Comparative study on milt quality features of different finfish species

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ABSTRACT

The aim of this research was to study the main sperm characteristics of three different finfish species. Twenty-one gilthead sea bream (*Sparus aurata*), 20 brown trout (*Salmo trutta*, morpha fario) and 15 rainbow trout (*Oncorhynchus mykiss*) male broodstocks, farmed under optimal conditions for each species and fed standard diets for broodstocks, were manually stripped. Brown trout yielded small amounts of sperm (4.5 *vs* 18.13 ml) that were very concentrated ($\cong$ 8.5 x 10^9 *vs* 1.24 x 10^9 Szoa/ml) with respect to the other species. The duration of spermatozoan motility for gilthead sea bream sperm was significantly longer (almost 50 min), in comparison to the one-minute motility of Salmonids. Single fatty acids of brown trout sperm were higher than in the other two species for almost all detected fatty acids. In particular, eicosapentaenoic acid (EPA) was at least three times more concentrated in brown trout than in rainbow trout or gilthead sea bream sperm (1238.3 μg/g *vs* 305.6 and 333.3 μg/g, respectively; P < 0.01). Saturated, polyunsaturated and total unsaturated fatty acid classes were significantly higher in brown trout sperm than in the other two species - almost double with respect to gilthead sea bream sperm and more than double in comparison to RT sperm (P < 0.01).

Key Words: Finfish, Milt, Fatty acid composition, Sperm motility, Sperm density.

RIASSUNTO
STUDIO COMPARATIVO DELLE CARATTERISTICHE QUALITATIVE DEL SEME DI SPECIE ITTICHE DIVERSE

*Lo scopo del presente lavoro è stato quello di analizzare le principali caratteristiche dello sperma di 3 diverse specie ittiche. Ventuno riproduttori di orata (*Sparus aurata*), 20 di trota fario (*Salmo trutta*, morpho fario) e 15 di trota iridea (*Oncorhynchus mykiss*), allevati in condizioni ottimali per ciascuna specie ed alimentati con diete standard per riproduttori, sono stati sottoposti a spremitura manuale. Le trote fario hanno prodotto volumi minori (4,5 vs 18,13 ml) di sperma, ma con una concentrazione maggiore di spermatozoi (≅ 8.5 x 10^9 vs 1,24 x 10^9 Szoa/ml) rispetto alle altre specie. Nell'orata, la motilità degli spermatozoi è risultata significativamente superiore (quasi 50 min) rispetto a quella dei Salmonidi (pari ad 1 minuto circa). Quasi tutti i singoli acidi grassi dello sperma di trota fario sono risultati più concentrati rispetto a quelli delle altre due specie ittiche. In particolare, l'acido eicosapentaenoico (EPA) è risultato almeno tre volte più concentrato nello sperma di fario rispetto a quello di trota iridea o di orata (1238,3 μg/g vs 305,6 e 333,3 μg/g, rispettivamente; P < 0,01). Similmente, le classi di acidi grassi saturi, poliinsaturi ed insaturi totali sono risultate significativamente più concentrate nello sperma di trota fario rispetto le altre due specie: quasi doppie rispetto a quelle di orata e più che doppie rispetto alla trota iridea (P < 0,01).*

Parole chiave: *Pesci, Liquido seminale, Composizione acidi grassi, Motilità spermatica, Densità spermatica.*

