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TRIPLOIDIZATION OF PERCID FISHES – A CHANCE FOR IMPROVEMENT AND DIVERSIFICATION OF EUROPEAN AQUACULTURE?*

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A b s t r a c t

The paper presents review of experimental triploidization trials in percid fishes, important for European aquaculture due to improve and diversification of fish production. The triploidization lead to obtain individuals with three sets of homologous chromosomes (3n) theoretically sterile and showing a faster growth rate compare to the normal diploid fish. Triploidization in aquaculture is usually performed with the use of thermal/pressure and chemical shocks. Parameters of environmental shocks are species specific and it is extremely important to optimize the exact conditions for procedure. In percids the efficiency of the pressure and thermal shocks is varied, and the survival rate of triploids relatively low. However the production of triploid percids stocks using a pressure shock, can be adapted widespread in the future in the fishery practice.

TRIPLOIDYZACJA RYB OKONIOWATYCH – SZANSA NA UDOSKONALENIE I DYWERSYFIKACJĘ AKWAKULTURY RODZIMYCH GATUNKÓW RYB?

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S ł o w a k l u c z o w e: triploidyzacja, ryby okoniowate, szok termiczny, szok ciśnieniowy, akwakultura.

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Abstrakt

W pracy przedstawiono wyniki eksperymentalnych zabiegów triploidyzacji u przedstawicieli ryb okoniowatych, ważnych dla akwakultury europejskiej ze względu na poprawę i dywersyfikację produkcji ryb. Celem triploidyzacji, jako manipulacji genomowej, jest uzyskanie osobników o powiększonym o 50% zestawie chromosomów homologicznych ($3n$) wobec osobników rodzicielskich, teoretycznie sterylnych i wykazujących szybsze tempo wzrostu niż typowe ryby diploidalne. W akwakulturze zabieg triploidyzacji przeprowadza się najczęściej z wykorzystaniem szoków termicznych oraz ciśnieniowych i chemicznych. Parametry stosowanych szoków środowiskowych są swoiste gatunkowo, dlatego niezwykle ważna jest doświadczalna optymalizacja ich warunków. U ryb okoniowatych efektywność szoków termicznych i ciśnieniowych jest bardzo zróżnicowana, a przeżywalność triploidów stosunkowo niska. Jednak metody produkcji triploidalnych stad okoniowatych z zastosowaniem szoku ciśnieniowego mogą znaleźć w przyszłości powszechne zastosowanie w praktyce rybackiej.

Percids aquaculture

During the last two decades, in the context of inland aquaculture diversification in Europe, percid fishes, namely Eurasian perch (*Perca fluviatilis* L.) and pikeperch (*Sander lucioperca* L.), are receiving increasing attention from scientists and fish farmers. Moreover, high flesh quality and reasonable market value made culture of both percid species economically justified (KESTEMONT and MÉLARD 2000). The main consumer markets for percid fish products (mainly fillets) concerns Finland and Sweden in Scandinavia and especially four European countries in Alpine region, Switzerland, Northern Italy, Germany and France (TONER 2015). Nowadays, EU aquaculture of percids is dominated by France, Netherlands and Denmark based on intensive rearing techniques as recirculation systems (RAS). Intensive culture in RAS provides optimal conditions for fish growth, high survival rate and shorter production cycle, but still needs huge economical inputs. A large part of the European fish farms are micro-enterprises, in most cases using rather extensive production technology (NIELSEN et al. 2015). Therefore, initial larval and juvenile perch culture under pond conditions has been combined with intensive ongrowing of fish to a commercial size in RAS. Combination of pond and RAS perch culture is successfully used mainly in countries of Central Europe where large pond area is available (POLICAR et al. 2013). The success of the comprehensive implementation of any fish species on a commercial scale production using RAS, depends on many factors. The key to the sustainable development of the production of percids was optimized methods for reproduction under controlled condition in both periods, spawning and out of reproductive season. Mastering techniques for conducting the controlled reproduction of percid species, by selecting the appropriate environmental combined with the use of hormonal stimulation have a significant impact on the effectiveness

