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Innovative methods in culture and reproduction of pikeperch (*Sander lucioperca*)

Inovativní metody v chovu a reprodukci candáta obecného (*Sander lucioperca*)

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CONTENT

CHAPTER 1

GENERAL INTRODUCTION

1.1. Pikeperch culture

Pikeperch (*Sander lucioperca*) is one of the most promising fish species for European freshwater aquaculture (Müller-Belecke and Zienert, 2008). Production of marketable pikeperch originating from intensive culture is expected to rise due to its excellent flesh quality, high growth rate, good market value (Uysal and Aksoylar, 2005; Wuertz et al., 2012; Kristan et al., 2013), suitability for intensive culture (Wang et al., 2009; Szczepkowski et al., 2011) and especially decreasing numbers of fish in wild populations (Policar et al., 2013). In the past, pikeperch was extensively farmed in ponds or lakes (Hilge and Steffens, 1996) but in the last few decades, it has been successfully reared under complete recirculation aquaculture system (RAS) conditions (Molnar et al., 2004) or in combination of pond and RAS conditions (Policar et al., 2014).

1.1.1. Pond culture

There are two final products of pikeperch pond culture. One of them is marketable fish, usually cultured in polyculture with common carp (*Cyprinus carpio*) and other fish species such as tench (*Tinca tinca*), grass carp (*Ctenopharyngodon idella*) and silver carp (*Hypophtalmichthys molitrix*). The main task of pikeperch in this culture system is to eliminate population of silver fish such us roach (*Rutilus rutilus*), rudd (*Scardiniuse erythrophthalmus*) or bream (*Abramis brama*) which are the main feed competitors of carp stock in ponds (Wedekind, 2008; Adamek et al., 2012; Policar et al., 2014). The whole production cycle of pikeperch takes about 3 to 4 years in the conditions of the Czech Republic (Čítek et al., 1998).

Another output of pikeperch pond culture is summer fry (Policar et al., 2011). To produce the summer fry, monoculture of pikeperch in small ponds is the most suitable culture method (Hilge and Steffens, 1996; Ruuhijarvi and Hyvarinen, 1996; Policar et al., 2011). In this case, artificial spawning nests with fertilized eggs (at three quarters of incubation time; Klimeš and Kouřil, 2003) or newly hatched pikeperch yolk sac larvae are stocked into ponds at about 200 000 individuals per hectare (Policar et al., 2013). It is necessary to prepare the ponds carefully. Technological steps such as wintering (keeping ponds drained during winter time) and fertilizing the ponds with compost or manure are essential to provide a sufficient amount of nutrition for zooplankton which is the main source of pikeperch larvae diet (Musil and Peterka, 2005; Policar et al., 2011). The pond outlet (monk) must be perfectly sealed so as to avoid juveniles from escaping (Blecha et al., 2015b).

A critical factor that affects survival and growth of fry is sufficient natural feed at the time of the first exogenous feeding (Musil and Kouřil, 2006). With pikeperch, it happens at the age of 5–6 days post hatching (dph) when the larvae is 6–8 mm long (Musil and Peterka, 2005; Ostaszewska et al., 2005). The most important components of the pikeperch larvae and juveniles' diet are daphnids (*Cladocera*, Musil a Peterka, 2005). In the past, it was supposed that rotifers were the main source of nutrition of pikeperch larvae (Coussement, 1978; Verreth, 1984) but they occurred in their diet only in situations when abundance of daphnia nauplii and copepod stages were low (Musil and Peterka, 2005; Blecha et al., 2015b). In conditions where rotifers zooplankton dominate, a slower growth rate of pikeperch early stages is observed compared to ponds with the dominance of daphnia zooplankton (Musil and Kouřil, 2006). In the later period, pikeperch juveniles feed daphnids or calanoids (*Calanoida* sp.) and when they reach the total length (TL) of 15 mm they can feed on benthic organisms such as chironomids (*Chironomus* sp., Steffens et al., 1996; Blecha et al., 2015b).

The length of the rearing period is from 35 to 60 days and depends on the amount of feed and water temperature (WT). The TL of harvested pikeperch juveniles varies from 35 to 40 mm at this time (Molnar et al., 2003, 2006; Policar et al., 2013). Some authors (Zakes, 1999; Ljunggren et al., 2003) recommend harvesting a pond when the TL of juveniles is about 20–30 mm. The main reason for that is easier converting of such small fish to pellet feed. A practical guideline to terminate the right time for summer fry harvesting is a decrease in abundance or total disappearance of zooplankton in a pond (Policar et al., 2011), otherwise, cannibalism may increase (Blecha et al., 2015b). The production of summer fry is variable but more or less stable in years (Peterka et al., 2003; Adamek and Opacak, 2005; Policar et al., 2011) and can reach from 50 000 to 150 000 individuals per hectare (Szkudlarek a Zakes, 2002; Blecha et al., 2015b). When the pond rearing lasts until autumn, average juvenile survival fluctuates between 12 to 36% and it strongly depends on pond productivity (occurrence and abundance of zooplankton and prey fish) and cannibalism (Steffens et al., 1996).

1.1.2. Recirculation aquaculture systems and pikeperch culture under intensive aquaculture conditions

1.1.2.1. Recirculation aquaculture systems

Recirculation aquaculture systems have been successful for the production of fresh water fishes as well as marine species (Martins et al., 2010). These systems reduce water consumption (Verdegem et al., 2006), control recycling of nutrients and waste management (Piedrahita, 2003), culture fish under better hygiene and improved disease management (Summerfelt et al., 2009; Tal et al., 2009), prevent escapes of fish from breeding facilities (Zohar et al., 2005), culture fish in high densities, culture fish continually all year round and completely control the entire rearing process (Martins et al., 2010). RAS are recognised as systems where water is reused after combination of several treatments. Basic and the most important parts of RAS, which in general make a very sophisticated and effective loop, are mechanical and biological filtration of water, oxygenation and disinfection of water with ultraviolet (UV) light and ozone treatment (Martins et al., 2010). I Insoluble particles (uneaten feed, excrements) must be removed from the water by mechanical filtration (screen filters, rotary microscreen filters), otherwise accumulation can lead to clogging of a biofilter and its decreased function and effectiveness, increased oxygen demands (Chen, 2000) and damage of fish gills (Chapman et al., 1987). The essential part of every RAS is a biofilter, where toxic ammonia (product of fish metabolism) is reduced to nitrate in a process called nitrification. The nitrification process consists of two parts. In the first part, *Nitrosomonas* bacteria are responsible for nitrifying ammonia to nitrite and in the second part, bacteria of the *Nitrobacter* genus change nitrite to nitrate (Stickney, 2000). In RAS, where the water exchange rate is low (i.e., 30 L per kg of feed in tilapia farms; Martins et al., 2009), a problem with nitrate cumulation can appear. In this case, denitrification has to be applied (van Rijn et al., 2006; Martins et al., 2010). Denitrification runs in an anaerobic environment where nitrate is converted to nitrogen gas (Martins et al., 2010). To disinfect and sterilize water in RAS, UV light and ozone treatment are usually used (Sharrer and Summerfelt, 2007). The UV light at the wavelength of 254 nm is an effective tool to kill bacterial and viral organisms. The effectiveness of the UV treatment depends on low concentration of suspended solids and very low turbidity of inflowing water. Under optimal conditions, the efficiency of UV treatment can achieve 99.9% of bacteria removal (Owsley, 2000). Ozone has been used in RAS for its bactericidal, parasiticidal and virucidal effects (Bullock et al., 1997; Liltved, 2002). Microbes are killed with ozone by oxidation of their lipid bilayer (Colberg and Lingg, 1978). Ozone treatment can also be applied for colour elimination,

nitrite reduction, algae control and turbidity removal (Tango and Gagnon, 2003; Summerfelt et al., 2009; Martins et al., 2010). The end product of the ozone reaction is dissolved oxygen (Sharrer and Summerfelt, 2007).

