.

Experiment	Treatment	Males No:	Females No:	Sperm/Ova or fertilized eggs amount
CHP	$2\male \times 4\female$	1, 2	1, 2, 3, 4	Different
BHP (trial 1)	$4\male \times 4\female$	1, 2, 3, 4	1, 2, 3, 5	Equal (300µl sperm, 150 ova)
BHP (trial 2)	$4\male \times 4\female$	1, 2, 5, 6	1, 2, 3, 5	Equal (300µl sperm, 150 ova)
BMF (trial 1)	$1\male \times 1\female$	3	1	Equal (300µl sperm, 150 ova)
	$1\male \times 1\female$	4	1	
	$1\male \times 1\female$	3	5	
	$1\male \times 1\female$	4	5	
BMF (trial 2)	$1\male \times 1\female$	5	1	Equal (300µl sperm, 150 ova)
	$1\male \times 1\female$	6	3	
	$1\male \times 1\female$	5	5	
	$1\male \times 1\female$	6	5	

CHP: current hatchery protocol; BHP: balanced hatchery protocol; BMF: balanced mixed families

Evaluation of sperm density, spermatocrit and sperm motility duration

Sperm density was determined using a haemocytometer. Duplicate dilutions and triplicate counts for each dilution were made for each milt sample (Tvedt *et al.*, 2001). Spermatocrit was determined in three replicates per sample using milt collected into microhaematocrit tubes and centrifuged for 195 s at 11,500 rpm (Wedekind *et al.*, 2007). Sperm motility was determined by diluting 10µl of milt in 1 ml non-activating solution and pipetting 1 µl of this diluted solution onto a

microscope slide. Activation was achieved by adding 25 µl of hatchery water. Using a video camera attached to the microscope the duration of sperm motility was evaluated in three replicates per sample as the time elapsed from activation until less than 5% of the sperm maintained forward swimming activity (Tvedt *et al.*, 2001). Duncan test was used to investigate differences in sperm traits of the six male breeders. Non-parametric Spearman's correlation coefficient was used to investigate the relationship between sperm parameters and the contribution of males in each trial ($p<0.01$).

DNA extraction and microsatellite analysis

Whole genomic DNA was obtained from caudal fin of adults using standard phenol-chloroform procedure (Sambrook *et al.*, 1989) or from whole larva using the Chelex® Resin procedure (Walsh *et al.*, 1991) at the Genetics Department of University of Santiago de Compostela, Lugo, Spain. Breeders were genotyped using previously reported primers for 9 microsatellite loci identified in *S. trutta*: Str15, Str58, Str60 and Str73 (Estoup *et al.*, 1993); Str85, Str543, Str591 (Presa and Guyomard, 1996); Ssa85 and SSOSL438 (Gene-Bank accession numbers: U43692 and Z49134, respectively (Laikre *et al.*, 1999)).

PCR reactions were performed in 15 µl volumes containing 0.66 µM of each primer, 100 µM dNTP, 1× PCR Gold Buffer PCR buffer with 1.5 mM MgCl$_2$, 0.5U of AmpliTaq Gold^{TM} DNA polymerase (Applied Biosystems) and 30 ng of genomic DNA. All PCR reactions were conducted in a MJ research PTC-100 thermocycler using the following cycle conditions: 10 min at 95 °C, 35 cycles of 45 s at 94 °C, 50 s at primer specific annealing temperature (60 °C for Str60 and Ssa85, 58 °C for Str15 and Str73, 56 °C for Str58 and 55 °C for Str85, Str543, Str591 and SSOSL438), 50 s at 72 °C and a final elongation for 10 min at 72 °C. Microsatellite profiles were obtained using an ABI PRISM® 3730 automatic sequencer (Applied Biosystems). Allele scoring was performed with GeneMapper 4.0 software (Applied Biosystems).

