www.trjfas.org
ISSN 1303-2712
Turkish Journal of Fisheries and Aquatic Sciences 16: 259-266 (2016) DOI: 10.4194/1303-2712-v16_2_05

RESEARCH PAPER

Semen Quality of Threatened Native Population of Mediterranean Brown Trout (*Salmo cettii,* Rafinesque 1810) in the Biferno River (Molise Region - South Italy)

Iaffaldano N.[1,*], Di Iorio M.[1], Manchisi A.[1], Gibertoni P. P.[2], Esposito S.[2]

[1] University of Molise, Environmental and Food Sciences, Department of Agricultural, via De Sanctis, snc 86100 Campobasso, Italy.
[2] Mediterranean Trout Research Group – Centro di ricerche "I Giardini dell'Acqua"- 42037 Collagna (RE), Italy.

* Corresponding Author: Tel.: +39.0874 404697; Fax: +39.0874 404855;
E-mail: nicolaia@unimol.it

Received 28 December 2015
Accepted 15 March 2016

Abstract

This research aimed to provide a characterization of fresh semen in wild and threatened Mediterranean brown trout (*Salmo cettii*), inhabiting the Biferno River basin and to study the variance of sperm parameters in a reproductive system with high male competition. The evaluated sperm quality parameters were: volume, density, viability, motility and sperm movement duration. High variability in sperm traits were found probably in response to the heterogeneity of individual condition, social status, spawning activity and migration costs. Duration of motility was positively correlated ($P<0.05$) with age class of breeders and motility. Stronger positive correlations ($P<0.001$) were observed between motility and viability and between sperm counts and viable sperms. This study reported the first data about semen quality of an Italian wild population of native trout. These results are useful for planning efficient artificial fertilization protocols in restoration programs involving supportive breeding together with other innovative conservation strategies as gamete cryopreservation.

Keywords: Mediterranean brown trout, sperm viability, sperm motility, native trout.

Introduction

The native trout population originally inhabiting the Biferno River basin such as the Adriatic and Tyrrhenian basins in the Molise region (South Italy) belongs to the Mediterranean brown trout species. It is reported as critically threatened and endangered under the taxonomical term *Salmo (t.) macrostigma* by IUCN (1996). Italian populations of Mediterranean brown trout are currently listed in the Italian IUCN Red List (Bianco *et al*., 2013) as "critically endangered" under the scientific name *Salmo cettii*. In this work, it was used the common name "Mediterranean Brown Trout" to identify this *taxon* to avoid confusion, a widely accepted name used to identify populations of brown trout inhabiting Mediterranean freshwater drainages. Native Mediterranean brown trout of Biferno River represent a local adaptation which show specific morphological and life-history features, as a distinctive migratory behaviour. Males and females reach the spawning grounds located at the headwaters of Bojano, after a spawning migration of several kilometres. The spawning period ranges from January to March.

The distribution range of native Mediterranean brown trout has suffered a gradual reduction over the last centuries. This could be largely attributed to the detrimental effects of anthropogenic disruptions, such as dam building, river straightening and local pollution. Hybridization of the native populations by domesticated Atlantic strains is considered to be one of the most serious threats to the long-term conservation of diversity within the species (Laikre, 1999; Ferguson, 2006).

After distinctive migratory patterns the native breeders reach the few suitable spawning sites resulting in high densities this is due to the loss of other optimal reproductive sites caused by pollution, water captations and anthropogenic barriers. Thus, the high breeder densities in the spawning grounds cause a strong male competition for female which strongly influences the individual and population fitness (Belmar-Lucero *et al*., 2012).

In such contest, sperm quality parameters are known to respond with plasticity among males depending on age, length, dominance and other life-history variables (Fleming and Gross, 1994; Liley *et al*., 2002; Bozkurt, 2006; Bozkurt *et al*., 2006a; Rudolsen *et al*., 2006; Janhunen *et al*., 2009; Pitcher *et al*., 2009).

Currently, in Molise region there is a growing interest by local administration and angling associations about conservation and restoration of autochthonous brown trout population. Together with

habitat reparation, the restocking of threatened and endangered fish involves selection of autochthonous wild spawners and artificial breeding programs.