of spawning, and in turn, on the quantity of stocking material produced (KUCHARCZYK et al. 1996, KOUŘIL et al. 1997, DEMSKA-ZAKĘŚ and ZAKĘŚ 2002, ZAKĘŚ and SZCZEPKOWSKI 2004, MIGAUD et al. 2004, 2006, ZAKĘŚ and DEMSKA-ZAKĘŚ 2009). In the case of the controlled reproduction of European percids, also major problems include synchronization and prediction of the time of ovulation, as well as variable quality of eggs. This may suggest, among others, that the procedure of artificial spawning itself can affect the quality of eggs. It has already been proven that in the process of artificial spawning, the quality of eggs was affected by water temperature and the type of hormonal preparation used for induction of ovulation (ŻARSKI et al. 2011a, 2011b). Progress of domestication of animals, including fish species seems to be a key factor for the development of further cultivation, however domestication of percid species still is progressing (TELETCHEA and FONTAINE 2014). Effects of domestication process on fish growth, low stress response, and reproduction have already been observed in other fish species. Therefore domestication of percids certainly allows to develop breeding programs similar to those that operate in the salmonids, consequently affecting the aquaculture of these species (FONTAINE et al. 2015).

Implementing culture of a given species in RAS is determined not only by developing reproductive technique, but also by creating effective larvae and fry rearing methods in this production system. The obstacles encountered at this stage of perch and pikeperch production are mainly connected with the size of larvae (belonging to the smallest freshwater ichthyofauna representatives), not fully developed gastrointestinal tract after hatching, necessity of filling the swim bladder in the first days of life and intra-cohort cannibalism. Undoubtedly, the stage of optimizing rearing of larvae and juveniles is the key to further development of both percid fish aquaculture (ZAKĘŚ et al. 2008). The phenomenon of intra-cohort cannibalism, mentioned before, is one of the major problems predatory fish farmers are confronted with at this stage of production. The moment of occurrence of cannibalism depends on how well the structures of the gastrointestinal tract are developed and whether an individual has obtained the ability to start exogenous feeding. As for predatory fish, the first cannibalistic attempts appear between 7 and 12 days post hatching (DPH) (BARAS et al. 2003, KESTEMONT et al. 2003, BABIAK et al. 2004, KRÓL et al. 2015, KRÓL and ZAKĘŚ 2016). At early stages of rearing larvae of predatory fish in RAS, the farmer is forced to use the first live food, usually *Artemia* sp. nauplii, which is a source of nutrients and, additionally, enriches fish intestinal environment with exogenous enzymes facilitating digestion process. At the further stages of rearing predatory fish larvae, co-feeding procedure (*Artemia* + dry diet) is used, mainly as a cost-cutting scheme, to be later entirely replaced with composed feed. Converting into serving solely

composed feed to larvae is a critical moment in rearing of predatory fish species causing the greatest losses as a result of cannibalism. In both cultured European percid species, several authors suggested that further studies are required for an optimization of the weaning protocol at the earliest possible stage of larval rearing (KESTEMONT et al. 2007, KRÓL and ZIELIŃSKI 2015). The latter is a result of the fact that the nutritional requirements of percid fish are still unknown and commercial diet dedicated to these species is no available on the market.

Growth heterogeneity is a main problem in larviculture especially in predatory species (KESTEMONT et al. 2003). Controlling the phenotypic sex of farmed fish is potentially one of the most promising strategies for improving production and profitability in aquaculture (STRÜSSMANN and NAKAMURA 2002). Reducing growth heterogeneity of the cultured stocks was one of the reasons for which methods of production percid monosex populations were developed (MALISON et al. 1986, ROUGEOT et al. 2002, 2005). Moreover, in some species, the use of monosex can provide additional benefits such as reducing aggressive interactions between conspecifics or controlling spontaneous reproduction in captivity. In case of percid species because of the faster growth and later maturation of females, the production of all-female stocks may have potential applications in culture management of this species (KESTEMONT and MÉLARD 2000). STEJSKAL et al. (2009) found that the significance differences in body weight between all-female and mixed sex stocks of perch began at age 144 days, when fish were over 13 g. The sexual growth dimorphism in perch probably appears markedly when sexual maturation reduces somatic growth. The large-scale production of percid species would be improved by producing sterile (triploid) fish. Sterility provides opportunities for increasing economic growth to use energy on the somatic growth rather than the development of the gonads. Triploidization, may cause sterilization of fish and therefore probably induced the improvement of growth performances by the reduction of the gonad development above 20% (ROUGEOT et al. 2003, STEJSKAL et al. 2009).