Parentage assignment and effective population size

Genetic diversity estimators (allele number, heterozygosity, polymorphic information content) and null allele frequency were obtained for each locus and for all loci from the 11 breeders using the allele frequency option of CERVUS 3.0 (Kalinowski *et al.*, 2007). Parentage assignment potential was estimated either when genetic information from the other parent was unknown (Excl1) or known (Excl2) using the same program. Parentage assignment was performed using an exclusion-based approach in FAP v.3.5 program (Taggart *et al.*, 2007) based on the 3 selected microsatellites described in Table 2.

The number of progeny produced by each male and female breeder in each trial was then determined and used to calculate their contribution to the total cohort in percentage. Chi-square tests were used to determine if the number of progeny produced by different females and males both overall and at each mating pair deviated from the null hypothesis of equal contribution ($p<0.05$). Effective population size of breeders was calculated according to Vandeputte *et al.* (2004) as $N_e = 4 (N-2) / ((Ks+Vs /Ks) + (Kd+Vd /Kd) -2)$, where N_e is the effective size, N is the number of sampled progeny, Ks and Kd are the mean number of progeny per male and per female, and Vs and Vd are the variances of male and female family sizes.

Results

Fertilization success, hatching rate and sperm parameters

Fertilization success in the present work was 92%. Eyeing of the fertilized eggs occurred at 224 degree/day and the alevins hatched at 434 degree/day with success rate of 90%. Complete yolk sac absorption of the alevins took long, 31 days from hatching at 7°C. Sperm density (3.97-7.76×10^9 sperm ml^{-1}), spermatocrit (33-62.66 %) and sperm motility duration (23.47- 29.91 s) were evaluated for the six males (Fig. 1). Sperm density and spermatocrit of male 1 were the highest ones and significantly different from all the other males. Male 4 showed the lowest values and males 2 and 3 were not significantly different ($p<0.01$). Regarding sperm motility duration, male 5 showed the highest value, males 3 and 4 were not significantly different and male 1 had the shortest duration time ($p<0.01$).
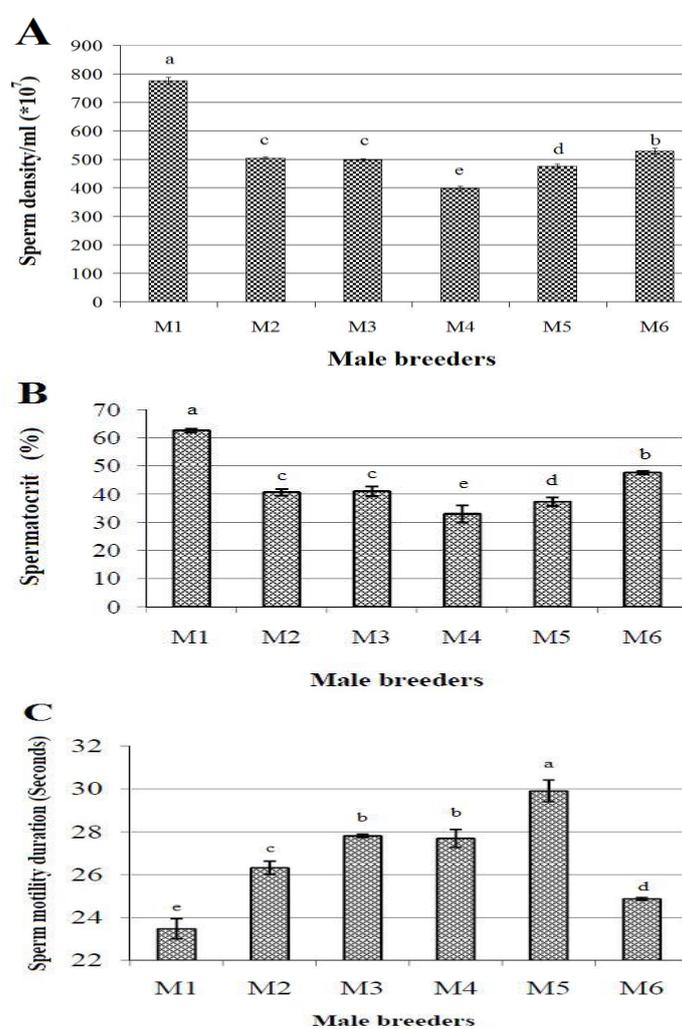

Figure 1: Values of sperm density (A), spermatocrit (B) and sperm motility duration (C) of the 6 male breeders of Caspian brown trout. Means sharing different alphabetical symbols differ significantly ($p<0.01$).

