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# REPRODUCTIVE PROCESS IN BULGARIAN TROUT FARMS IN RELATION TO THE PREVENTION OF M74 SYNDROME

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## Abstract

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Currently, the term syndrome M74 combines all reproductive disorders of hydrobionts (including their gametes, embryos and yolk-sac fry) with similar symptoms caused by xenobiotics transposed in aquatic organisms from polluted environment.

Because in Bulgaria there are only few publications on the M74 and the syndrome is not known to fish farmers in the branch, the aim of the present study was to analyze the reproductive process in Bulgarian trout farms, located in different mountains regions and make recommendations for preventing M74 syndrome.

This is why for the first time in the country were developed criteria for assessing the risk of syndrome M74 and questions were formulated in a special „Questionnaire“. The survey data from 14 Bulgarian trout farms were calculated to analyze the reproductive process and investigate the possibility for spreading of the syndrome M74 mainly on farms located in the following mountains: Rila, Rhodope, Stara Planina and Sredna Gora. Additionally were investigated commonly used biological parameters for assessment of the reproductive syndrome M74 – eggs fertilization, hatchability and survival rate of the rainbow trout fry. Better reproductive parameters were registered in 1$^{st}$ rank trout farms (0-5 points) which have no problems with the M74 syndrome. In the rest farms the unsuitable environmental conditions, which lead to occur M74 syndrome can be partly compensated using a sperm activation medium №49397 for artificial insemination of trout eggs.

*Key words:* salmonidae; artificial insemination; sperm activation medium; M74 syndrome

## Introduction

Multifaceted human activity often causes environmental pollution of different nature. Along with the traditional problems accompanying biotechnology of breeding fish in recent decades a global problem also appears primarily affecting salmonids, known as M74 syndrome. Called the name of the river Indalsalven (Swedish “Miljo”) – power hatchery Bergefors in Sweden, where it was first found in salmonids in 1974 (Ackefors et al., 1991). “Miljobetingad” in Swedish means the environment around the river “Miljo”. In the 90s of the previous century trout farms in Sweden and Finland were massively affected by the M74 reproductive syndrome. The observed mortality clutch in some places reaches up to 80%. In 2003-2005 it was below 10% and in 2005-2010 increased again, but did not exceed 30% (Keinänen et al., 2012). Mortality of the larvae in some rivers was up to 96% (Soivio, 1996; Karlström, 1999).

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The syndrome M74 is widespread in Baltic salmon and sea trout (Bengtsson and Westernhagen, 1996; Amcoff et al., 2002; Pickova et al., 2003; Vuori et al., 2006). A number of studies have linked the syndrome with xenobiotic attacks, production systems and expression of biotransformation enzymes (Lundström et al., 1999; Pesonen et al., 1999; Zhelyazkov, 2014; Zhelyazkov et al., 2015; Stoyanova et al., 2016). Not all studies, however, failed to prove the link between xenobiotic death of embryos and their larvae (Mac and Schwartz, 1992; Smith et al., 1994; Fitzsimons, 1995; Asplund et al., 1999). This syndrome is primarily associated with the depletion of thiamine (vitamin $B_1$) in adults and larvae (Amcoff et al., 2000; Koski et al., 2005; Wolgamood et al., 2005). The etiology of M74 is also associated with Cayuga – syndrome (Fisher et al., 1995a, b). Both syndrome clinical symptoms can be ameliorated or prevented by administration of thiamine (Amcoff et al., 2000; Brown et al., 2005; Fitzsimons et al., 2005). The vitamin $B_1$ is an essential cofactor in various basic, metabolic or neurological reactions (Koski et al., 2005) and its absence in egg is due to thiaminase dietary parents. In this aspect, a number of studies dealing with the diet of nutrition and the link between population during the years of sprat (*Sprattus sprattus L.*) and herring (*Clupea harangus membras L.*), and the mortality rate due to reproductive syndrome M74 (Karlsson and Karström, 1994; Mikkonen et al., 2011; Keinänen et al. 2012) were elaborated.