Introduction

The evaluation of milt quality in the reproduction of finfish species is an important tool for establishing its potential fertility. Unfortunately, its quality varies, depending on endogenous and exogenous clues: male age, season, exposure to female hormones, frequency of stripping, feeding level of broodstocks, quality of their diet or rearing conditions (Springate *et al.*, 1985; McNiven *et al.*, 1992). Lahnsteiner *et al.* (1998) observed a high correlation between sperm fertility and spermatozoa motility (percentage of motile spermatozoa, their velocity, type of swimming and flagellar beating frequency) and between seminal plasma composition and spermatic cell metabolism. Glogowski *et al.* (2000) found a negative correlation (- 0.41; P <0.05) between the fertilization rate of semen with protein concentrations of spermatozoa (expressed as g L⁻¹), but cryopreserved sperm was used in the study. Tvedt *et al.* (2001) observed a strict correlation between density and fertility in halibut (Hyppoglossus *hyppoglossus*). In fact, as noted also by Poole and Dillane (1998), poorly concentrated sperm usually has a higher percentage of immotile spermatozoa. Since density tends to reduce with male aging, a possible explanation of the fertility reduction can be related to that natural physiological phenomenon of density decrease (Buyukhatipoglu and Holtz, 1984). Consequently, according to Poole and Dillane (1998), qualitative evaluation of gametes should consider, not only motility and fertility rates, but also sperm concentration.

The lack of knowledge regarding sperm features of fish species also limits the possibility of drawing a general protocol for fish sperm cryopreservation. This technique would allow a more efficient and economic management of male brood-stocks, with consequential genetic improvement (Piironen, 1993). Until now, the methods to determine milt quality after thawing and to quantify the damage resulting from cryopreservation have not yet been standardised in aquaculture (Piironen, 1993; Lahnsteiner, 2000).

The fatty acid composition of cells has an important role for membrane function as well as fluidity (Aloya and Boggs, 1985; Muriana and Ruiz-Gutierrez, 1992). Differences in fatty acid composition can influence membrane functions, such as permeability and active transport (Hazel, 1972; Finstad and Thomassen, 1991), that are essential characteristics of special cells like spermatozoa. The extent to which their fatty acid composition can differ, according to species, could also provide useful indications with respect to sperm resistance to damage from freezing and thawing, and therefore as regards the viability of spermatozoa after cryopreservation (Pustowka *et al.*, 2000).

The aim of the present work was to compare the volume, the concentration, the motility (rate and duration) and the fatty acid composition of milt in two fresh water species: brown trout (*Salmo trutta*, morpha fario) and rainbow trout (*Oncorhynchus mykiss*), and a marine species: gilthead sea bream (*Sparus aurata*). The choice of these species is based on their importance in Southern Europe and in the Mediterranean Sea, both from an ecological and a commercial point of view.

Material and methods

All fish species were farmed under optimal rearing conditions and fed standard broodstock diets. They were hand-stripped, without any previous hormonal treatment, when it was full ripening season for the species: mid-December for rain-

Table 1. Average temperature and dissolved oxygen of the water during broodstock rearing phase.

Water parameters		GSB	BT	RT
Temperature	°C	18.0 ± 2.4	14.0 ± 0.9	13.0 ± 1.2
Dissolved oxygen	mg l⁻¹	7.5 ± 1.3	9.3 ± 0.9	7.6 ± 1.0

GSB: gilt-head sea bream; BT: brown trout; RT: rainbow trout.

bow trout, mid-January for brown trout and gilt-head sea bream. For each species the semen collection was made on two consecutive days. Table 1 reports water temperature and dissolved oxygen levels during farming phase of broodstocks up to ripening.

Fish and facilities

Gilthead sea breams (GSB) were farmed in a commercial hatchery (Ca'Zuliani, Monfalcone, Italy). Broodstocks were held in indoor concrete tanks (20 m³, water flow: 10 L sec⁻¹) with an artificial, decreasing, photophase. Twenty-one fish (two years old, 1130 g a. bw) that were ready to spawn were sampled.

Brown trout (BT), of the autochthonous strain *Judrio* (a local river of Friuli), were farmed in a public hatchery of Ente Tutela Pesca in the Friuli region (Flambro, Udine). Twenty specimens that were ready to spawn (three years old, 879.9 g abw), were sampled from indoor concrete tanks (2 m³, water flow: 2 l sec⁻¹).