Methods of triploidization

Triploidization is a genomic manipulation leading to obtain organisms with one additional chromosome sets. It is achieved by prevent the extrusion of the second polar body in the egg shortly after egg fertilization, but before the first mitotic division of the zygote (CHOURROUT 1988, MALISON et al. 1993). The fertilized egg has then three haploid nuclei derived from the egg, the sperm and the second polar body, which after the fusion constitute the nucleus of the triploid (autotriploid) zygote. Sterility of organisms created as the result of

triploidization is caused by the fact that dividing three sets of chromosomes equally under subsequent phases of meiosis becomes impossible. Although there are a few naturally occurring triploid species of fish that exist as all-female populations with unique reproductive strategies (PURDOM 1984), for most species triploidy is not a natural condition (BENFEY 2001). Triploidization of fish can be achieved by several methods include physical (thermal or pressure shocks) and chemical treatments (with colchicine or cytochalasin B). Generally physical methods are the most successful used to induce triploidy in fish (THORGAARD 1986, IHSSEN et al. 1990, MALISON et al. 1993, PIFERRER et al. 2009). Parameters of thermal shock are determined experimentally and individually for each fish species. The most important parameters are: temperature of the shock, time of its implementation – the time after fertilization of the egg – and the duration of the thermal shock itself application (PANDIAN and KOTEESWARAN 1998). The most common principle to determine the temperature of the shock is that high temperature (26–32°C) so called hot shock is used for cold-water species (ARAI and WILKINS 1987), while low temperature (4–7°C) so called cold shock is used for warm-water species (BASAVARAJU et al. 2002, DIAS DA SILVA et al. 2007). The most crucial parameter of triploidization seems to be the moment of shock initiation. As it has been already mentioned, shock has to be applied before the second polar body leaves the egg, after egg fertilization, but before the first mitotic division of the zygote (CHOURROUT 1988). In salmonids during triploidization procedures thermal shocks were used in 5 to 45 minutes (ARAI and WILKINS 1987). For cyprinids the time between 1–4 minutes after fertilization of eggs was adapted (BASAVARAJU 2002). The duration of shock is also dependent on the species and is most often performed in 5 to 25 minutes. Triploidization with use of the thermal shock itself is relatively easy to made. Usually, fertilized gametes placed on the sieves are kept for a few minutes in water bowls at significantly higher or lower temperature compared to the temperature of the water where egg fertilization was made (ROUGEOT 2005). For the use of pressure shock to induce the triploidization, two parameters, i.e. time of initiation and duration of the shock, are determined analogously to the described thermal method. It also requires the establishment of the third parameter, which is the condition of the high pressure shock to which the fertilized eggs are subjected (PANDIAN 1994). Triploidization with the use of this method requires a special device with the possibility to regulate and stabilize the condition of the pressure shock for at least several minutes, mostly between 58–85 Mpa (MALISON et al. 1993, PRESTON et al. 2013).

Verification of the triploidization effectiveness

The first one is to compare the size of the nuclei of erythrocytes in groups of fish subjected effected by shocks to the ones in the control group (unshocked group). For this purpose blood is collected from the tail vein of the fish, a drop of blood is then applied to the microscope slide and stained with Wright's dye (WOLTERS et al. 1982) or Giemsa method (FELIP et al. 1997). Then diameter of several dozens erythrocytes from each sample that has been made is measured and the results are verified by statistical analysis. This method is often used to estimate the ploidy since it easily identifies differences in the nuclear volume of erythrocytes of triploid and diploid organisms (CHERFAS et al. 1994). However it is limited by an adequately large size of the examined organisms from which we can take intravitally blood samples. Second method of verification of triploization's efficiency is analysis of the number of chromosomes (karyotyping). For this method was used fragments of fish tissues, for example spleen, gill epithelium or kidney of which we make cytological preparations. This method is not commonly used in analytics due to long time and high costs needed to perform it (JANKUN et al. 2008). The most reliable and relatively quick diagnostic test verifying the ploidy of the fish offspring is analysis by flow cytometry. This method is based on the measurement of the content of the nuclear DNA, isolated from small fragments of fish fins or muscles. The biological material is incubated with a fluorescent DNA-specific dye DAPI (4',6-diamidino-2-phenylindol), which crosses the nuclear membrane and binds to the DNA nucleotides, which therefore corresponds to the level of ploidy of the examined organisms (LECOMMANDEUR et al. 1994).The fluorescence intensity for each nucleus is proportional to the content of the DNA and which therefore corresponds to the level of ploidy of the examined organisms (LECOMMANDEUR et al. 1994).