These fish serve as the main food of Baltic salmon and have very low thiamine, especially young sprat, which is higher in fat and unsaturated fatty acids. It has been found that low levels of vitamin $B_1$ in fresh fertility caviar grains of Atlantic salmon (0.19 $nmol.g^{-1}$) and newly hatched fry (0.09 $nmol.g^{-1}$) are responsible for the expression of the syndrome M74 (Amcoff et al., 1996). For comparison, the content of thiamine in unaffected syndrome fertilized eggs and fry were usually 1.70 $nmol.g^{-1}$ and 1.27 $nmol.g^{-1}$ respectively. The authors also managed to prevent mortality of fry site contains them in the thiamine bathroom with a concentration of vitamin $B_1$ 500 ppm. It has been shown that from the 44 familial groups whose thiamine concentration in the eggs is below to 0.33 $nmol.g^{-1}$, 43 develop syndrome M74. The egg color of salmonid depends on ketokarotens content, mainly astaxanthin and canthaxanthin (Czeczuga, 1975, 1976; Craik, 1985). The low level of these and other antioxidants can cause oxidative stress and peroxidation of lipids (Pettersson and Lignell, 1996; Atanasoff et al., 2012). Based on the content of the carotenoids in eggs attempts have been made to diagnose the syndrome M74 of salmon (Lignell, 1994; Pettersson and Lignell, 1999; Keinänen et al., 2008)

Currently, the term syndrome M74 combines all metabolic effects and pathologies of hydrobionts (incl. their gametes, embryos and yolk-sac fry) with similar symptoms caused by xenobiotics caught in aquatic organisms from polluted environment.

In Bulgaria there are only few publications on the M74 and the syndrome is not known to fish farmers in the industry (Atanasov, 1995, 2005; Atanasov et al., 2015).

The aim of the present study was to analyze the reproductive process in Bulgarian trout farms, located in different mountains regions and make recommendations for preventing M74 syndrome.

## Material and Methods

For analyze of the reproductive process and the risk assessment for the Bulgarian trout farms in order to identify Syndrome M74, for the first time detailed questionnaire has been developed and used. Survey was conducted by especially skilled team (Table 1).

***Investigated trout farms.*** The survey for risk assessment of syndrome M74 of rainbow trout *(Oncorhinchus mykiss W.)* was carried out by means of a questionnaire and on a sample of 14 fish farms which were selected from following mountain regions: Rila, Rhodope, Stara Planina and Sredna Gora.

***Investigated reproductive indicators.*** Additionally were investigated commonly used biological parameters for assessment of the M74 reproductive syndrome – eggs fertilization rate, hatchability rate and survival rate of the rainbow trout fry (Dimitrov et al., 2000; Atanasov et al., 2006).

***Investigated period.*** The study was conducted during 2014 – 2016 year.

***Used sperm activation medium***. For the purposes of the survey were used results from this and previous investigations, where for the artificial insemination of trout eggs besides fresh water, a sperm activating medium №49397 was used (Atanasov, 2005; Atanasov et al., 1991b; 2015).

***Statistical analysis.*** Statistical processing of the results was computed by the program STATISTICA 6 using ANOVA test.

## Results and Discussion

The following Figure 1 presents data from the risk assessment of syndrome M74 of rainbow trout *(Oncorhinchus mykiss W.)* in ranked Bulgarian trout farms.