In restocking programs, the hatchery conduction is also very important: the key factor is to plan artificial breeding avoiding induced selection and domestication in order to ensure the maintenance of biodiversity (Campton, 2004).

Therefore, the availability of semen with desirable quality is one of the critical factors necessary to increase the efficiency of artificial breeding of threatened fish species (Hajirezaee *et al*., 2009), allowing to increase genetic variability and effective size of broodstock, together with fertilization practices which minimize sperm competition and artificial selection of non-adaptive sperm traits. Currently, nothing is known about the semen quality of autochthonous Mediterranean brown trout of Biferno River.

Thus, the aim of our pilot study was to evaluate for the first time, the semen quality of the Biferno Mediterranean brown trout to inform artificial breeding programs for the most appropriate breeding practises. A preliminary correlation model between sperm parameters and individual age is also investigated.

Materials and Methods

Specimens Selection

Males (N=11) of Mediterranean brown trout were captured using electro-fishing during the peak of the spawning period (February 2014) from Biferno River at Bojano springs. The native Mediterranean brown trout were identified according to their phenotypic characteristics (Gibertoni *et al*., 1998; Jelli and Gibertoni, 1999; Penserini *et al*., 2006). Specimens were aged as 1+ to 4+ class by scalimetry (Lux, 1971). Individuals were then assigned to II, III, IV, V age classes corresponding to the number of winters passed from birth.

Collection of Sperm

Fish abdomens were dried before stripping in order to avoid contamination of semen with urine, mucus and blood cells. Afterwards, the semen was collected into 10 mL graduated glass tubes by gentle abdominal massage. Each male was stripped only once and released quickly back into the river. The total amount of expressible milt was collected individually and the sperm volume was expressed as mL. The semen samples were held in a mobile fridge (4ºC) before analysis.

Evaluation of Sperm Quality

The evaluated sperm quality parameters include sperm volume (mL), spermatozoa density ($\times 10^9$/mL), viability (%), motility (%) and spermatozoa movement duration (s). Sperm volume and motility of sperm were evaluated immediately and within forty minutes after sperm collection. 10 µL of the activation solution (0.3% NaCl) (Bozkurt, 2006) was added onto a glass microscope slide containing 5 µL of sperm at room temperature (20°C). Spermatozoa motility was observed under × 40 magnification and the percentage of motile spermatozoa were assessed. Only forward moving sperm were assessed as motile, whereas simply vibrating sperm were assessed as immotile.

The duration of spermatozoa movement was assessed using a chronometer that was started simultaneously with the addition of activation solution into the sample (Bozkurt, 2006).

Spermatozoa density was determined by using a Neubauer chamber. The semen was diluted 1/1000 and sperm counts were done in duplicate, at a magnification of 400 × and expressed as × 10^9/mL. The total number of sperm cells for ejaculate was calculated by multiplying sperm concentration ml^{-1} by ejaculate volume (Bozkurt, 2006).

Sperm viability was assessed using the LIVE/DEAD Sperm Viability Kit (Molecular Probes, Inc.) containing the fluorescent stains SYBR-14 and propidium iodide (PI). This procedure was performed on 1 µL of fresh semen, which were added to 40 µL of immobilizing medium (80 mM NaCl, 40 mM KCl, 0.1 Mm $CaCl_2$, 30 mM Tris-HCl, pH 9.2) (v/v). Subsequently, 2.5 µL of SYBR-14 working solution (diluted 1:100 in DMSO) were added to the cell suspension. After 10 min of incubation at room temperature in the dark, 3 µL of working PI solution (PI solution diluted 1:100 in phosphate buffered saline "PBS" diluent), was added to the cell suspension. Spermatozoa were incubated for a further 10 min under the same conditions (Martínez-Páramo *et al.*, 2009). Next, 10 µL of this suspension were placed on microscope slides, covered with coverslips and examined at a magnification ×1000 using a ×100 oil immersion objective under epifluorescence illumination. For each sample, approximately 200 spermatozoa were examined in duplicate aliquots.