Rainbow trout (RT) were farmed in the experimental farm of the Animal Husbandry Department, University of Torino, Italy. Fifteen specimens (two years old, 940 g abw) were randomly sampled from indoor fiberglass tanks (3 m³, water flow: 0.2 l·sec⁻¹).

Diets

The diets fed to the different broodstocks were standard for each species and contained 25-30 % fish meal and 10-12 % fish oil. In particular, GSB were fed a commercial diet for marine species (Vitalis Repro), while diets for BT and RT were formulated and manufactured, respectively, at the Animal Science Department, University of Udine, Italy, and at the Zootechnical Science Department, University of Torino, Italy. Table 2 reports the proximate composition of the diets, determined according to AOAC (1995) methods. Broodstocks were fed once every two days at 0.5 % a.b.w.

Reproductive variables

Ready to spawn males were sampled and the following variables determined:
1. Body weight, before stripping (g);
2. Total and relative sperm volume (ml and ml/kg bw);
3. Sperm density (Szoa x 10^9/ml and Szoa x 10^9/Kg bw);
4. Spermatozoan motility (in % e min) of fresh or stored samples (for 18 h at 4°C, in refrigerator);
5. Sperm fatty acid composition. For extraction and methylation of fatty acids the Folch method (1957) and external marker (C19:0) were adopted. The detection of methyl fatty acids was carried out by gas-chromatography (trace GC 2000 Thermo Finnigan, USA) with the following operative conditions: Owen: 2 min at 160°C, from 160°C to 240°C at +5°C/min; Carrier: H_2, 1.2 ml/min; Detector: FID, 250°C; Injector: 1ml, split 100:1, 250°C; Column: Omegawax 320 Supelco, 30m x 0.25mm ID x 0.25 mm film.

Fish handling and milt analysis

All fish were anaesthetised with tricaine methane sulfonate (Argent Laboratories, USA; 100 mg L⁻¹ diluted in the tank water for 5 minutes) and were subject to manual abdominal pressure (as for standard gamete collection in dry fertilization, commonly used for Salmonids) and the total

Table 2.   Chemical composition of the diets fed to male broodstocks of the three finfish species (data expressed as % DM, except dry matter, %).

	GSB	BT	RT
Dry Matter	90.1	92.3	88.5
Crude Protein	52.9	47.6	52.6
Ether extracts	19.5	18.3	24.1
Ash	10.9	6.6	9.5
Crude fibre	1.9	2.5	2.0
N-free extract	14.8	25.0	14.8

amount of sperm was collected in volumetric glass tubes. During manual stripping, small samples of sperm were sampled in order to avoid any contamination with organic fluids. The glass tubes were kept on ice and samples analysed immediately. One μl of milt by each male was mixed with a drop of distilled water (for Salmonids) (Mims, 1991) or salt water (for gilthead sea bream) (Sorbera *et al.*, 1996) and then observed on a microscope slide (400 X magnification), without the addition of any specific extender. The rate of spermatozoan motility (immediately after the water) and the duration of flagellar movements (of at least 5 % of the observed spermatozoa in the sample) were evaluated immediately after milt collection and after 18 h storage period at +4°C (overnight storage in refrigerator).

Concentration of sperms was determined by counting the number of spermatozoa in a sample diluted with distilled water (10501 x) in a Bürker hemocytometer, under 400 X magnification (Rainis *et al.*, 2003). To avoid subjective bias, all tests on sperm quality were carried out by the same person.

Statistical analysis

Data were analysed using a 1-way ANOVA. For motility variables a 2-way ANOVA was made, considering species and type of storage (fresh *vs* refrigerated sample). Differences among means were compared with LSD test (Snedecor and Cochran, 1982).