Genetic diversity in Caspian trout breeders

The eleven breeders were genotyped at the 9 microsatellite loci presented in Table 2. The number of observed alleles per locus in the breeders ranged from 1 (Str60) to 11 (Str58), with a mean of 4.55. The average expected heterozygosity ranged between 0 (Str60) and 0.918 (Str58) with a mean of 0.498. Remarkable excess of heterozygotes was observed at Str15 and Str543 loci, which could be explained by the small sample size managed in our study.

According to these values and the particular crossing scenario at the different trials, three loci (Str58, Str73 and Str591) were finally selected for parentage assignment of progeny. The average number of alleles and expected heterozygosity for these three loci were 8.0 and 0.790, respectively. Nearly all estimates of null allele frequency from CERVUS 3.0 algorithm were negative, suggesting no evidence of null alleles in our breeders at these loci (Table 2).

Table 2: Estimates of genetic diversity and frequency of null alleles for the 9 loci analyzed in the 11 Caspian
brown trout breeders using CERVUS 3.0 (Kalinowski *et al.*, 2007).

Locus	A	Ho	He	PIC	Excl1	Excl2	Null alleles
Str15	3	0.909	0.558	0.432	0.142	0.238	-0.2747
*Str58**	11	1	0.918	0.864	0.602	0.753	-0.0693
Str60	1	-	-	-	-	-	-
*Str73**	4	0.636	0.606	0.491	0.172	0.293	-0.0455
Str85	2	0.182	0.173	0.152	0.014	0.076	-0.0405
Ssa85	4	0.727	0.766	0.683	0.308	0.483	0.0041
SsoSl438	2	0.091	0.091	0.083	0.004	0.042	-0.0141
Str543	5	0.636	0.528	0.475	0.140	0.304	-0.1629
*Str591**	9	0.909	0.848	0.787	0.463	0.637	-0.0687
Overall	4.555	0.565	0.498	0.440	0.911	0.985	-
3 loci*	8	0.848	0.790	0.714	0.823	0.936	-

A: number of alleles; Ho: observed heterozygosity; He: unbiased expected heterozygosity; PIC: polymorphic information content), probabilities of exclusion (Excl 1: unknown parents and Excl 2: one parent known).

Parentage assignment

Combined probabilities of exclusion for the 3 selected loci estimated from the genotypes of the 11 breeders were 0.823 (Excl1) and 0.936 (Excl2). However, the actual potential was higher because only subsamples of breeders were involved in each cross. Microsatellite profiles for the 3 selected loci were obtained for all progeny in the different trials performed and were used to trace back their paternity. After

exclusion based approach, it was possible to assign most progeny to a single parental couple: 98.4% in CHP trial, 99.8% in BHP trial 1, 93.1% in BHP trial 2 and 100% in BMF trials. Using these 3 microsatellites, paternity could be assigned unambiguously in 447 out of 480 analyzed progeny in the worst performing BHP trial 2.