SYBR-14, a membrane permeable nucleic acid stain, emits green fluorescence, whereas PI, a non-permeable probe, emits red fluorescence (Martínez-Páramo *et al.*, 2009).

Thus, spermatozoa showing green fluorescence are scored as alive and those showing red fluorescence as dead. The percentage of viable spermatozoa was calculated as the number of green cells × 100 divided by the total number of sperm counted. To calculate the total number of viable sperm cells per ejaculate we multiplied the sperm count by the percentage of viable cells.

Statistical Analysis

Results are presented by mean and standard deviation (mean ± S.D.) of each seminal parameter

value. Pearson's correlation among qualitative seminal parameters and the age class factor were investigated. These analyses were performed with SPSS (SPSS 15.0 for Windows, 2006; SPSS, Chicago, Ill). Multiple Regression Analysis was also performed to investigate additive and interaction effects among factors using R Project open sources program, testing only the simplest and more coherent *a priori* models in order to avoid data dredging (Burnham and Anderson, 2002).

Results

Fresh semen quality parameters of native population of Mediterranean brown trout (*Salmo cettii*) in the Biferno River (Molise Region - South Italy) are shown in Table 1. A large variability among the eleven specimens is apparent. The volume of semen collected ranged from 0.2 to 5 mL, while the sperm concentration ranged between 6.5×10^9 and 14.7×10^9 sperm cells per millilitre of semen. Significant heterogeneity was also evident for motility and its duration, ranging from 48% to 98% and from 52 sec to 113 sec, respectively. The viability range showed a minimum value of 53% and a maximum of 87%. The total number of sperm cells and viable spermatozoa per individuals, range from 2.9×10^9 to 63.2×10^9 and from 2.4×10^9 to 45.5×10^9, respectively.

Results of Pearson's correlation between sperm parameters are shown in Table 2. Significant correlation coefficients were found among duration and age ($P<0.05$), duration and motility ($P<0.05$), motility and viability ($P<0.001$) and sperm counts and viable sperms ($P<0.001$). Both the sperm counts and the viable sperms are strongly correlated with the ejaculate volume ($P<0.001$) but not with the concentration of sperm.

In the analysis of regression, the factors age class and viability explains respectively 49% and 38% of duration variance ($P<0.05$) (Figure 1).

Results of multiple regression show additive effect of the two factors age and Motility on duration of motility. The model with both age and motility (Duration *vs* Age + Motility) explains 69% of variance ($R^2=0.69$, $P<0.01$). No interaction effect between these two variables was detected ($P>0.05$). No significance additive or interaction effects were found among any of the other factors ($P<0.05$).

In this study the sperm motility values were similar to those obtained in Brown trout (Bozkurt *et al*., 2006, 2006b, 2011; Hatipoğlu and Akçay, 2010) and in Rainbow trout (Bozkurt *et al*., 2005, 2006c; Aral *et al*., 2007; Canyurt and Akhan, 2008), while higher values were found in Brown trout by Rainis *et al*. (2005) and in *O. mykiss* (Glogowski *et al*., 2000; Rainis *et al*., 2005; Şahin *et al.,* 2013).

In comparison to other salmonid species, the

Table 1. Sperm quality parameters in 11 Mediterranean brown trout individuals of native population in the Biferno river (Molise Region - South Italy)

Age class	Ejaculate volume (mL)	Sperm Concentration (10^9/mL)	Motility (%)	Duration of motility (s)	Viability (%)	Sperm count ($\times 10^9$)	Viable sperm count ($\times 10^9$)
II	4	13.0	85	52	79	52.0	41.4
II	2.5	13.9	82	65	71	34.8	25.0
II	0.2	14.6	95	68	86	2.9	2.4
III	2.5	14.7	50	75	67	36.8	24.9
III	1.5	9.2	48	55	53	13.7	7.3
IV	1.9	9.4	85	104	84	17.8	15.0
IV	0.5	11.1	80	93	84	5.5	4.3
IV	2	12.4	78	69	78	24.8	19.4
IV	5	12.6	78	80	71	63.2	45.5
IV	3.3	6.5	72	57	68	21.5	14.6
V	2.5	12.6	98	113	86	31.5	27.4
Mean ± SD	2.35 ± 1.4	11.81 ± 2.6	77.36 ± 15.9	75.55 ± 20.2	74.63 ± 9.4	7.7 ± 18.5	20.7 ± 14.1