1.1.2.2. Pikeperch culture under intensive aquaculture conditions

Current research is focused on an improvement of pikeperch intensive aquaculture because there are still many obstacles related to low quality of gamete originating from RAS-cultured broodstock which results in low fertilisation and hatching rate, low viability of hatched larvae and high occurrence of larvae deformations (Policar et al., 2011; Lund et al., 2012; Zakes, 2012). These obstacles are mainly connected with a non-optimal nutrition composition of pellet feed (Zakes, 2012). Another problem in pikeperch culture under RAS is an inability of pikeperch larvae to inflate their swim bladder (SB) which leads to high losses, body deformations (Demska-Zakes et al., 2003; Szkudlarek and Zakes, 2007) and increased cannibalism (Kindschi and MacConnell, 1999; Szczepkowski et al., 2011). The inability to inflate the SB has been regarded as one of the major obstacles for intensive aquaculture of many different freshwater and marine fish species (Chatain, 1994; Summerfelt, 1996; Martin-Robichaud and Peterson, 1998; Demska-Zakes et al., 2003).

Pikeperch are physoclistous and must inflate their SB by breaking the water surface and swallowing an air before the pneumatic duct closes (Chatain and Ounais-Gushemann, 1990; Rieger and Summerfelt, 1998; Craig, 2000). Pikeperch begin to inflate the SB at 5 or 6 dph and this procedure must be finished before 11–12 dph (Demska-Zakes et al., 2003). SB inflation fails during intensive larviculture because of a high viscosity of the water surface caused by oils from feed (Chatain and Ounais-Gushemann, 1990), pollutants on the water surface which represent a potential source of infection (Marty et al., 1995), sub-optimal water temperature (Trotter et al., 2003) and other abiotic factors such as light intensity, photoperiod, salinity, turbidity and tank colour (Ronzani Cerqueira and Chatain, 1991; Barnabé and Guissi, 1993; Rieger and Summerfelt, 1997; Martin-Robichaud and Peterson, 1998). To increase SB inflation, water surface spraying can be applied (Barrows et al., 1993; Moore et al., 1994; Bristow et al., 1996; Summerfelt, 1996).

About 10 new farms were built in Europe to produce pikeperch under RAS in the last decade and many others are being constructed across Europe (Fontaine et al., 2015). If certain technological steps are followed, marketable pikeperch can be produced in RAS all year round. One of the most important aspects of successful intensive pikeperch farming is broodstock management and out-of-season spawning leading to a continual larval production (Ronyai, 2007; Zakes, 2007; Müller-Belecke and Zienert, 2008; Policar et al., 2014). At the beginning of the intensive culture, pikeperch larvae are fed with *Artemia salina* nauplii directly after their mouth opening (Ostaszewska et al., 2005; Szkudlarek and Zakes, 2007) until 15–21 dph when weaning (transition from live food to formulated feed) starts (Kestemont et al., 2007; Szkudlarek and Zakes, 2007; Ljubobratovic et al., 2015). Artemia nauplii enriched with HUFA and vitamin C improve the growth and significantly reduce the deformity rate (Kestemont et al., 2007). Initial larval stocking rate is highly variable ranging from 25 to 100 individuals per litre (Mani-Ponset et al., 1994; Szkudlarek and Zakes, 2007). In post-larval culture, RAS systems stock juveniles in high densities of 30–50 $kg.m^{-3}$ (Wedekind, 2008) and for older fish categories even at 80–100 $kg.m^{-3}$ (Dalsgaard et al., 2013). To produce a marketable fish (1.2 kg) within 15 to 18 months, a constant water temperature of 24–26 °C (Fontaine et al., 2015) and feeding with appropriate pellet feed is required (Wang et al., 2009).

1.1.3. Combination of pond and intensive culture of pikeperch

A combination of pond and RAS culture has been used in pikeperch culture for a long time (Zakes and Demska-Zakes, 1998; Molnar et al., 2004; Altun et al., 2008; Policar et al., 2013) and can provide production of high-quality juveniles (Policar et al., 2014) which can be used for further rearing in RAS or as a stocking material for fishing grounds and other open waters (Rennert et al., 2005). This culture technique is especially applicable for countries with large pond areas such as Czech Republic, Hungary, Germany etc. (Policar et al., 2014).

The transition of pond reared or wild-caught pikeperch juveniles to dry feed (using different types of feed) has been described by many authors (Zakes and Demska-Zakes, 1998; Molnar et al., 2004; Altun et al., 2008; Policar et al., 2013). The most thorough description is provided by Policar et al. (2014). They divided the entire procedure and the following culture into three basic parts: a) stocking of pond-reared pikeperch juveniles to RAS, b) space and feed adaptation to a new environment of RAS and pellet feed, c) culture of fully adapted pikeperch juveniles in RAS up to 8 grams of body weight.

a) Pikeperch juveniles of 30 to 50 mm TL are stocked into RAS immediately after pond harvesting and size sorting. At the beginning of RAS culture, a preventive antiparasitic bath (0.015 ml of 35% formaldehyde per 1 litre for 8 hours) must be applied to prevent later health complications. A preventive antifungal bath in salt (3 $g.l^{-1}$ for 20 minutes) should also be used, because a higher concentration of water salinity has a positive effect on health condition of reared fish (Kestemont et al., 2008). Recommended initial fish density is 8.6 individuals per litre and the WT of 19 °C (Policar et al., 2014).
b) Space adaptation means that juveniles adjust a new environment and water temperature is being gradually increased up to 23 °C within 48 hours. Photoperiod should be established at 15L:9D and light intensity at 100 lux. Fish should not be fed for a two-day period so as to increase their subsequent feeding activity. Next, the most important phase of this culture, so-called co-feeding, begins. The entire process takes ten days and results in pikeperch feeding on only pellet feed. Initially, the fish are fed only with frozen chironomids and every two days, the rate is reduced by 25% and the rate of pellet feed (BioMar, INICIO Plus 1.1 mm) is increased by 25% until only the pellets are fed. Fish are fed during the light period by hand every thirty minutes on an *ad libitum* basis (according to the feeding behaviour of fish). About 97% should accept the pellets. The survival rate of pikeperch juveniles after co-feeding achieves 78±5.5%.
c) After accommodation to pellet feed, pikeperch should be about 8 g and can be stocked in farms. The initial stocking density is 6.7 individuals per litre, the daily feeding rate ranges from 2 to 5% of fish biomass and the WT=24 °C. The average survival rate reaches 88.7±5.3% at the end of the culture.

In the past, several different kinds of live food were used in the pikeperch weaning such as *Daphnia magna* and *Moina branchiata* (Zakes, 1999), *Neomysis integer* and yolk (Ljunggren et al., 2003), *Tubifex tubifex* (Molnar et al., 2004), *Gambusia affinis,* minced fish meat and minced *Parapenaeus longirostris* (Altun et al., 2008) and chironomid larvae suggested as the most effective variant (Wedekind, 2008; Policar et al., 2013). Other factors which can affect the success of the weaning in general are water temperature (Zakes, 1997b, 1999), fish size and stocking density (Zakes, 1997a; Policar et al., 2009, 2013).

1.2. Reproduction of pikeperch

1.2.1. Natural spawning

Pikeperch sexually mature in its third (male) and fourth (female) year of life. Natural spawning of pikeperch takes place from April to June when the water temperature ranges between 10 to 16 °C (Ruuhijärvi and Hyvärinen, 1996; Schlumberger and Proteau, 1996; Lappalainen et al., 2003). Natural pond spawning of pikeperch is the oldest method of propagation of this species and the first information is dated to the nineteenth century (Zakes and Demska-Zakes, 2009). Spawning is carried out in small ponds where two pairs of broodstock per hectare are stocked until the harvest of summer fry or juveniles in autumn (Raat, 1991; Steffens et al., 1996). Usually, no hormonal treatment is used (Wojda et al. 1994). Relative fecundity of pikeperch females is about 100 000–200 000 egg.kg^{-1} of body weight and 1g of eggs contains 1 500–2 000 eggs (Schlumberger and Proteau, 1996; Kucharczyk et al., 2007; Ronyai, 2007). Eggs incubation lasts 8 to 9 days at the water temperature of 16 °C (Lappalainen et al., 2003), the size of newly hatched larvae is 4.5–5.5 mm and depends on the egg diameter (Schlumberger and Proteau, 1996). Natural spawning of pikeperch is the simplest propagation method, however, the final result is rather unpredictable (Musil and Kouřil, 2006).