From the results it becomes apparent that there is a wide range of trout farms having from 3 to 14 points and one is with 18 points. In 21.43% from these farms (I rank) surveyed to assess the syndrome M74 of reproduced rainbow trout have no problems with it and 50.00% from them (II rank)

**Table 1**
**Questionnaire – for analyze of the reproductive process and the risk assessment for the Bulgarian trout farms in order to identify Syndrome M74**

| № | A sign | Yes, points | No, points |
|---|---|---|---|
| 1 | Farm imports fertilized eggs from Scandinavian and Baltic countries | 1 | 0 |
| 2 | Broodstock have not typical bright color during the breeding season and pronounced signs of sexual maturity and timely readiness for breeding | 1 | 0 |
| 3 | Milking eggs is problematic with accompanying hemorrhages | 3 | 0 |
| 4 | Yielded eggs are pale yellow | 2 | 0 |
| 5 | In the eggs grains there is fat drop or precipitate | 2 | 0 |
| 6 | Milking sperm is problematic with accompanying hemorrhages | 3 | 0 |
| 7 | The activity of the spermatozoa in water is less than 35 sec. | 3 | 0 |
| 8 | Fertility of eggs is below 50% | 6 | 0 |
| 9 | Embryonic mortality is over 50% | 6 | 0 |
| 10 | Hatchability is below 50% | 6 | 0 |
| 11 | Yolk sac of fry has gray precipitate | 2 | 0 |
| 12 | Yolk sac of fry has fat drop | 2 | 0 |
| 13 | Yolk sac of fry is swollen | 2 | 0 |
| 14 | Fry have exophthalmos – bulging eyes | 2 | 0 |
| 15 | Fry carried uncoordinated, subdued movements and rapidly depleting | 3 | 0 |
| 16 | Fry have grayish color due to the discoloration of the skin | 2 | 0 |
| 17 | Fry have visible hemorrhage | 3 | 0 |
| 18 | Feeding fry is problematic | 4 | 0 |
| 19 | Survivability of fry is below 50% | 6 | 0 |
| 20 | Water supply of the farm is carried out by catchment region with anthropogenic pressure | 2 | 0 |

***Ranking in surveyed fish farms:***
I rank (0-5 points) – the farm has no problems with the M74 syndrome;
II rank (6-10 points) – the farm has potential problems with the M74 syndrome;
III rank (11-17 points) – the farm has partial problems with the M74 syndrome;
IV rank (18-30 points) – the farm has serious problems with the M74 syndrome;
V rank (31-45 points) – the farm has very serious problems with the M74 syndrome;
VI rank (46-60 points) – the farm has extreme problems with the M74 syndrome

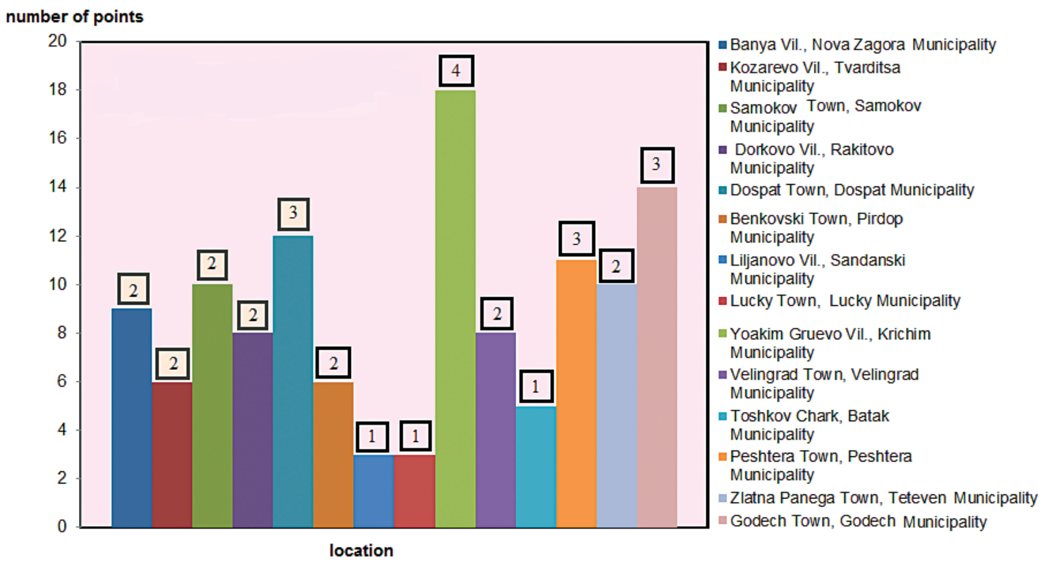


**Fig. 1. Ranking of the surveyed trout farms**

have potential problems. The rest 21.43% fish farms are in III rank, having partial problems and only one is IV rank and has serious problems with the M74 syndrome.