Differential contribution of breeders
CHP trial

DNA parentage analyses showed different contributions between females overall, regardless of mating pair ($\chi2$ $_{d.f.}$ $_3$= 50.52, p=0); between males overall, regardless of mating pair ($\chi2$ $_{d.f.}$ $_1$=9.36, p=0.002); between males mating with female 1 ($\chi2$ $_{d.f.}$ $_1$= 12.93, p=0); and between females mating with male 1 ($\chi2$ $_{d.f.}$ $_3$= 31.86, p=0) and male 2 ($\chi2$ $_{d.f.}$ $_3$= 26.74, p=0). Male 2 sired close to 60% of the sampled alevins (Fig. 2). Among females, female 4 had the highest contribution which corresponded to more than 42% of the sampled alevins. N_e for the hatchery protocol was found to be 4.71, compared to the census size of 6 and N_e/N_c ratio was 0.78. The correlation analysis of sperm parameters with male contributions, showed a positive and significant value only with sperm motility duration (r=1, p= 0).
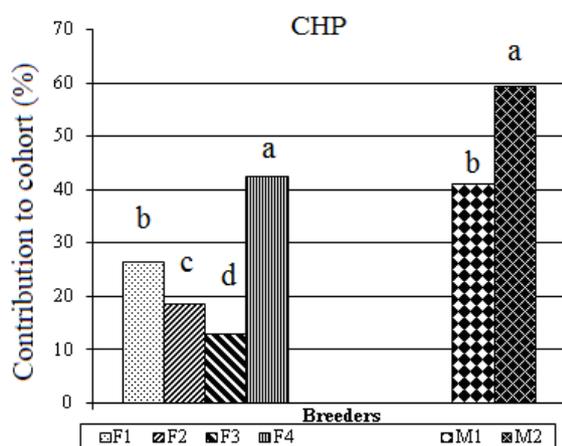

Figure 2: Contribution of female and male breeders to progeny in the Caspian brown trout current hatchery protocol (CHP). Means sharing different alphabetical symbols differ significantly (*p*<0.01).

BHP (trial 1)
Contributions to this trial were significantly different between females overall, regardless of mating pair ($\chi2$ $_{d.f.}$ $_3$= 25.12, p =0); between males overall, regardless of mating pair ($\chi2$ $_{d.f.}$ $_3$= 116.01, p=0); between males mating with female 1 ($\chi2$ $_{d.f.}$ $_3$= 31.48, p=0), female 2 ($_{d.f.}$ $_3$=14.17, p=0.02), female 3 ($_{d.f.}$ $_3$=72.50, p=0) and female 5 ($_{d.f.}$ $_3$= 39.25, p=0); and between females mating with male 1 ($\chi2$ $_{d.f.}$ $_3$= 24.30, p=0), male 2 ($\chi2$ $_{d.f.}$ $_3$= 11.13, p=0.011), male 3 ($\chi2$ $_{d.f.}$ $_3$= 23.21, p= 0) and male 4 ($\chi2$ $_{d.f.}$ $_3$= 38.0, p= 0) (Fig. 3A). The best performing male (male 3) sired over 40% of progeny, but specially, two of the males (1 and 4) sired only around 10% of progeny (Fig. 3). Among females, the contributions were more balanced. N_e was found to be 6.72, compared to the census size of 8 and N_e/N_c ratio was 0.84. There was a positive correlation between males contribution and sperm motility duration but this was not significant (r=0.632, p=0.368) due to a single outlier. The correlation between male contributions and sperm density (r= -0.105, p=0.895) and spermatocrit (r= 0.105, p=0.895) were not significant.

BHP (trial 2)
Contributions to this trial were significantly different between females overall, regardless of mating pair ($\chi2$ $_{d.f.}$ $_3$=11.13, p= 0.011); between males overall, regardless of mating pair ($\chi2$ $_{d.f.}$ $_3$= 640.62, p=0); between males mating with female 1 ($\chi2$ $_{d.f.}$ $_3$= 139.42, p= 0), female 2 ($_{d.f.}$ $_3$= 159.06, p= 0), female 3 ($_{d.f.}$ $_3$= 137.14, p= 0), and female 5 ($_{d.f.}$ $_3$= 214.11, p= 0); and between females mating with male 6 ($\chi2$ $_{d.f.}$ $_3$= 15.64, p= 0.001). Produced progeny from male 5 alone, dominated the cohort compromising >75% of the sampled alevins. Females 3 and 5 had the same