1.2.2. Nest spawning

Nest spawning can be performed in cages, ponds, concrete or plastic tanks and RAS systems (Steffens et al., 1996; Schlumberger and Proteau, 1996; Kucharczyk et al., 2007; Zakes and Demska-Zakes, 2009; Policar et al., 2016). Females lay eggs on an artificial spawning substrate which can be made from on of several kinds of materials (natural or plastic) such as branches of coniferous trees, roots, mats, carpets or artificial grass. The size of the nest is very important because of high fecundity of pikeperch. The nest should not be smaller than 0.5x0.5m or it should cover the whole bottom of the tank (Kucharczyk et al., 2007; Policar et al., 2016). To stimulate the maturation of broodstock and synchronize spawning time, hormonal treatment can be applied (see in 1.2.3). The broodstock are removed from spawning tanks or cages after the spawning, or the male is left in the tank during the incubation time (Kucharczyk et al., 2007; Policar et al., 2016). The nests with fertilised eggs can be moved to ponds or a hatchery one day before the hatching (Salminen et al., 1992; Kucharczyk et al., 2007; Zakes and Demska Zakes, 2009). This method of pikeperch propagation usually produces 80 000–90 000 larvae.kg^{-1} of female. According to Policar et al. (2016), the use of RAS for the nest pikeperch spawning provides several advantages. It is possible to control and adjust the water temperature to stimulate the broodstock before the spawning as well as manage the incubation time (heating or chilling the water in the system). In RAS, a preventive antifungal bath in formaldehyde (long-term bath, 0.015 ml.l^{-1}) can be applied to protect the eggs during incubation. Also, a good estimate larvae production per nest (pair of spawners) can be accomplished; viable egg count is done by using a volumetric method.

1.2.3. Artificial spawning, egg stripping and artificial fertilisation

Artificial spawning is a reliable method to obtainin a high number of pikeperch larvae (Kucharczyk et al., 2007). Broodstock are kept in tanks and must be hormonally stimulated (Salminen et al., 1992; Zakes a Szczepkowski, 2004). Eggs and sperm are collected by manual stripping and fertilized eggs are incubated in Zug jars (Kucharczyk et al., 2007).

Usually, the broodstock (used for artificial reproduction) are wild specimens obtained from open waters or production ponds captured in fall or spring during the spawning migration (Schlumberger and Proteau, 1996; Steffens et al., 1996; Zakes and Demska-Zakes, 2005, 2009; Zakes, 2009). Because of a different age and origin of the broodstock, different maturity of oocytes occurs. Therefore, the investigation of an oocyte maturity stage is needed to improve the spawning effectiveness and enhance synchronisation of the artificial spawning (Demska-Zakes and Zakes, 2002; Kucharczyk et al., 2008; Zakes and Demska-Zakes, 2009; Zarski et al., 2012). To assess the oocyte maturity stage, a sample of oocytes must be taken from female via urogenital papilla with a plastic catheter. Pikeperch eggs are not transparent and, therefore, the exposure in Serra fluid (ethanol: formaldehyde: acetic acid; mixture rate 6:3:1) is needed (Policar et al., 2016). According to Zarski et al. (2012), seven categories of oocyte maturity from the end of vitellogenesis to ovulation are recognised in pikeperch. Identification of the exact oocyte category in each female represents an essential step in preparation and stimulation of the broodstock for the artificial reproduction. If the oocyte category is known, the decision about hormonal treatment, postponed stimulation or immediate egg stripping can be provided (Kucharczyk et al., 2007; Zarski et al., 2012).

Captive pikeperch females usually do not ovulate without the hormonal treatment (Zakes and Szczepkowski, 2004; Kucharczyk et al., 2007). They are sensitive to stress which leads to a heightened level of cortisol (Schreck et al., 2001) and this hormone affects the function of the hypothalamus-pituitary-gonadal axis (HPGA), leading to problems with oocyte maturation and ovulation (Carragher et al., 1989). Several different hormonal preparations have been used for pikeperch male and female stimulation, such as carp pituitary extract (CPE), human chorionic gonadotropin (hCG), luteinizing hormone-releasing hormone (LH-RH), Gonadotropin Releasing Hormone analogs (GnRHa) and pregnant male serum gonadotropin (PMSG0, Antila et al., 1988; Kucharczyk et al., 2001; Demska-Zakes and Zakes, 2002; Kucharczyk et al., 2007; Ronyai, 2007; Zakes and Demska-Zakes, 2005, 2009; Kristan et al., 2013; Policar et al., 2016). Other gonadotropins (CPE and hCG) can be used for pikeperch hormonal stimulation (Tab. 1). Currently, the hCG at one dose of 500 IU.kg^{-1} is preferred and mostly used with pikeperch (Zarski et al., 2012; Kristan et al., 2013; Blecha et al., 2015a; Policar et al., 2016). If the dose of hormone treatment is divided into two injections, the initial dose should be 20–50% in the case of chorionic gonadotropins and 10–20% in the case of other spawning agents (Kucharczyk et al., 2007).

Table 1. *The most used hormones and hormonal preparations in pikeperch hormonal stimulation.*

Hormone/ preparation	Dose per kg of body weight	Doses	References
CPE	2.4–5 mg	2	Ronyai, 2007; Zakes and Demska-Zakes, 2009
hCG	100–700 IU	1–2	Steffens et al., 1996; Zakes and Demska-Zakes, 2005, 2009; Zarski et al., 2012; Kristan et al., 2013; Policar et al., 2016
PMSG	400 IU	2	Zakes and Demska-Zakes, 2006
GnRHa	25 µg	1	Kristan et al., 2013
Ovopel	0.75–1.25 pellet	2	Zakes and Demska-Zakes, 2005

The latency time (injection to ovulation) usually ranges from 10 to 70 hours when the hCG is used (Zakes and Demska-Zakes, 2009), but varies depending on the oocyte maturity stage (Kucharczyk et al., 2007; Zarski et al., 2012). Pikeperch eggs obtained by stripping of female

must be inseminated artificially with milt collected with syringes from several males (Steffens et al., 1996; Zakes and Demska-Zakes, 2005; Blecha et al., 2015a). Average concentration of pikeperch spermatozoa range from 15–20 million.ml^{-1} (Teletchea et al., 2009; Kristan et al., 2013; Blecha et al., 2015a). To fertilize 100g of pikeperch eggs, the use of 1–2 ml of semen is recommended (Steffens et al., 1996; Zakes and Demska-Zakes, 2005). Spermatozoa motility is relatively divergent because many authors have described a very different percentage of motile cell from 50 to 90% (Zakes and Demska-Zakes, 2005, 2009; Cejko et al., 2008; Teletchea et al., 2009; Blech et al., 2015a).

Because pikeperch eggs become sticky after activation, the adhesiveness must be removed by bathing them in a desticking solution such as alcalase, protease, tannic acid, talc/chloride or talc/milk mixture, milk and clay suspensions (Schlumberger and Schmidt, 1980; Billard et al., 1995; Gela et al., 2003; Demska-Zakes et al., 2005; Zakes et al., 2006; Kristan et al., 2015) (Tab. 2). Incubation of eggs and hatching of larvae take place in Weiss jars at the WT of 15–18 °C (Kucharczyk et al., 2007).

Table 2. *Different un-stick solutions used for removing of the adhesiveness in pikeperch eggs.*

Un-stick solution	Bath composition	Treatment duration (min)	References
Alcalase	1.5 ml.l^{-1}	2	Kristan et al., 2015
Protease	0.5%	2	Zakes et al., 2006
Tannic acid	0.5–1 g.l^{-1}	5	Demska-Zakes et al., 2005
Talc/sodium chloride	100g talc+25 g NaCl+10 l water	45–60	Schlumberger and Schmidt, 1980
Talc-milk	100 g talc to 10 l water + 100 g milk powder to 1 l water; 1:1	60	Kristan et al., 2015
Milk	20–25 g.l^{-1}	30	Billard et al., 1995
Clay	20 g per L of hatchery water	60	Gela et al., 2003

1.2.4. Out-of-season spawning

Out-of-season spawning is one of the key approaches of successful and profitable year-round fish production (Blecha et al., 2015a) and allows fry to be produced for further intensive rearing in a prolonged period of the year (Zakes and Szczepkowski, 2004; Ronyai, 2007). In nature, the process of vitellogenesis begins in October or November and the oocytes achieve their full size after 5–6 months (Zakes and Demska-Zakes, 2009). This process can be shortened by adjustment of photo-thermal conditions (Zakes and Szczepkowski, 2004). Thermal stimulation of spring spawning fish species, such as pikeperch, includes three phases: a cooling phase (CP), a chilling phase (CHP) and a warming phase (WP) (Zakes and Demska-Zakes, 2009). The water temperature should be lowered to 8 °C in the CP, kept between 4–8 °C in the CHP and increased to 15–16 °C in the WP (Zakes, 2007). Zakes and Szczepkowski (2004) applied a 6-week CP, a 6-week CHP and a 4-week WP and changed the photoperiod gradually from 16D:8L (CP), to 8L:16D (CHP) and to 14L:10D (WP). Spawning condition can be achieved 3–4 months prior to the natural spawning season. Many authors say that photoperiod does not have any significant impact on the processes of gametogenesis and spawning itself in percid species which spawn in spring (Kayes and Calbert, 1979; Dabrowski et al., 1994; Malison et al., 1998b; Migaud et al., 2002; Ronyai, 2007; Zakes and Demska-Zakes, 2009).