With the serious risk of M74 reproductive syndrome is characterized only 1 trout farm subjected to anthropogenic pressures. This farm is located nearly to the village of Joakim Gruevo, Krichim Municipality enjoying water from the lower reaches of the river Vatcha is a favorite in points. It is due to the anthropogenic pressure, including three big dams built along the river and the large net cages farms situated in their aquatory. Probably frequent eutrophication of the water and the accumulation of various xenobiotics in the body can cause reproductive disorders at trout in the farm.

Furthermore, the study itself showed that the fish farmers are not at all aware of the existence of this problem and its nature. It required the publication of a scientific paper for introducing the fish farming branch with the syndrome M74 (Atanasov et. al., 2015).

In many Bulgarian farms the activity of trout spermatozoa in water is less than 35 s and the eggs fertility is below 50% (see questions number 7 and 8 in "Questionnaire"). The following figures show the results on conducted reproductive experiments in different ranks studied fish farms (Figure 2, 3).

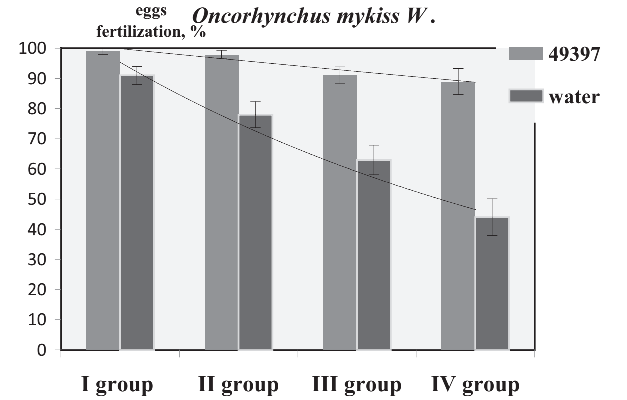


**Fig. 2. Rainbow trout eggs fertilization rate after artificial insemination with semen, activated with fresh water and sperm activating medium № 49397**

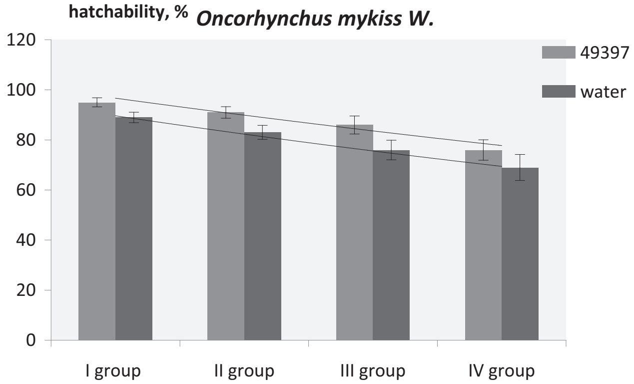


**Fig. 3. Rainbow trout eggs hatchability rate after artificial insemination with semen, activated with fresh water and sperm activating medium № 49397**

It could be seen that the sperm activating medium № 49397 have excellent effect on trout eggs fertilization in farms from all ranks. Furthermore, the slope of the exponential curve which depended on the ranks was much higher. This proves that the unsuitable environmental conditions which lead to M74 syndrome, which can be partly compensating using sperm activation medium 49397 for artificial insemination of trout eggs. In contrast, using fresh water, as a natural sperm activator, fertilization rate depended on physic-chemical parameters of water. These studies confirm our previous investigations on salmonids semen used for artificial insemination (Atanasov et al., 1991a, 1991b, 2004; Atanasov, 1995, 2005; Georgiev et al., 1991).