contribution of 29%, which was significantly higher than female 1 and 2, although contributions of females were much more balanced than those of males (Fig. 3B). N_e for trial 2 was found to be 4.11, compared to the census size of 8 and N_e/N_c ratio was 0.51. There was only a positive significant correlation (*r = 1, P= 0*) between males contribution and sperm motility duration among the analyzed sperm parameters.
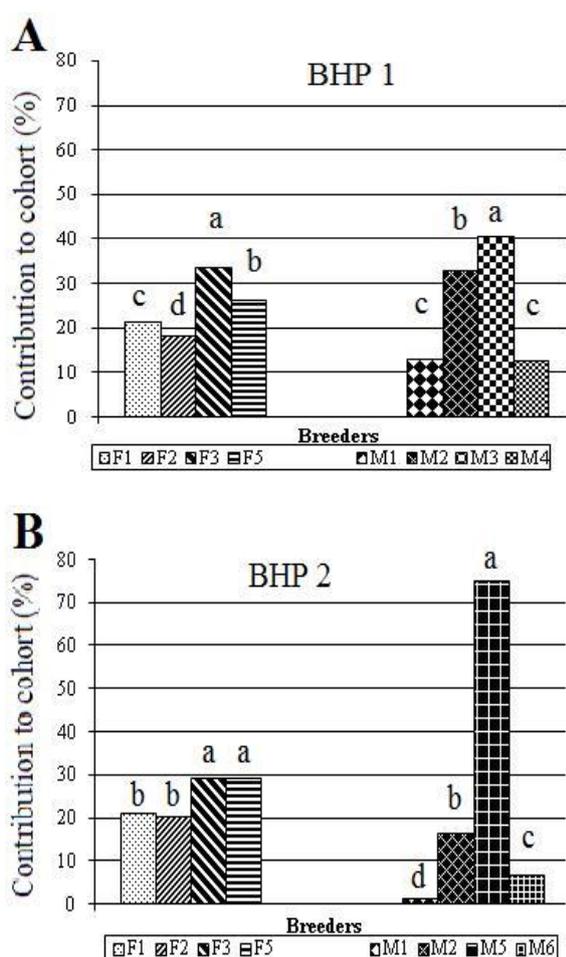

Figure 3: Contributions of female and male breeders to progeny in Caspian trout balanced hatchery protocol (BHP) trial 1 (A) and trial 2 (B). Means sharing different alphabetical symbols differ significantly (*p<0.01*).

BMF trials 1 and 2

Parentage assignment and the subsequent chi-square tests, showed non-significant differential contributions of the founded families to progeny in the non-sperm competition situation performed in these two trials (Fig. 4). N_e for control trial 1 and 2 was determined as 3.99 and 3.98, respectively compared to N_c=4 also indicating no differences in viability between created families.
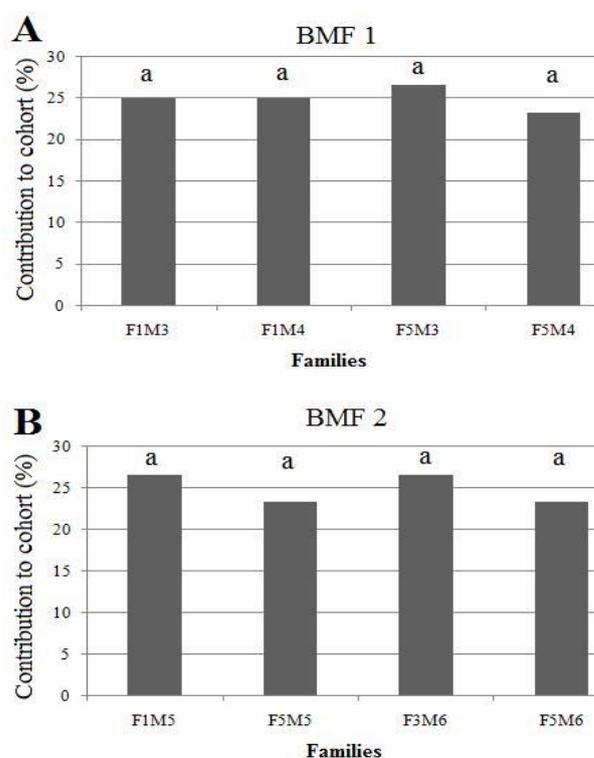