On the other hand, hormonal injection of broodstock is considered to be the key factor for oocyte maturation, spermiation and ovulation in the out-of-season pikeperch reproduction (Zakes and Szczepkowski, 2004; Zakes and Demska-Zakes, 2009). Szczepkowski (2004) and Ronyai (2007) and Zakes (2007) reported that fish which were injected only with sodium chlorine (0.9% NaCl) did not ovulate and no progress in oocytes maturation was observed. Mostly hCG (200–400 IU.kg^{-1} in one or two doses) and/or CPE (6 mg.kg^{-1} in one dose) were tested for stimulation of the out-of-season spawning in pikeperch (Zakes and Demska-Zakes, 2009). In case of the out-of-season spawning, males are not captured from ponds or lakes in the pre-spawning period and they are not as mature as wild individuals, therefore, hormonal stimulation (200 IU hCG.kg^{-1}) is needed (Zakes, 2007).

1.3. Modern methods used in fish culture and reproduction

A current aquaculture trend is to produce marketable fish in the shortest possible time with low production costs. Therefore, modern and innovative methods and farming procedures such us domestication, culture of monosex populations or triploid fish are applied. These methods can lead to more effective and profitable farming, lower production costs, better flesh quality and can protect wild stocks against genetic contamination from fish escaped from farms (Piferrer et al., 2009; Flajšhans et al., 2013).

1.3.1. Domestication

Domestication means a consistent control of the reproduction process year after year in generations of fish (animals) which are held, bred and cultured in captivity (Liao and Huang, 2000; Bilio, 2007; Williams, 2008; Mylonas et al., 2010). To be considered as a domesticated species, the entire life cycle must controlled in captivity and must be totally independent of wild conditions and sources of eggs, larvae, juveniles or broodstock (Liao and Huang, 2000; Bilio, 2007). It requires complete and perfect broodstock management and the use of the most effective methods for rearing larvae and juveniles under controlled conditions (Bilio, 2007; Teletchea and Fontaine, 2011). The broodstock management involves steps such us culture of high quality broodstock individuals, controlled induction of oocyte and spermatozoa maturation, artificial spawning and egg incubation and larvae hatching (Liao et al., 2001). Larval and juvenile culture requires development and the use of adequate culturing systems and feeding practices (Teletchea and Fontaine, 2014).

The number of domesticated fish is not clear and strongly depends on authors' view, opinion and estimation. For example, Duarte et al. (2007) consider all finfish species which are listed in the FAO aquaculture production database (about 300 species or groups of species) as domesticated. On the contrary, Balon (2004) thinks that only two fish species are truly domesticated: common carp and goldfish (*Carassius auratus*). According to Bilio (2007), domesticated fish species are those where the first efforts of selective breeding had been made or at least three successful reproduction cycles were finished. This statement could include about 42 different fish species. Teletchea and Fontaine (2014) has presented a theory or thought which divides domestication process and fish used in aquaculture into several levels from 0 to 5 (Tab. 3). According to this source, pikeperch belongs to level 4. It means that application of some selective breeding programme is needed for its complete domestication.

Table 3. *Levels of domestication in fish species used in aquaculture.*

Level of domestication	Evaluation of domestication level	Examples of fish species
0	Captured fisheries (fish from open waters)	
1	First trials of acclimatization to captivity	*Blicca bjoerkna*
2	Part of the life cycle is controlled in captivity	*Anguilla anguilla; Thunnus thynnus*
3	Full life cycle in captivity is achieved	*Rutilus rutilus; Thunnus orientalis*
4	Full life cycle is obtained in captivity independently of wild input	*Perca fluviatilis; Sander lucioperca*
5	Selective breeding programmes focusing on specific goals are applied	*Cyprinus carpio; Salmo salar*

1.3.2. Sex manipulation and monosex population production

The main aim of monosex population induction is to take advantage of sexually dimorphic characteristics (including flesh quality), to control reproduction or to prevent establishment of exotic species in non-native areas. In the past, several different strategies such us sex reversal, interspecific crossing and gynogenesis have been used for induction of partly or completely male or female populations (Gomelsky et al., 1998, 1999; Dunham et al., 2001). All-female populations have been successfully developed for salmonids, carps, tilapias (Dunham, 1996), as well as percids (Malison et al., 1986, 1988, 1998a; Stejskal et al., 2009).

Sex reversal is usually induced by applying direct and indirect methods. The direct methods include exposure of incubating eggs to a hormonal bath (Bye and Lincoln, 1986; Donaldson and Benfey, 1987) or peroral application of hormonal treatment in feed during a critical period of larval development (Horváth and Orbán, 1995; Gomelsky, 2003; Kocour et al., 2005). The indirect methods of sex reversal are based on exposure of fish to a bath which contains steroid hormones or their metabolites (Hulák et al., 2006). The most used hormone for feminisation is 17-beta-estradiol (Pandian and Koteeswaran, 1998) and for masculinisation it is 17-alfa-methyltestosterone. The success of such treatment strongly depends on the water temperature, beginning of treatment, feeding intensity, total dose of hormone, culture system (recirculating or flow-through), fish density and health conditions (Flajšhans et al., 2013).

To produce a monosex population, an interspecific crossing of two different kinds of fish can be applied. Crossbreeding of *Oreochromis niloticus* and *O. aureus* leads to production of almost a complete all-male population which shows faster growth compared to parental populations (Gjedrem, 2005).

Induced meiotic gynogenesis in combination with sex reversal leads to a complete 100% all-female population and has been used for many different species such as rainbow trout (*Oncorhynchus mikiss*), blue tilapia (*Oreochromis aureus*), common carp or tench (Lincoln and Scott, 1983; Linhart et al., 1995; Cherfas et al., 1996; Kocour et al., 2005).

In percids, there are several possibilities of all-female population induction. One of them is the gynogenesis with progenies obtained by controlled egg fertilization with genetically inactivated sperm and restoration of diploidy by thermal or physical shock (Piferrer, 2001; Rougeot et al., 2005). The second way is direct feminization by natural or synthetic estrogens (Malison et al., 1988; Piferrer, 2001; Gorschkov et al., 2004) and the last possibility is the

use of a 17-alfa-methyltestosterone bath treatment (Malison et al., 1986, 1998; Pandian and Sheela, 1995; Malison and Garcia-Abiado, 1996; Blázques et al., 1998; Rougeot et al., 2002, 2005; Stejskal et al., 2009).

1.3.3. Polyploidisation

Polyploidy individuals can be defined as organisms with one or more additional chromosome sets with respect to the number most frequently found in nature for a given species (Piferrer et al., 2009). Triploids, organisms with three sets of homologous chromosomes, are found spontaneously in wild and cultured populations and can be easily induced in many commercially relevant species of fish and shellfish (Legatt and Iwama 2003; Maxime, 2008). Differences from a standard ploidy level can occur naturally (Aegerter and Jalabert, 2004) or can be induced artificially (Chourrout, 1980). Primary sources of natural polyploidisation are oocyte ageing and over-ripening (Samarin et al., 2015) which leads to failure of second polar body extrusion and ploidy anomalies in larvae (Flajshans et al., 2007). Triploidisation can be induced in fish via inhibition of the second meiotic cell division through blocking the second polar body extrusion by shocking the eggs shortly after the fertilisation (Malison et al., 1993). Artificially induced polyploidisation is usually made by a heat shock (Rougeot et al., 2003), a cold shock (Flajshans et al., 1993), a hydrostatic pressure shock (Garcia-Abiado et al., 2001) or an electric shock (Taranger et al., 2010). The most important variables of triploidisation are: the time of the shock treatment initiation (in minutes post eggs and sperm activation), intensity of the shock treatment and duration of the shock treatment (Piferrer et al., 2009).