Results

Brown trout spawned a smaller amount of milt (4.54 ml), almost 75 % less than rainbow trout and gilthead sea bream (18.13 ml; p<0.01; Table 3). Also, the relative sperm volume of BT was significantly lower, almost 1/3rd than the other two species (5.69 *vs* 17.5 ml/Kg; p<0.01; Figure 1). On the contrary, sperm density was significantly higher: 8.49x109 for BT *vs* 1.24x109 Szoa/ml of the other two species (p<0.05). When density was correlated to the male weight, RB and BT showed similar values (35 x 109 Szoa/Kg and 46.10x109 Szoa/Kg, respectively), markedly higher than GSB males (11.57 x 109 Szoa/Kg; p<0.01; Figure 2).

Motility of fresh samples did not show significant inter specific differences: values ranged from

Table 3.   Volume, density and motility (% and duration in min) of fresh sperm and after refrigeration (+4°C for 18h) of the three finfish species.

		GSB	BT	RT	SEM	df
Milt volume: Total	ml	17.70[A]	4.54[B]	18.57[A]	11.86	52
Density: Szoa/ml x 10⁹		0.72[B]	8.49[A]	1.76[B]	3.07	52
Motility (min): Fresh samples After 18 hrs		49.51[A] 32.52[B]	1.31[C] 1.14[C]	0.99[C] 0.92[C]	10.56 10.56	99 99
Motility (%): Fresh samples After 18 hrs		92.73[A] 79.00[B]	94.00[A] 59.00[C]	100.00[A] 25.00[D]	25.89 "	99 "
Reduction of the % motility[1]:						
	min	44.60	3.40	7.00	52.43	49
	%	14.80[B]	37.4[AB]	75.00[A]	53.20	49

*Means on the same line not sharing a common superscript letter are significantly different; A,B,C: P <0.01.*
Motility: comparison on vertical and horizontal lines. Means not sharing a common superscript letter are significantly different; A,B,C: P <0.01.
df: degree of freedom

[1][(Motility on fresh milt- Motility of stored milt)/Motility on fresh milt]*100.

Figure 1.   Relative volume (ml/Kg) of sperm yielded by the three finfish species
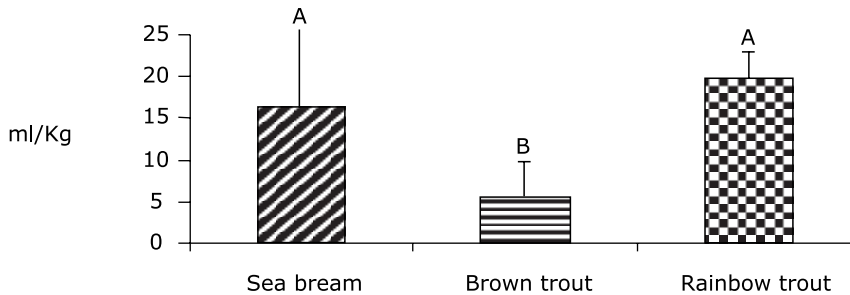
Figure 2.   Relative density (Szoa/Kg*109) of sperm yielded by the three finfish species
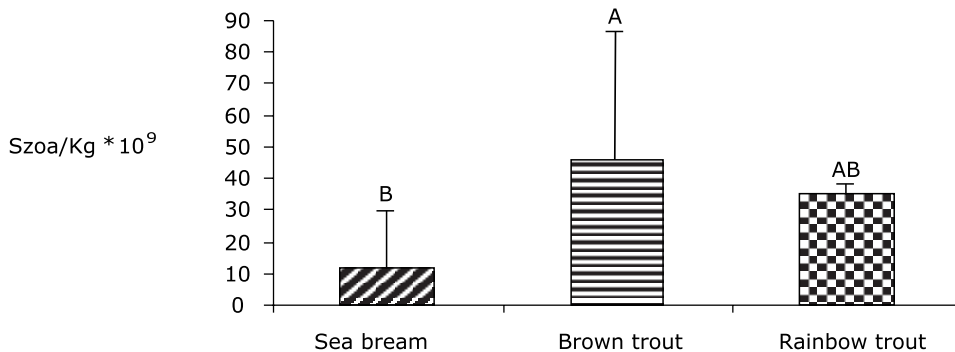
100% (RT) to 92.73% (GSB) (Table 3). Overnight storage significantly affected motility of sperms in all species, but GSB sperm motility was significantly higher than BT and RT sperm motility (79 > 59> 25 %, respectively, p < 0.01, Table 3). Consequently, the reduction of motile spermatozoa rates was higher for RT (75 %) than for GSB (~15 %) (p<0.01; Table 3).