Figure 4: Contributions of families to progeny in Caspian brown trout balanced mixed families (BMF) trial 1 (A) and trial 2 (B). Means sharing the same alphabetical symbols do not differ significantly (*p<0.01*).

Discussion

Using the least possible number of microsatellite markers to determine the pedigrees of progeny produced from mixed milt fertilization is important to the cost effectiveness of programs dealing with conservation of genetic resources in

aquatic animals (Castro *et al.*, 2007; Martínez and Fernández, 2008). In the present study, high percentages of parentage assignment were achieved in the Caspian brown trout with only 3 microsatellites. More than 98% of progeny in the CHP trial, BMF trials and BHP trial 1, and around 93% of progeny in BHP trial 2 were assigned to single parental couples using a parentage exclusionary method. These percentages of assignment are similar and even higher than those described for other fish species (Sourinejad *et al.*, 2010). The selection of the most polymorphic microsatellite markers based on the primary screening of a large microsatellite set suitable for population analysis in Caspian brown trout, the low number of breeders and their subdivision at specific crosses (maximum of 16 possible couples in competition trials) was effective in achieving such high percentages of parental assignment. The rate of fishes assigned and the number of loci used in this study show how small sets of microsatellites might best be used in parentage assignment in hatcheries while focusing on the cost effectiveness of the research (Sourinejad *et al.*, 2010).

The maintenance of high levels of genetic diversity and low levels of inbreeding is a major objective in conservation programs (Frost *et al.*, 2006). In Caspian brown trout, our data demonstrated that the genetic contribution of breeders to progeny was unequal in CHP and BHP trials. In the CHP, contribution of males was unbalanced and male 2 contributed about 20% higher to progeny than male 1. Among female breeders, a single female (female 4)

produced 42% of progeny. These unequal parental contributions determined the N_e/N_c ratio of 0.78 in this trial. In the BHP, the amount of gametes per breeder was balanced and this determined the more balanced contribution of females in both BHP 1 and 2, especially when compared to males. Only two males sired 74% of progeny in BHP 1 and a single one sired 75% in BHP 2. This strong unbalanced contribution determined N_e/N_c ratios of 0.84 in trial 1 and only 0.51 in trial 2. The highly skewed contribution of males observed in our study is in agreement with Bekkevold *et al.* (2002) and Brown *et al.* (2005) who reported that broodstock contribution to progeny is more variable in male breeders than in females.

Unbalanced contribution of male breeders to progeny seems to be the result of sperm competition, which exists among male breeders in mixed fertilization procedures (Campton, 2004; Frost *et al.*, 2006) and is a determinant of the differential male reproductive success. In agreement with our study, unequal contribution of males to progeny in artificial breeding of barramundi (*L. calcarifer*) and the decrease of effective size were assigned to sperm competition (Frost *et al.*, 2006). When we analyzed different sperm traits in our experiment, it appeared that the variance in sired larvae within CHP and BHP trials could be explained largely by differences in sperm quality. In our experiments, sperm density was not a predictor of fertilization success. In different salmonid species sperm number was not the primary determinant of sperm competition success (Gage *et al.*, 2004) and even a negative relationship was

reported in rainbow trout (Tuset *et al.*, 2008). The application of large sperm/ova ratio determined that even in mixed fertilization, each male had the opportunity to fertilize all the eggs and contribute to 100% of progeny. Sperm number should be less than that needed to fertilize all eggs in order to reveal differences in fertilization ability (Fauvel *et al.*, 1999; Kime *et al.*, 2001) and maybe this is the reason that sperm density in this study did not affect sperm competition success. Therefore, it can be concluded that differences due to variation in the density of sperm but not of sperm motility can be masked by the quantity of sperm.