In fish, the induction of triploidy is mainly used to avoid problems associated with sexual maturation such as lower growth rates, increased incidence of diseases and deterioration of organoleptic properties (Piferrer et al., 2009), minimising ecological effect of fish escaped from farms on wild populations (Cotter et al., 2000) and plan management and control (Conover et al., 2007). Polyploidisation has been made with varying results also in percid fish. Optimal triploidisation procedure for Eurasian Perch is the heat shock at 30 °C for 25 min initiated 5 min after the fertilisation at 16–17 °C which results in 100% triploids (Rougeot et al., 2003). For Yellow Perch, the heat shock at 28 °C for 25 min initiated 5 min after the fertilisation at 11 °C results in 100% triploids (Malison et al., 1993). With walleye, the hydrostatic pressure shock of 55.7 MPa for 30 min initiated 4 min after the fertilisation at 11 °C creates 100% triploids (Malison et al., 2001).

THE AIM OF THIS THESIS WAS TO:

1. Improve the success and effectivity of pikeperch reproduction, especially with the focus on the pre-spawning broodstock management.
2. Try to apply the new methods in the pikeperch egg management and larvae culture under the controlled conditions.
3. Find possibilities to combine pond and RAS culture for a sustainable pikeperch culture.
4. Artificially induce triploidy in pikeperch.

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CHAPTER 2

QUALITY AND QUANTITY OF PIKEPERCH (*SANDER LUCIOPERCA*) SPERMATOZOA AFTER VARYING COLD WATER TREATMENTS

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Quality and quantity of pikeperch (*Sander lucioperca*) spermatozoa after varying cold water treatments

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Summary

Three groups of seven pond-cultured pikeperch males, held under controlled conditions of low water temperature and for a varying photoperiod were injected with 500 IU kg^{-1} human chorionic gonadotropin. Following a latent period of 72 h, sperm was collected. Stripping was on 26 March (Group A), 21 April (Group B), and 13 June (Group C). Spermatozoa was obtained from 85% fish in group B and from 42% of fish in group C. Mean volume of stripped semen for Group A was 0.64 ml, for Group B 1.07 ml, and for Group C 1.80 ml, while the mean concentration of spermatozoa was similar in all groups (15.73 ± 2.68–19.34 ± 3.87 10^9 ml^{-1}). Group A spermatozoa showed the longest motile period (89.93 ± 10.20 s) and Group B the shortest (55.18 ± 10.46 s). The highest velocity at 15 s post-activation was recorded in group A (220 ± 22.3 μm s^{-1}) and the lowest in Group B (159 ± 35 μm s^{-1}). Group C showed velocity of 187 μm s^{-1}. The results of our study showed that the length of the cold water period had no influence on spermatozoa quality, but did have an influence on the ability of males to produce sperm.

Introduction

Pikeperch (*Sander lucioperca*) inhabit most rivers, lakes, and reservoirs of continental Europe (Hilge and Steffens, 1996). It is a valuable species for intensive aquaculture, having excellent flesh quality, high growth rate, and good market value (Wuertz et al., 2012; Kristan et al., 2013).

Artifficial reproduction of pikeperch is difficult because of their high sensitivity to stress and manipulation (Demska-Zakes and Zakes, 2002). A hormonal treatment is necessary before artificial reproduction of pikeperch (Zakes and Szczepkowski, 2004; Kristan et al., 2014). The main ingredient of hormonal preparations, such as carp pituitary extract (CPE) or human chorionic gonadotropin (hCG), is gonadotropin that supports the gamete maturation, ovulation and spermiation (Zakes and Demska-Zakes, 2009). One of the most important factors in successful artificial and natural spawning is sperm quality (Teletchea et al., 2009). In fish with an annual spawning cycle, gametes change qualitatively and quantitatively during the season (Billard et al., 1995; Linhart et al., 2000; Rurangwa et al., 2004; Alavi and Cosson, 2006; Alavi et al., 2008). Assessment of spermatozoa quality can help to determine the optimal time for sperm stripping and artificial fertilization of eggs (Billard et al., 1995; Linhart et al., 2004). Spermatozoa concentration and motility determine fertilizing ability (Teletchea et al., 2009). The characteristics of seminal plasma and spermatozoa motility in pikeperch are reported in Cejko et al. (2008).

The aim of this study was to compare pikeperch spermatozoa quality (motility rate and duration and velocity) following artificial stimulation of maturation by varying periods of coldwater treatment.

Materials and methods

Preparation of broodstock for stripping

Twenty-one pond cultured pikeperch males (total length: 510 ± 23 mm; weight: 1202 ± 156 g) were divided into three Groups (A, B, and C), experiencing different lengths of cold water periods. All fish were subjected to the same protocol prior to stripping. Fish were held in 0.6 m^3 tanks for a cold water period (CWP) that varied in duration per group at water temperatures <5°C (mean 2.2–2.5°C) (Table 1). Length of the cold water period was 122 days for Group A, 149 days for Group B and 223 days for Group C. At the end of the CWP, water temperature was increased over the course of 21 days by 1°C per day to reach 14–15°C. Subsequently, fish were anesthetized with clove oil at 0.03 ml L^{-1} (Kouril and Hamackova, 2005) and human chorionic gonadotropin (hCG) (Chorulon, Intervet International B.V.) was injected into the dorsal muscle at 500 IU kg^{-1} (Kristan et al., 2013). The broodstock were then held in circular 0.3 m^3 tanks. Water temperature and oxygen saturation were measured (InoLab, WTW) twice daily at 07.00 and 15.00 hours throughout the trial. The photoperiod was not the same among groups. The process used for preparation and stimulation of broodstock before sperm stripping followed common practices to induce the out-of-season spawning (Zakes and Szczepkowski, 2004; Ronyai, 2007).

Sperm stripping and assessment of spermatozoa quality

Collection and assessed parameters. All males were stripped 72 h after hormone injection. Sperm samples were collected

Table 1
Water temperature, light intensity (lux; water surface), and photoperiod during the trials. Temperature values represent means and standard deviation based on two readings per day

	Group A	Group B	Group C
WT during the cold water period (°C)	2.2 ± 1.6	2.45 ± 1.59	2.55 ± 1.64
Duration of the cold water period (CWP; days)	122	149	223
Photoperiod during the CWP	9 : 15 L:D	9.5 : 14.5 L: D	10 : 14 L:D
Light intensity during the CWP (lx)	30	30	30
WT during latency (°C)	14.42 ± 0.83	15.37 ± 1.1	14.73 ± 0.11
Photoperiod after CWP	11 : 13 L:D	13 : 11 L:D	15 : 9 L:D
Light intensity after cold water period (lx)	100	100	100
Sperm stripping date	March 26	April 21	June 13

by syringes without an addition of immobilization solution. The syringes were placed on ice (2–4°C) and immediately transported to the laboratory for analyses.

Variables assessed were ability to produce sperm, volume of stripped sperm, spermatozoa concentration, percent motile cells (motility rate), spermatozoa velocity at 15, 30, and 45 s post-activation, and duration of motility.

Sperm production and spermatozoa concentration. Volume of sperm was defined as the volume in the sampling syringe. Ten μl of sperm was diluted in the immobilizing solution Kurokura 220 (Rodina et al., 2002) to a final spermatozoa concentration of 1 μl of sperm in 10 000 μl of Kurokura. The counting was performed in 16 squares of a Burker chamber after spermatozoa sedimentation (Teletchea et al., 2009). The concentration of sperm was expressed as billions of spermatozoa per ml sperm.