The data from the figure show that the eggs hatchability rate depends mainly on the ranks of fish farms (environmental conditions), not only from the artificial insemination, improved by using sperm activating medium № 49397. In this aspect the methods of assisted reproduction cannot compensate entirely the negative influence of xenobiotics on embryonal survival. In other words, successful fertilization does not guarantee successful hatching, especially for eggs in trout farms from IV and higher rank. This is evidenced by the same slopes of the exponential curves of this figure.

Additionally, in some fish farms where a few batches imported fertilized eggs were hatched, it was noticed that the orange eggs have better hatchability in comparison with the yellow ones (see questions 1 and 4 in a "Questionnaire"). This fact confirmed our previously found effects of specialized trout fish feed with astaxsantin and cantaxantin on color and hatchability of eggs (Atanasov et. al., 2015; Tzanova et al., 2016). According to Maoka (2011) carotenoids in marine animals have protective effects on their reproductive functions. Raposo et al. (2015) similarly studied the carotenoids from marine microalgae as a valuable natural source for the prevention of chronic diseases.

At the next Figure 4 is shown a yolk sac fry survival after hatching of Rainbow trout in studied fish farms.

The slope of the extrapolation curve on Figure 4 proves that the survivability of the yolk-sac fry depends on the quality of eggs derived from different trout farms. The presented

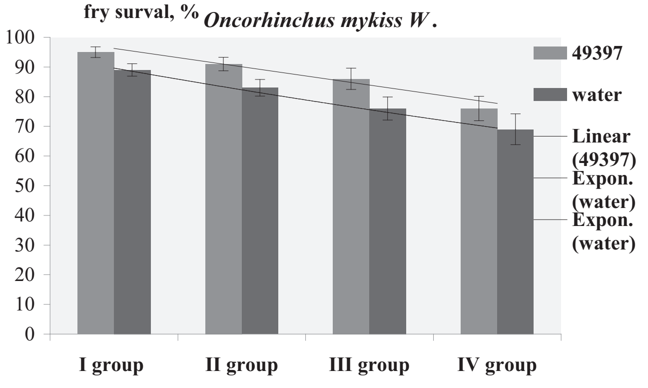


**Fig. 4. Rainbow trout yolk sac fry survival after hatching**

results are not surprising because newly hatched trout fry powered its self-energy and metabolic needs from yolk sac (Atanasov et al., 1999a, 1999b). Exactly post-hatching development (generally early developmental stages of *Salmonidae*) is the most critical period due to thiamine deficiency and the accumulated xenobiotics in yolk sac.

Moreover, survival of the larvae largely depends on the ambient conditions (Armstrong and Nislow, 2006; Viant et al., 2006; Finn, 2007; Keinanen et al., 2012). According to Vuori et al. (2004), Baltic salmon (*Salmo salar*) yolk-sac fry mortality is associated with disturbances in the function of hypoxia-inducible transcription factor (HIF-1alpha) and consecutive gene expression. After yolk sac utilization and switching to exogenous feeding, the M74 symptoms disappear under normal conditions (Atanasov et al., 2015).

## Conclusion

For the first time in Bulgaria criteria for risk assessment of M74 syndrome were developed and questions were formulated in a special "Questionnaire". With serious risk of M74 reproductive syndrome is characterized only one trout farm subjected to anthropogenic pressures. The unsuitable environmental conditions, which lead to M74 syndrome, can be partly compensating using sperm activation medium 49397 for artificial insemination of trout eggs, but successful fertilization does not guarantee successful hatching, especially of eggs in trout farms from IV and higher ranks.

***Recommendation for practice***

For compensating the unsuitable environmental conditions, which lead to occur M74 syndrome and partly neutralizing it, the fish farmers should use sperm activation medium 49397 for artificial insemination of trout eggs.

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