In the Caspian brown trout, sperm motility duration seems to highly influence the sperm competition success. In the CHP trial and the BHP trial 2, this effect was obvious. Even in the BHP trial 1, the proportion of sired progeny in competition could be explained completely by differences in sperm motility duration if we removed a single outlier. The results reported here are in agreement with recent findings in rainbow trout by Tuset *et al.* (2008), who reported that fertilization success depended on duration of sperm movement, but not on percentage sperm motility. In addition, an effect of motility on the outcome of sperm competition was reported in common carp (*Cyprinus carpio*) (Linhart *et al.*, 2003). Nevertheless, other studies have found that fertilization success in cod (*Gadus morhua* L.) (Trippel and Neilson, 1992) and walleye (*S. vitreus*) (Casselman *et al.*, 2006) were not correlated with sperm motility. Velocity and percentage sperm motility have also been recognized as

important sperm traits for male fertility in some species (Vermeirssen *et al.*, 2004). In Atlantic trout, the same was observed and the milt containing faster sperm was the most efficient (Gage *et al.*, 2004).

The contribution of families in the BMF was balanced in both trials 1 and 2. This balanced contribution rendered N_e/N_c ratios of 0.99. These results show the benefit of using balanced mixed family trials in producing progeny retaining most genetic diversity. According to our data, for example, the family consisting of male 3 and female 5 produced 32% more progeny than that consisting of male 4 and female 5 in BHP trial 1, meanwhile this percentage was not significantly different in BMF trial 1.

Overall, our data indicate that applying equal volumes of milt for the fertilization of Caspian brown trout ova does not produce equal number of progeny per male in a competition situation. In accordance with our data, equalization of sperm by volume did not produce equal number of progeny per male in a competitive situation in common carp and left a wide range of unexplained variability in competition success (Kaspar *et al.*, 2007).

The maintenance of the natural populations of this species mainly depends on artificial breeding in hatcheries and the unbalanced contribution of broodstock determines the decrease of genetic diversity in natural populations. The major reasons for loss of genetic diversity in hatchery populations most often are bottlenecks and small effective population sizes, due to inappropriate mating designs (Aho *et al.*, 2006). In order to reduce the risks of genetic diversity loss in breeding

programs of Caspian brown trout, N_e within the hatchery population needs to be increased by raising the number of parents, and especially by equalizing their contributions. In the difficulty of obtaining more breeders, it seems that improvements should focus on fertilization schemes to increase the number of families and their balanced contributions to the next generation. For this, random crosses of breeders from one sex with the maximum number of breeders of the other sex are recommendable using one to one crosses by stripping method. Although this task is not feasible in many fish species due to large number of available breeders, it can be applied to Caspian brown trout hatcheries considering the small number of breeders during one spawning season. Other protocols like dividing the sperm of one male or more to different parts and crossing each part with various parts of mixed eggs from different females are also recommendable and need more investigation.

In conclusion, polymorphic microsatellites together with paternity analysis served as a powerful tool to determine the parentage of communally reared progeny from different breeders in Caspian brown trout. Parentage analysis demonstrated unequal parental contributions to progeny in the CHP, and also in the BHP, where equal amounts of gametes were used for fertilization. The results indicate that contribution of males to the progeny was more variable than females, which seems a consequence of sperm competition in this species caused by differences in sperm quality. We observed a positive association between sperm motility duration and percentage of sired progeny. Sperm density and spermatocrit were not correlated with fertilization ability of breeders. Our data indicate that increasing N_e should be performed by equalizing the genetic contribution of breeders through the application of appropriate fertilization designs in the hatchery. This will be essential to maintain genetic diversity and to conserve Caspian trout natural resources.

Acknowledgments

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