Spermatozoa motility variables. Spermatozoa motility variables included velocity, motility rate, and duration of motility. Spermatozoa motility was observed and recorded (DVD video-recorder SONY SVO-9500 MDP, Japan) immediately after activation with hatchery water (Alavi et al., 2008; Kristan et al., 2013) and continued until cessation of motility. Velocity and motility rate were assessed according to the position of spermatozoa heads. Microscopy using dark-field optics (Olympus BX50, magnification × 200) illuminated by a stroboscopic LED illumination unit Exposure Scope 0.1 (FROV, JU, Czech republic) combined with video recording with a CCD video camera (SONY SSC-DC 50 AP, Japan) provided images for analysis. Video recording provided 50 half-frames s^{-1} with 720 × 576 pixels (PAL 4 : 3) spatial resolution per frame. Motility rate and velocity were measured by tracking positions of the spermatozoa head on five consecutive video frames using red for frame 1, green for frames 2–4, and blue for frame 5 (Hulak et al., 2008). Thirty spermatozoa were observed in each frame. Moving spermatozoa were visible in the three colours, and stationary spermatozoa were white. The velocity of spermatozoa was calculated as μm s^{-1} based on length of traces of spermatozoa heads from red to green to blue. Motility rate was calculated from red vs white cells.

Data analysis

Assessment of spermatozoa velocity and motility rate was replicated three times for each male. Statistical analyses were made with Statistica 10.0 (StatSoft, Inc.). One-way ANOVA with Tukey s post-test was used to investigate differences between the observed groups. The level of significance was set at $P < 0.05$.

Results

Presence of sperm was highest in Group B fish lowest in Group C (Table 2). The volume of sperm differed significantly among groups. The highest volume of sperm was obtained in Group C and the lowest in Group A. There were no differences in spermatozoa concentration among groups. The longest motility time was observed in Group A. Duration of motility in Group B and Group C was similar (Table 2).

The lowest spermatozoa velocity was recorded in Group B. Spermatozoa velocity decreased with time post-activation in all groups. No differences among groups were observed 45 s post-activation (Fig. 1).

Spermatozoa motility rate decreased with the length of the post-activation time. Significant differences among groups were observed only at 15 s post-activation. The highest motility rate at 15 s post-activation was observed in Group A, and the lowest in Group B. No difference was recorded among groups 45 s post-activation (Fig. 2).

Discussion

A primary aim of intensive aquaculture is the year-round production of market-size fish. Continuous production is dependent on multiple or out-of-season reproduction of

Table 2
Parameters of spermatozoa quality and quantity obtained from the same males at difference stripping dates. Different letters indicate significant difference ($P < 0.05$)

Stripping date	A (March 26)	B (April 21)	C (June 13)
Presence of sperm	5 of 7 (71%)	6 of 7 (85%)	3 of 7 (42%)
Volume of sperm [ml]	0.64 ± 0.26^{a}	1.07 ± 0.53^{b}	1.8 ± 0.2^{c}
Spermatozoa concentration [mld ml^{-1}]	19.34 ± 3.87^{a}	15.73 ± 2.68^{a}	16.47 ± 2.29^{a}
Motility duration [s]	89.93 ± 10.2^{a}	55.18 ± 10.46^{b}	59.5 ± 8.69^{b}

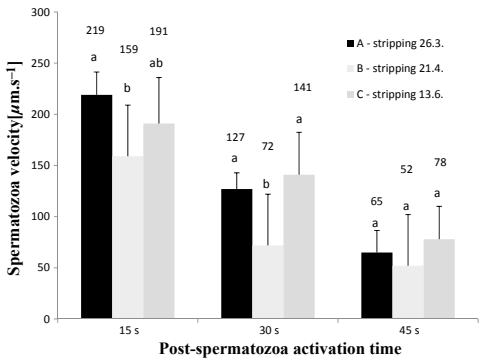

Fig. 1. Pikeperch spermatozoa velocity at 15, 30, and 45 s post-activation for spermatozoa obtained at different stripping dates. Columns represent means and bars standard deviation. Sample size (n) indicated above each column ($P < 0.05$)

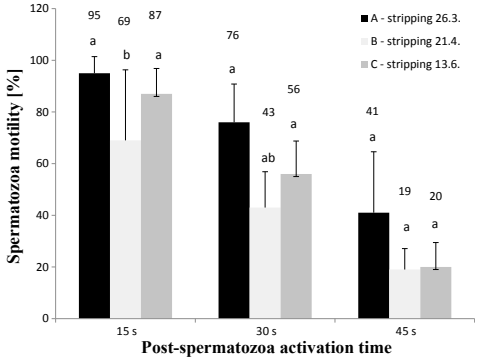

Fig. 2. Pikeperch spermatozoa motility rates at 15, 30, and 45 s post-activation for spermatozoa obtained at different stripping times. Columns represent means and bars standard deviation. Sample size (n) indicated above each column ($P < 0.05$)

broodstock. Two sperm collections (Group A, 3/26 and Group C, 6/13) were made outside the natural pikeperch spawning season in the Czech Republic (end of April and beginning of May). Spawning of broodstock was conducted by the method commonly used for out-of-season spawning of pikeperch (Zakes and Szczepkowski, 2004; Ronyai, 2007). Water temperature and photoperiod are important factors in natural spawning, especially in out-of-season spawning. Mueller-Belecke and Zienert (2008) used a period with temperature below 10°C and controlled photoperiod to achieve the out-of-season spawning of pikeperch; they reported that a 31–61 day cold water period is sufficient to stimulate spawning. Ronyai (2007) suggests that 3 months of cold water is sufficient for maturation of pikeperch gonads. In our experiment, the shortest coldwater period was 122 days (Group A) and the longest 223 days (Group C). The longer CWP was associated with a lower ability to produce sperm.

For reasons of photo/thermal regime, stress, or lack of spawning substrate, it is not possible to induce spermiation in all pikeperch without hormone injection (Zakes and Szczepkowski, 2004; Ronyai, 2007). Therefore fish in the three groups were injected with hCG prior to sampling (Schlumberger and Proteau, 1996; Ronyai, 2007; Zakes and Demska-Zakes, 2009).

Spermatozoa concentration can change during the reproductive season (Alavi et al., 2008) and among fish species. In pikeperch in the present study, spermatozoa concentrations ranged from 15.73 ± 2.68 (Group B) to 19.34 ± 3.87 10^9 ml^{-1} (Group A). Teletchea et al. (2009) reported a spermatozoa concentration of 14.8 ± 4.1 10^9 ml^{-1} and Kristan et al. (2013) 14.87 ± 1.3 10^9 ml^{-1} in pikeperch, similar to our result. However, Cejko et al. (2008) observed a pikeperch spermatozoa concentration of only 4.28–5.26 10^9 ml^{-1}.

Spermatozoa velocity has been recognized as a primary determinant of fertilization success in number of fish species (Urbach et al., 2007). The highest spermatozoa velocity at 15 s post-activation was observed in Group A (219 ± 22 μm s^{-1}). This is higher than observed by Teletchea et al. (2009), who reported pikeperch spermatozoa velocity of 148 ± 9 μm s^{-1}, similar to our finding of 159 ± 34 μm s^{-1} in Group B. Spermatozoa velocity decreased with time after the activation (Kristan et al., 2014). The observed motility rate at 15 s post-activation is comparable with findings of Teletchea et al. (2009). Cejko et al. (2008) observed motility of pikeperch sperm in a range of 14.72–25%.

The results of our study showed that the length of the cold water period had no influence on spermatozoa quality, but influenced the ability of the males to produce sperm. In group C (CWP = 223 days), sperm was obtained from only three fish. According to our results, we suggest the use of a greater number of males for artificial reproduction if the cold water period is longer, to ensure sufficient amounts of sperm.

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CHAPTER 3

BENEFITS OF HORMONE TREATMENT OF BOTH SEXES IN SEMI-ARTIFICIAL REPRODUCTION OF PIKEPERCH (*SANDER LUCIOPERCA* L.)

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Benefits of hormone treatment of both sexes in semi-artificial reproduction of pikeperch (*Sander lucioperca* L.)