Triploidization of percid fishes

As part of the experimental work so far were tested effects of environmental shocks on perch fish eggs. For the yellow perch *Perca flavescens* (MITCHILL 1814) the most efficient variants were these with the use of the heat shock (MALISON et al. 1993, MALISON and GARCIA-ABIADO 1996) – Table 1. REUGEOT et al. (2003), performing triploidization of the Eurasian perch *Perca fluviatilis* L. also obtained 100% of triploids with very similar shock parameters (Table 1). For the yellow perch the use of the shock resulted in the lack of any effects of the procedure (MALISON et al. 1993). However for the walleye *Stizostedion vitreum* (MITCHILL 1818) results of the experiments with thermal shocks were

not as good as for the aforementioned perch species. MALISON et al. (2001), analyzed the efficiency of the selected options, as a best variant obtained only 44% of triploid organisms (Table 1). In the triploidization of the European seabass *Dicentrarchus labrax* L., cold shocks were used, which resulted in a stock consisting entirely of triploids while maintaining a relatively high survival rate and obtaining around 80% of triploids (FELIP et al. 1997). The induction of the triploidization with the use of the short cold shocks was highly dependent on water temperature (FELIP et al. 1997). Generally it was observed, that the shorter exposure of eggs time for environmental factor caused the greater efficiency of triploidization. Similarly good results, were also obtained by PERUZZI and CHATAIN (2000) who used short cold shocks on European seabass.

Table 1

The most effective results of triploidization in percids fishes with use of thermal shock

Species	Shock conditions (initiation time/ /water temp./ /duration time)	Percentage of triploids [%]	Survival rate [%]	Source
Yellow perch *Perca flavescens* (Mitchill, 1814)	5 min AF / 30°C/25 min	100.0	16.7 ± 6.7	MALISON et al. (1993)
	5 min AF/ 31°C/25 min	100.0	3.3 ± 3.3	
	2 min AF/ 30°C/10 min	100.0	30.0 ± 15.3	
	5min AF/ 30°C/10 min	93.3 ± 6.7	43.3 ± 14.5	
Walleye *Stizostedion vitreum* (Mitchill, 1818)	2 min AF/ 31°C/25 min	25.0 ± 14.4	13.3 ± 8.8	MALISON et al. (2001)
	5 min AF/ 31°C/25 min	35.3 ± 19.2	16.7 ± 12.0	
	2 min AF/ 30°C/25 min	44.3 ± 29.4	13.3 ± 8.8	
	5 min AF/ 30°C/25 min	30.3 ± 19.2	16.7 ± 8.8	
Eurasian perch *Perca fluviatilis* L.	5 min AF/ 30°C/10min	88.0 ± 6.0	36.0 ± 3.0	REUGEOT et al. (2003)
	7 min AF/ 30°C/10 min	98.0 ± 2.0	38.0 ± 7.0	
	5 min AF/ 30°C/25 min	100.0	43.0 ± 34.0	
	7 min AF/ 30°C/25 min	93.0	27.0 ± 17.0	
Pikeperch *Sander lucioperca* L.	10 min AF/29°C/40 min	75.0	–	BLECHA et al. (2016)
	5min AF/31°C/20 min	100.0	–	
European sea bass *Dicentrarchus labrax* L.	5 min AF/ 0°C/5 min	87.0	70.0	FELIPI et al. (1997)

During experiments related to the production of percid triploids hydrostatic pressure shocks were also used (Table 2). However, the effectiveness of these procedures was strongly varied. MALISON et al. (1993) obtained 50% of triploid yellow perch, but at a very high mortality rate. For the walleye the use of the pressure shocks resulted 100% of triploids. The problem, similarly to the

situation of the Eurasian and yellow perch, was a high mortality rate of larvae (MALISON et al. 2001). However, PERUZZI and CHATAIN (2000) using pressure shocks in European seabass, obtained 100% of triploids.