Significant differences were observed also in the duration of flagellar movement. In fact, while sperm motility in Salmonids was approximately one minute (1.31 - 0.99 min for BT and RT respectively), in GSB the sperm motility lasted almost 50 min (p<0.01) (Table 3). The duration of sperm motility, after overnight storage, confirmed the differences observed for fresh milt: the GSB milt lasted more than 30 min, significantly more than the flagellar movement of Salmonids (~ 1 min) (p< 0.01, Table 3).

The main fatty acid concentrations in milt of the different finfish species are reported in Table 4. In general, single fatty acids of BT milt were higher than in the other two species, for almost all detected fatty acids. In particular, palmitic acid, which reached a concentration of 1989.2 μg/g in BT, was significantly higher than in RT (348.0 μg/g) or GSB milts (814.4 μg/g) (p<0.01). Also C18:2 n-6, a precursor of arachidonic acid (AA), reached a concentration in BT (355 μg/g) higher than in salmonid milt (186.9 - 31.1 μg/g) (p<0.01). In addition, linolenic acid, a precursor of n-3 PUFA, showed a similar trend, with the highest concentration in BT milt (26.1 μg/g) and the lowest in RT (5.4 μg/g) (p<0.05). On the contrary, stearic acid and oleic acid did not show significant different concentrations among species (Table 4). Eicosapentaenoic acid (EPA) was at least three times more concentrated in BT than in RT or GSB

Table 4.   Fatty acid composition of sperm yielded by the three finfish species (μg f.a./g tissue).

	GSB	BT	RT	SEM (23 df)
C10:0	0.1[B]	0[B]	1.1[A]	0.3
C12:0	0.7[B]	11.2[A]	0.4[B]	2.9
C14:0	42.6[B]	231.3[A]	23.6[B]	41.6
C15:0	16.5[B]	27.4[A]	4.6[C]	8.5
C16:0	814.4[B]	1989.2[A]	348.0[B]	502.8
C16:1 n-7	136.9[a]	127.6[b]	21.7[c]	62.4
C16:2 n-4	20.5[a]	11.5[ab]	0.2[b]	10.5
C17:0	21.9[a]	21.5[a]	4.7[b]	10.4
C16:3 n-4	19.9[A]	13.7[A]	2.2[B]	8.4
C16:4 n-1	8.9	9.50	3.6	6.1
C18:0	280.3	237.0	86.0	154.0
C18:1 n-9	789.4	665.7	154.2	397.0
C18:1 n-7	128.4[B]	466.2[A]	90.0[B]	8.5
C18:2 n-6	186.9[B]	355.1[A]	31.1[B]	100.9
C18:2 n-4	6.2[B]	25.7[A]	0.2[B]	5.11
C18:3 n-6	4.9	3.2	3.7	2.6
C19:0	3.9	1.1	0.0	6.5
C18:3 n-4	5.9	11.2	2.0	5.7
C18:3 n-3	18.1[b]	26.1[a]	5.4[c]	8.3
C18:4 n-3	6.8[b]	12.9[a]	6.1[b]	4.4
C18:4 n-1	1.7[B]	10.9[A]	4.4[B]	2.9
C20:0	7.3	3.4	2.1	4.6
C20:1 n-9	69.2[a]	55.7[ab]	17.4[b]	31.4
C20:3 n-9	6.7	10.5	2.9	4.8
C20:2 n-6	14.1[b]	34.2[a]	7.0[b]	14.9
C20:3 n-6	14.6	27.1	4.0	21.5
C20:4 n-6	137.2[B]	269.0[A]	73.6[B]	94.1
C20:3 n-3	4.8	7.9	2.3	33.1
C20:4 n-3	19.1[B]	48.3[A]	10.4[B]	12.1
C20:5 n-3	333.3[B]	1238.3[A]	305.6[B]	70.0
C22:0	1.9[B]	9.9[A]	3.3[B]	2.3
C22:1	16.9	9.7	7.8	8.2
C21:5 n-3	8.2[B]	31.9[A]	6.0[B]	7.5
C22:4 n-6	28.8	65.8	21.2	29.8
C22:5 n-3	114.1[B]	221.6[A]	50.0[B]	66.8
C24:0	4.5	2.8	3.5	4.8
C22:6 n-3	1048.4[B]	2691.4[A]	627.6[B]	687.2
C24:1	18.4	12.6	3.1	21.6