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ABSTRACT: Propagation of pikeperch *Sander lucioperca* by semi-artificial management was investigated using hormonal induction to better understand the effect of hormonal treatment of both sexes. Fourteen pairs of pikeperch brood fish were divided into two groups (seven pairs in each group). Seven females in Group A and both sexes in Group B were hormonally injected with 500 IU of hCG per kg. Each pair of brood fish was held separately in a 300-l circular tank with an added spawning material on the bottom. Brood fish were removed from the tanks on the day after spawning; three-hundred randomly selected eggs were removed from the spawning nests and transferred to 250 ml plastic incubators. Sperm samples were collected from males in individual 1-ml syringes. Significant differences in fertilization rate (Group A 59.5%, Group B 80.4%), hatching rate (Group A 51.2%, Group B 71.6%), and number of larvae produced per female (Group A 49 429, Group B 122 000) were observed. Differences were attributable to sperm quality, primarily volume (Group A 0.16 ml, Group B 0.64 ml), and duration of spermatozoa motility (Group A 59.5 s, Group B 97.7 s). Hormone treatment of both sexes is beneficial for pikeperch semi-artificial reproduction by inducing ovulation and improving milt production and spermatozoa quality, fertilization and hatching rate, all contributing to a higher number of produced larvae.

Keywords: hCG; spermatozoa; fertilization; hatching; spawning nest; larvae

INTRODUCTION

Pikeperch (*Sander lucioperca* L.) is important in reservoir ecology as the natural predator of small cyprinids (roach – *Rutilus rutilus* L., rudd – *Scardinius erythrophthalmus* L., or bream – *Abramis brama* L.), and as a valuable freshwater game fish in Europe due to its delicate flesh (Schulz et al. 2007; Kubecka et al. 2009; Kristan et al. 2013). Unfortunately, the natural populations of pikeperch are affected by over-fishing in many areas and by human-related environmental degradation (Dil 2008). Pikeperch have been farmed in ponds or lakes through natural spawning and recruitment (Hilge and Steffens 1996). As an alternative to this traditional method, a combination of pond and recirculation aquaculture system (RAS) is currently being used (Policar et al. 2014). This technique is based on rearing larvae and juveniles in ponds to produce advanced fingerlings (total length 30–50 mm) and then using intensive culture and artificial pellet feeding (Ruuhijarvi and Hyvarinen 1996; Zakes and Demska-Zakes 1998; Policar et al. 2014). A new development which is being applied in West European countries is the rearing of pikeperch production under complete

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RAS conditions (Policar et al. 2014; Blecha et al. 2015). The demand for pikeperch to restock natural waters and for intensive farming is increasing and will require more efficient methods of reproduction (Zakes and Demska-Zakes 2009).

The oldest method of pikeperch propagation is by natural spawning in ponds (Steffens et al. 1996). The main disadvantages of this method are low efficiency and inability to predict the final production (Demska-Zakes and Zakes 2002). Improved results are obtained through the semi-artificial spawning (Schlumberger and Proteau 1996) which can be conducted under hatchery conditions (Kucharczyk et al. 2007) where artificial spawning substrata are added such as coniferous tree branches, mats, carpets or artificial grass (Skrzypczak et al. 1998; Demska-Zakes and Zakes 2002). The spawning material can be checked several times per day for eggs. After spawning, brood fish are removed from the cages or tanks; the eggs can be left in the spawning tanks to incubate or transferred on the spawning material to special incubation tanks (Kucharczyk et al. 2007). Also, brood fish can be hormonally stimulated using the semi-artificial reproduction (Zakes and Demska-Zakes 2009).

Non-hormone-stimulated pikeperch females held in captivity usually do not ovulate (Zakes and Szczepkowski 2004) or ovulation occurs only in few individuals (Salminen et al. 1992). Kucharczyk et al. (2007) suggest that pikeperch do not spawn in captivity because of stress, lack of spawning substrate, or unsuitable photo-thermal regime. To circumvent these problems, hormonal induction can be used. The substances that stimulate fish maturation and reproduction have been extensively investigated in recent years (Steffens et al. 1996). These substances stimulate either the hypothalamus, hypophysis or the gonads (Demska-Zakes and Zakes 2002). Final maturation and synchronization of the ovulation in pikeperch can be achieved by administering carp pituitary extract (CPE), luteinizing hormone-releasing hormone (LH-RH) (Ronyai 2007), mammalian gonadotropin releasing hormone analogue (GnRHa) or human chorion-gonadotropin (hCG) (Kristan et al. 2013). Chorulon, a formulation with hCG, has been used for artificial reproduction of different fish species, such as goldfish (*Carassius auratus auratus* L.) (Targonska and Kucharczyk 2011), Eurasian perch (*Perca fluviatilis* L.) (Kucharczyk et al. 1996), and also pikeperch (Kristan et al. 2013). Currently, there is no information on hormone treatment effects for male pikeperch.

The main aim of this study was to compare reproductive success, and gamete and offspring traits between hormonally injected pikeperch pairs where both sexes and pairs with only females were hormonally treated. Parameters measured were latency (time between hormone injection and ovulation), percentage of successfully spawned females, diameter of fertilized eggs, fertilization and hatching rates, egg incubation time, numbers of free-swimming larvae, total length of free-swimming larvae, larval survival after a 90-min osmotic shock and for males, volume of stripped sperm, spermatozoa concentration, spermatozoa motility rate, motility duration, and velocity.

MATERIAL AND METHODS

Fish groups and spawning conditions. Fourteen females and fourteen males were used in this study. All the brood fish were captured from the ponds of Rybářství Nové Hrady s.r.o. and then transferred to the South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses in Vodňany, Czech Republic. Holding tanks (600 l capacity) were supplied with water from a recirculating system (water temperature (WT) = 13.6 ± 0.2°C). Oocyte maturation stage was assessed at the beginning of experimentation (Zarski et al. 2012). Fish were randomly divided into seven male/female pairs and then into two groups – A (total length (TL) = 458 ± 33 mm; weight (W) = 1056 ± 112 g) and B (TL = 442 ± 41 mm; W = 988 ± 153 g). In Group A, only the females were injected with hCG at 500 IU per kg, and in Group B both sexes were injected at the same dosage. Each fish pair was held separately in 300-l circular tanks with spawning substrate constructed of artificial grass with fibre length 50 mm placed on the tank bottom. The 14 tanks were part of a recirculation aquaculture system that provided stable and optimal conditions for pikeperch spawning (14.8 ± 1.4°C and oxygen saturation 10.8 ± 2.3 mg O_2/l). Brood fish were removed from the tanks after spawning. Fertilized eggs were incubated on the spawning substrate. On day 4 after spawning, water inflow was blocked to prevent larvae from escaping (the hatching started on day 5 after spawning). Newly hatched free-swimming larvae were counted using the volume method: all larvae in the tank were

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concentrated in 10 l of water, and five samples of 25 ml of water with larvae were removed. Larvae were counted in each subsample and the total number was calculated from the mean count in the sub-samples. WT and oxygen saturation were measured twice daily with an oximeter (OxyGuard International A/S, Farum, Denmark) during the latency and incubation time.

Assessment of the eggs and larvae quality. On the first day after spawning, 300 randomly selected eggs were removed from the substrata and 100 eggs were placed in each of the three 250-ml plastic incubators to measure diameter of fertilized eggs, fertilization rate (FR), and hatching rate (HR). Incubators were held in a Styrofoam frame floating on the surface of the spawning tank; water in the incubators was changed twice daily. FR was calculated as the proportion of fertilized eggs in each incubator and HR as the number of larvae relative to the initial number of eggs. Egg diameters were measured 24 h after spawning to the nearest µm using a stereo microscope SMZ745T (Nikon, Tokyo, Japan) with Quick PHOTO MICRO 3 program. Length of freshly hatched larvae was measured at hatching by the same method used for the measurement of eggs diameter. 25 eggs and 25 larvae from each incubator were used for diameter and length measurement, respectively.

Twenty larvae in four repetitions from each spawned pair were used to assess resistance to osmotic shock. The test was carried out in 2% sodium chloride solution. The number of larvae surviving after 15, 30, 45, 60, 75, and 90 min of exposure was recorded.

Collection of the spermatozoa samples and assessed parameters. On the day following spawning, milt samples were collected (4 individuals from Group A and 5 from Group B) into individual 1-ml syringes without addition of immobilization solution. Syringes were placed on ice and immediately transported to the laboratory for analyses.

From spermiating males, the following parameters were taken: volume of stripped milt, spermatozoa concentration, percentage of motile cells (motility rate), duration of spermatozoa motility, and spermatozoa velocity. Milt volume was defined as the amount in the sampling syringe. For assessment of spermatozoa concentration, 10 µl milt was diluted to a final concentration of 1 µl sperm in 10 000 µl of KUROKURA-220 immobilizing solution (Rodina et al. 2002). Counting was performed according to Teletchea et al. (2009) and concentration was expressed as billions of spermatozoa per ml of milt.