Table 2
The most effective results of triploidization in percids fishes with use of hydrostatic pressure shock

Species	Shock conditions (initiation time/ /water temp./ /duration time)	Percentage of triploids [%]	Survival rate [%]	Source
Yellow perch *Perca flavescens* (Mitchill, 1814)	5 min AF /9000PSI/12 min	54.4 ± 27.2	80.0 ± 5.8	MALISON et al. (1993)
	5 min AF /11000PSI/12 min	50.0 ± 16.7	63.3 ± 6.7	
Walleye *Stizostedion vitreum* (Mitchill, 1818)	4 min AF/7000PSI/15 min	93.0 ± 3.0	88.3 ± 4.4	MALISON et al. (2001)
	4 min AF/7000PSI/30 min	73.1 ± 4.4	56.7 ± 8.8	
	4 min AF/8000PSI/15 min	72.2 ± 11.1	73.3 ± 6.7	
	4 min AF/8000PSI/30 min	100.0	63.3 ± 8.8	
European sea bass *Dicentrarchus labrax* L.	6 min AF/8500PSI/2 min	100.0	41.0–89.0	PERUZZI and CHATAIN (2000)

Conclusions and possibilities of using the triploid percids

Genetic manipulations techniques, including triploidization, are gaining more and more interest of world aquaculture. This is due to the fact, that their use creates potential possibilities of fish production with features of gigantism or sterile stocks, which can indirectly contribute to improve the economic efficiency of fish aquaculture (PURDOM 1983, PANDIAN and KOTEESWARAN 1998). An adequate scaling-up of the method from laboratory to hatchery is a key step if the triploidization is to be applied at the large scale required for mass production. It should be noted that polyploids are not considered to be genetically modified organisms (GMOs). This method has many useful applications to aquaculture. The major consequence of triploidy is gonadal sterility, which is of advantage in the aquaculture with supporting of superior growth. In fish, the induction of triploidy is mainly used to avoid problems associated with sexual maturation such as lower growth rates, higher aggressive and territorial behavior, increased incidence of diseases and deterioration of the organoleptic properties. Triploidy can also be used to increase the viability of some hybrids, and is regarded as a potential method for the genetic containment of farmed fish (PIFERRER et al. 2009).

In the initial stage of rearing diploids perch grow faster than triploid fish. After reaching a weight of about 20 g, the triploid perch start to gain weight faster than the diploids due to the beginning of sexual maturation process as well as the initiation of ovo- and spermatogenesis (MALISON et al. 1986). In this stage the diploid gonads are significantly more developed than triploid fish gonads (MALISON et al. 1993). These results confirm the thesis that the triploids use more energy for somatic growth than diploids, which formed their gonadal structures (MALISON et al. 1993). It was observed the differences in the ovaries development between the diploid and triploid yellow perch. With the total length of the fish being approx. TL = 75 mm, the oocyte diameter of the diploids was TL = 110 μm, while the triploids had the oocyte diameter of only 60 μm. After the fish achieved the total length of TL = 125 mm, the ovaries of the diploid females contained the vitellogenic oocytes while in the gonads of the triploids only few single previtellogenic oocytes were discovered. The diploid males (with the total length of 75 mm) had in testis numerous spermatogonia and several scattered spermatocytes. When the fish reached the total length of approx. TL = 100 mm, testes already contained primary and secondary spermatocytes as well as the spermatids. In total length of TL = 125 mm, all germ cells of spermatogenesis including spermatozoids were observed. In the same time triploid males (range 100–125 mm of total length) had only spermatogonia in seminiferous lobules (MALISON et al. 1993). Among the salmonids, triploid males develop much larger gonads than triploid females and often produce functional spermatozoa, but these spermatozoa are aneuploid (BENFEY et al. 1986)

Parameters of applied physical shocks used to induce triploidy are fish species dependent. For high procedure effectiveness extremely important is to determine their optimal conditions. Alternative production methods of Eurasian perch triploids with the hydrostatic pressure shock can be commonly used in the innovative aquaculture and fishery practice since, theoretically it's easier to optimize the standard conditions for such procedure.

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