Table 2. Pearson's correlation among sperm quality parameters and the age of Mediterranean brown trout native population in the Biferno river (Molise Region - South Italy)

	Age	Motility	Duration of motility	Viability	Volume	Concentration	Sperm count
Motility	0.11						
Duration of motility	**0.68*	0.43					
Viability	0.24	****0.88*	**0.62*				
Volume	0.11	-0.06	-0.18	-0.08			
Concentration	-0.44	0.22	0.05	0.30	-0.04		
Sperm count	-0.02	-0.02	-0.11	0.01	****0.93*	0.30	
Viable sperm count	0.02	0.10	-0.03	0.14	****0.91*	0.32	****0.99*

In bold significant correlation values: * at the 0.05 level; *** at the 0.001 level.

sperm concentration found in this study was higher in respect to that obtained in previous paper by different authors (Table 3), but was lower compared to sperm concentration found in Brown trout by Hatipoğlu and Akçay (2010) and Sahin *et al*. (2013a).

As reported in the Table 3 the sperm volume resulted less in respect to those found by the most of the authors, except by Aral *et al*. (2007) and Şahin *et al*. (2013).

To better evaluate the sperm quality parameters of Mediterranean brown trout inhabiting the Biferno River, after standardization, our data are graphically compared in Figure 2 with those of other brown trout populations or broodstocks. Sperm motility is similar to the mean values measured in other studies.

Discussion

In this pilot study, the semen quality of an Italian autochthonous Mediterranean brown trout population was evaluated for the first time. Semen quality exhibited high variance among the eleven specimens examined. This is expected in spawning ground frequented by a wild population characterized by consistent variability of biological, life-history that is, the extant of migration, and growth-rate among individuals (Rudolfsen *et al*., 2006). Previous studies have shown that sperm quality, depends on male age, season, exposure to female hormones, frequency of stripping (or reproductive events), feeding level and quality of their diet or rearing conditions both in wild and farmed stocks (Springate *et al*., 1985; McNiven *et al*., 1992; Rainis *et al*., 2005).

For comparison, the results of previous papers regarding sperm motility, concentration and ejaculate volume in trout are shown in Table 3. Sperm parameters of Brown trout (*Salmo trutta* complex) and rainbow trout (*Onchorynchus mykiss*) are taken into account as both represent important salmonid species of economic and zootechnical interest, also subject of conservation and restocking programs concerning the endangered wild populations.

Comparing with other brown trout stocks, the present study revealed concentration as partially in accordance with Stockley *et al*. (1997) that reported an increase in the sperm count and ejaculate volume in fish with greater sexual competition. The lower observed volume could be explained by our sampling methodology that relied on the capture of specimens directly at the spawning grounds during the reproductive peak. The volume of collected ejaculate may be largely dependent on the number of reproductive acts performed by each male especially for fish with multiple fertilization in high density spawning sites. Sperm volumes were highly variable, ranging from 0.2 to 5 mL, and represented the main factor affecting both the sperm number/ejaculate and the viable sperm/ejaculate, that were not correlated with the sperm concentration.

Semen quality, which includes measures of sperm motility, longevity and density, are considered the primary determinants of fertilization success (Casselman *et al*., 2006; Tuset *et al*., 2008). Sperm motility, for instance, has been found to predict fertilization efficiency (Rurangwa *et al*., 2004) and sperm velocity is probably the decisive factor for fertilization success under sperm competition (Levitan, 2000; Gage *et al*., 2004; Rudolfsen *et al*., 2008). Levitan (2000) observed that duration of motility is negatively correlated to spermatozoa velocity, assuming the existence of a trade-off between these two parameters. More recent discordant studies did not find negative correlations or evidence about trade-off between duration of motility and spermatozoa velocity in Atlantic salmon (Gage *et al*., 2004), or reported a positive relationship between the two considered parameters in *Arctic charr* (Janhunen *et al*., 2009) and *Coho salmon* (Pitcher *et al*., 2009).