Means on the same line not sharing a common superscript letter are significantly different;
a,b,c: P<0.05; A,B,C: P<0.01.

Table 5. Main fatty acids classes and ratios of milt yielded by the three finfish species (μg f.a./g tissue).

	GSB	BT	RT	SEM (23 df)
Σ SFA	1211.20[B]	2544.30[A]	477.30[B]	709.10
Σ MUFA	1159.20[a]	1337.60[a]	294.30[b]	569.20
Σ PUFA	2009.60[B]	5126.70[A]	1171.07[B]	1288.00
Σ USFA	3168.80[B]	6463.70[A]	1466.00[B]	1837.00
SFA/USFA	0.38	0.39	0.33	0.03
Σ n-3	1552.80[b]	4278.40[a]	1013.40[b]	1031.00
Σ n-6	386.60[B]	754.50[A]	140.50[B]	233.90
Σ n-9	865.20[A]	731.90[A]	174.50[B]	425.80
n-3/n-6	4.01[C]	5.67[B]	7.21[A]	0.55
DHA/EPA	3.20[A]	2.17[B]	2.04[B]	0.39
DHA/AA	7.85	10.07	8.54	2.32

Means on the same line not sharing a common superscript letter are significantly different;
a,b,c: P<0.05; A,B,C: P<0.01.

milt (1238.3 μg/g *vs* 305.6 and 333.3 μg/g, respectively). The same was true for DHA: 2691.4 *vs* 627.6 e 1048.4 μg/g for BT, RT and GSB respectively; p<0.01).

Saturated, polyunsaturated and total unsaturated fatty acid classes were significantly higher in BT sperm than in the other two species: almost double GSB sperm and more than double RT sperm (p< 0.01; Table 5). Saturated/unsaturated fatty acids ratio was unexpectedly similar for all species, ranging from 0.33 to 0.38 (Table 5). N-3 and n-6 fatty acid series were also more concentrated in BT than in the other species, while n-9 series fatty acids, did not differ significantly between *Salmo trutta* and *Sparus aurata* milts. The highest n-3/n-6 ratio was found in RT milt (7.21), the lowest in gilthead sea bream (4.01) (p<0.01, Table 5).

Significant differences were observed for DHA/EPA ratio, which was higher in GSB milt (3.20 *vs* 2.10), while DHA/AA ratio in the sperm did not differ among species (7.85 - 10.07).

Discussion

According to Poole and Dillane (1998) and Lahnsteiner (2000), we observed that brown trout yields small amounts of sperm, but with high concentration. This is probably due to the evolution of the species, whose reproduction in nature happens in small spaces (the sources and the upper areas of streams) and whose males induce females to spawn by aggressive raps on their abdomens. Consequently it is not necessary for sperm to have a long duration motility to reach spawned eggs, as was effectively observed.

The rate of motile fresh sperm was high for all three species (> 92%), but surprisingly the duration of the sperm movement for GSB was very long: approximately 50 min. Further research is needed to confirm this phenomenon, eventually considering the type of energetic metabolites characterising sea bream spermatozoa. Also in this case there is probably a species adaptive-strategic explanation (opposite to BT), related to the type of environment where fecundation occurs: open sea and large spaces to go through by sperm to reach the eggs.