A positive correlation between the age of the spawners and the duration of motility was identified in this study, whereas in previous studies on salmonids no difference were observed in the sperm

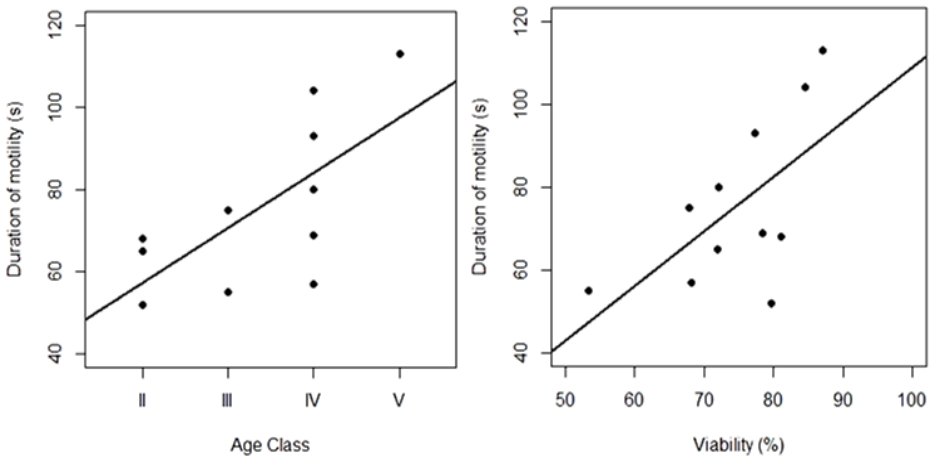

Figure 1. Graphical representations of linear model regressions of Duration *vs* Age Class (R^2=0.47, P<0.05) and Duration *vs* Viability (R^2=0.38, P<0.05).

Table 3. Sperm motility, concentration and volume of some salmonid species

	Motility (%)	Sperm Concentration (10^9/mL)		Ejaculate Volume (mL)			
		Mean	SD	Mean	SD	Mean	SD
BROWN TROUT (*Salmo trutta* complex)							
Present study	*S.cettii (S.t.macrostigma)*	77.36	15.9	11.81	2.6	2.35	1.4
Rainis *et al.*, 2005	*S.trutta*	94	-	8.49	-	4.54	-
Bozkurt *et al.*, 2006	*S.t.fario*	81	10.74	9.43	3.76	3.9	1.48
Hatef *et al.*, 2007	*S.t.caspius*	-	-	3.3	-	-	-
Hajirezaee *et al.*, 2009	*S.t.caspius*	61.475	-	2.775	-	13.825	-
Bozkurt *et al.*, 2011	*S.t.macrostigma*	80.37	2.36	6.02	0.46	13.93	0.84
Bozkurt *et al.*, 2006b	*S.t.abanticus*	75.2	3.24	-	-	-	-
Hatipoğlu and Akçay, 2010	*S.t.abanticus*	81.8	1.7	17.9	0.4	7.4	0.3
Sahin *et al.* 2013	*S.coruhensis*	-	-	13	4.93	1.6	0.64
RAINBOW TROUT (*Onchorynchus mykiss*)							
Glogowski *et al.*, 2000	*O.mykiss*	90	0	10.39	2.13	-	-
Rainis *et al.*, 2005	*O.mykiss*	100	-	1.76	-	18.57	-
Bozkurt *et al.*, 2005	*O.mykiss*	72.29	10.79	-	-	18.17	2.74
Bozkurt, 2006c	*O.mykiss*	75.3	15.05	7.7	4.31	9.3	3.33
Aral *et al.*, 2007	*O.mykiss*	73.25	5.15	6.06	0.9	1.22	0.22
Canyurt and Akhan, 2008	*O.mykiss*	78.25	3.63	-	-	-	-
Şahin et al., 2013	*O.mykiss*	96.7	8.16	8.4	4.75	17	4.56

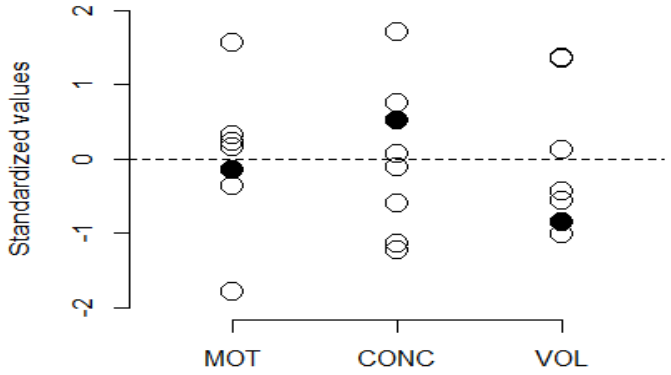

Figure 2. Graphical comparison between the results of present study on Biferno river Mediterranean brown trout (solid dots ●) and other papers (empty dots ○) in brown trout (*Salmo trutta* complex). Sperm quality parameters: MOT=motility, CONC=sperm concentration, VOL=ejaculate volume. Results are standardized with mean=0 and SD=1. Dotted horizontal line represents the centred mean.

longevity among age classes (Liley *et al.*, 2002) or, in general, no significant relationship between age classes and sperm quality was found (Campton, 2004). The positive correlation identified in the present work is not in accordance with life-history theories that assume an inverse relationship between age at sexual maturity and sperm competitiveness that occurs when small subordinate males compete with a high number of dominant males during spawning (Fleming and Gross, 1994).

High variance in sperm quality and sexual competition at high density results in greater variance in individual reproductive success and a greater reduction in the effective size of population (N_e) relative to the census size (N) (Belmar-Lucero *et al.*, 2012). High density of breeders in relation to the loss of suitable spawning grounds increases the risk of red superimposition (McNeil, 1964; Champigneulle *et al.*, 2003; Rubin *et al.*, 2004), further reducing the N_e/N ratio (Belmar-Lucero *et al.*, 2012). Moreover, the strong competition of the early stages after emergence results in further loss of population fitness by density-dependent mortality (Milner *et al.*, 2003).

Mediterranean brown trout populations in Biferno River are threatened by various types of human activities, namely environmental degradation,

recreational fishing and stocking of non-native population that often result in the erosion or extinction of local wild gene pools (Leary *et al*., 1993; Hansen and Loeschcke, 1994; Ryman *et al*., 1995a; Allendorf and Waples, 1996). In order to obtain an effective restoration program of the native trout, the protection and rehabilitation of habitats, the improvement in water quality, the removal of barriers for spawning migration and the control of fishing pressure under conscious regulation plans are necessary. Habitat improvement is the most desirable option because it should lead to long-term sustainable improvements with minimal deleterious ecological impacts.

Supportive breeding is also an important measure to reduce the short-term probability of population extinction, but artificial breeding programs should be designed so as not to further reduce the effective population size (Ryman *et al*., 1995b; Campton, 2004). Wedekind *et al*. (2007) suggests that hatchery-induced sperm competition not only increases the loss of genetic variation but may also induce artificial selection, depending on the fertilization protocols. Thus, the minimization of genetic risks linked to the *in vitro* fertilization is crucial.

Typical fertilization protocols have to be well designed for producing progeny from a relative limited number of eggs (Johnson *et al*., 2013) due to the possible difficulty in finding females of endangered pure autochthonous trout. However, the knowledge of sperm quality parameters can be used to minimize, at the same time, the probability to reduce genetic variability of the wild population.

In conclusion, the relationship between sperm quality parameters in a wild population of Mediterranean brown trout and we provide important information on fresh semen quality of native trout of Biferno River are described. These findings are crucial for planning efficient artificial fertilization design in restoration programs involving artificial breeding and other innovative conservation strategies such as gamete cryopreservation, considered an effective method to save endangered fish species by the storage of their gametes in gene banks (Gausen, 1993; Akcay *et al*., 2004; Martínez-Páramo *et al*., 2009; Iaffaldano *et al*., in press) and the utilization in crossed fertilization protocols.

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