At the moment, they are available only laboratory methods, rather complicated, consisting of demembranation and further reactivation of spermatozoa to evaluate the movement and the recovery activity of spermatic cells. Unfortunately, they are not feasible at farm level (Bromage and Roberts, 1995). McNiven *et al.* (1992) concluded

that, from the operative and practical point of view, motility evaluation by microscope and Bürker hemocytometer, is quick, sufficiently accurate, and relatively easy to apply at farm level. Motility made it possible to analyse some "indefinite" spermatozoa properties, which are important for egg fertilization (Munkittrick and Moccia, 1987). Obviously, some operative limits of the motility determination by microscope must be considered, as the method is not as objective as a CASA system and it is affected both by the experience of the laboratory technician and by the operative conditions. Moreover, the brief duration of the cell movements is an important source of error (Labbe and Maisse, 2001). According to Lahnsteiner *et al.* (1999) the method would be suitable only for determining the initial motility, but not for the evaluation of the total duration of the flagellar movement.

Sperm is characterised by many intra- and inters- specific differences in its chemical composition. Inorganic ions play a fundamental role in the osmotic pressure and in spermatozoa activation. In the case of fresh water species, the activation of the sperm motility is determined by the reduction of the hydrostatic pressure and of the extra-cellular K^+, but with a relative increase in Ca^{++}. In marine species, this physiological process is triggered in a different way, because spermatozoa need a hyper-osmotic environment to be activated, with respect to the inter-testicular fluids of the animal (Ciereszko *et al.*, 1996).

Milt concentrations that were observed in brown trout (8.49×10^9 Szoa/ml) fall within the range reported by Poole and Dillane (1998) for the species ($2.2\text{-}26.7 \times 10^9$ Szoa/ml), while for rainbow trout our data (1.76×10^9 Szoa/ml)) were lower than those of Campbell *et al.* (1994) ($13\text{-}16 \times 10^9$ Szoa/ml). Very few data are available for gilthead sea bream semen concentrations. Barbato *et al.*, (1998) observed $15\text{-}25 \times 10^9$ Szoa/ml densities in GSB milt (irrespective of male weight) higher than those of the present work (0.72×10^9 Szoa/ml), but the same authors pointed out the high variability and the irregular trends of data concerning milt volume and spermatozoa count. Many other factors, except weight, can cause wide ranges in milt concentration. We should consider different rearing systems (cages or inland concrete tanks), farm-

ing temperature, different periods of the reproductive cycle for gamete sampling and so on. This field still lacks of standardisation for broodstock farming and nutrition, to detect sure effects of certain variables on sperm traits.

Conclusions

Milt characteristics in finfish vary according to the investigated species. Brown trout males spawn small amounts of sperm that is very concentrated. Gilthead sea bream milt has opposite traits: relatively large volumes but less concentrated than BT sperm and with long duration of motility (approx. 50 min). These differences could be related to the type of environment where these species naturally spawn.

BT milt shows significantly higher concentrations of the main fatty acids or classes (n-3, n-6, SFA, MUFa and PUFA) than RT or GSB milt. In particular, linolenic (26.1 *vs* 11.75 μg/ g tissue), arachidonic acid (269 *vs* 105.4 μg/ g tissue), EPA acid (1238 *vs* 319.5 μg/ g tissue), and DHA (2691.4 *vs* 838 μg/ g tissue) were all significantly more concentrated in BT milt than in the other two species.

In conclusion, it should be noted that, both researchers and farmers (in commercial hatcheries) fail to sufficiently take into consideration the importance of male broodstocks and the quality of their milt for aquaculture production. Standardised procedures should be developed, also as respects the cryopreservation of finfish milt, based on the practical consequences that would result for products used in frying.

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