Spermatozoa motility variables. Spermatozoa were assessed by motility variables, including duration of motility, motility rate, and velocity 15, 30, and 45 s post-activation. Immediately after activation in hatchery water spermatozoa motility was recorded with a DVD video-recorder SVO-9500 MDP (SONY, Tokyo, Japan) (Kristan et al. 2013) until motility ceased. Microscopy used dark-field optics (Olympus BX50, magnification ×200; Olympus, Tokyo, Japan) illuminated by a stroboscopic LED illumination unit Exposure Scope 0.1 (University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, Czech Republic) combined with video recording on a CCD video camera SONY SSCDC 50 AP which provided recorded images for spermatozoa motility and velocity analysis. Video recording provided 50 half-frames with 720 × 576 pixels (PAL 4 : 3) spatial resolution per frame. Motility rate and velocity were measured according to Hulak et al. (2008). The velocity of spermatozoa was calculated as µm/s based on length of traces of spermatozoa heads from red to green to blue using MicroImage facility (Olympus). Motility rate was calculated as a percentage of red cells among all cells.

The data are presented as mean ± standard deviation. Statistical analysis was based on one-way Analysis of Variance (ANOVA) (STATISTICA, Version 12, 2013) including *F*-statistics. Significant differences between groups were estimated using Tukey's *post-hoc* test. The level of significance was set at $P < 0.05$.

RESULTS

All parameters were significantly different between treatment groups (fertilization rate: Group A 59.5 ± 17.9%, Group B 80.4 ± 9%; hatching rate: Group A 51.2 ± 17.7%, Group B 71.6 ± 9.4%; production of free-swimming larvae per female: Group A 49 429 ± 32 544, Group B 122 000 ± 15 311; volume of stripped sperm the day after spawning: Group A 0.16 ± 0.09 ml, Group B 0.64 ± 0.26 ml). Concentration of spermatozoa was $37.6 \pm 8.3 \times 10^9$ per ml in Group A and $19.3 \pm 3.9 \times 10^9$ per ml in Group B. Significant differences were also observed in motility percentage at 30 and 45 s post-activation (Figure 1A) and motility duration – Group A: 59.5 ± 31.8 s, Group B: 97.7 ± 7.1 s (Table 1).

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Table 1. Fertilization and hatching rate – eggs, larvae, and spermatozoa quality and quantity

	Group A	Group B	*F*-statistics
Latency (h)	96.4 ± 9.0[a]	92.4 ± 1.5[a]	4.543
Successful spawning (%)	57.1	71.4	
Fertilization rate (%)	59.5 ± 17.9[a]	80.4 ± 9[b]	5.293
Hatching rate (%)	51.2 ± 17.7[a]	71.6 ± 9.4[b]	5.054
Incubation time (degree days)	89.9 ± 7.8[a]	90.4 ± 1.8[a]	3.018
Diameter of fertilized eggs (mm)	1.15 ± 0.026[a]	1.26 ± 0.068[a]	8.772
Number of free-swimming larvae per female	49 429 ± 32 544[a]	122 000 ± 15 311[b]	21.11
Total length of free-swimming larvae (mm)	3.55 ± 0.16[a]	4.2 ± 0.12[b]	88.38
Larva survival after a 90-min osmotic shock (%)	97.2 ± 0.6[a]	96.6 ± 1.2[a]	5.273
Ability to produce sperm (%)	57.1	71.4	
Volume of stripped sperm (ml)	0.16 ± 0.09[a]	0.64 ± 0.26[b]	10.49
Spermatozoa concentration (10^9/l)	37.6 ± 8.3[a]	19.3 ± 3.9[b]	19.81
Spermatozoa motility duration (s)	59.5 ± 31.8[a]	97.7 ± 7.1[b]	11.56

[a,b]different superscript letters indicate significant difference ($P < 0.05$)

There were no differences between groups in ovulation latency: Group A 96.4 ± 9 h, Group B 92.4 ± 1.5 h); successful spawning: Group A 57.1%, Group B 71.4%; incubation time: Group A 89.9 ± 7.8 degree days (°D), Group B 90.4 ± 1.8 °D (Table 1), and spermatozoa velocity: 15, 30, or 45 s post-activation (Figure 1B). The osmotic shock used to test the larvae viability and resistance (Policar et al. 2010) showed no differences between Groups A and B (Table 1).

DISCUSSION

This study demonstrated the importance of hormonal treating both sexes in pikeperch semi-artificial spawning. Water temperature was 14.8°C during the experimental spawning study. Zakes and Demska-Zakes (2009) recommend 14–16°C as optimal water temperature for pikeperch reproduction. Pikeperch females usually do not ovulate in captivity without hormone treatment (Zakes and Szczepkowski 2004; Kucharczyk et al. 2007), but there has been no information published about the influence of hormone treatment on male pikeperch. All hormone-treated fish were injected with hCG at 500 IU per kg, which is the commonly used concentration for pikeperch reproduction in captivity (Ronyai 2007; Sosinski 2007; Kristan et al. 2013).

Benefits of hormone treatment for males in semi-artificial spawning were indicated by the treatment group differences. Spermatozoa concentration was 37.6 ± 8.3 × 10^9 per ml in Group A (non-hormone treatment) and 19 ± 3.9 × 10^9 per ml in Group B.

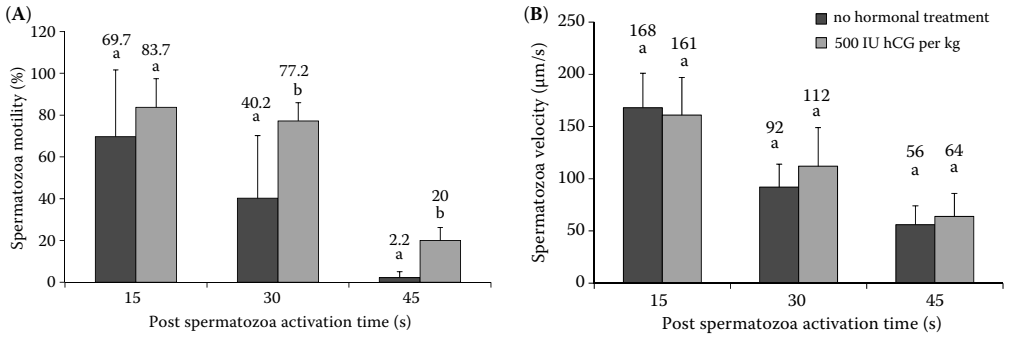

Figure 1. Spermatozoa motility (**A**) and velocity (**B**) at 15, 30 and 45 s post-activation

[a,b]different letters indicate significant difference ($P < 0.05$)

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Kristan et al. (2014), who also used 500 IU of hCG per kg for hormone stimulation of brood fish males, reported pikeperch spermatozoa concentration of $14.83 \pm 1.3 \times 10^9$ per ml, similarly to our results for Group B. Cejko et al. (2008) observed pikeperch spermatozoa concentration of only $4.28–5.26 \times 10^9$ per ml, different from both groups in our study. No difference was observed in spermatozoa velocity between groups A and B; velocity at 15 s post activation was 168 µm/s in Group A and 161 µm/s in Group B. Similar results (spermatozoa velocity 135–162 µm/s) obtained from untreated males have been reported by Teletchea et al. (2009). We measured motility rate in Group A at 69.7% and in Group B at 83.7% 15 s post-activation, which was comparable to the findings of Teletchea et al. (2009) who observed 59–85% of spermatozoa were motile. Cejko et al. (2008) found that only 14.7–25.0% of spermatozoa were motile following the injection of 450 IU of hCG per kg.

Latency of ovulation after induction with 500 IU/kg·hCG at 15.0 ± 0.5°C water temperature was 96.4 h (Group A) and 92.4 h (Group B) compared to 78.05 ± 6.93 h (Kristan et al. 2013). Fertilization and hatching rates were highly influenced by spermatozoa quality. Fertilization rate in Group B (80.4 ± 9%) was similar to 87 ± 8% reported by Ronyai (2007) after hCG treatment of both sexes, but 71.6 ± 9.4% in Group B was lower than that of 84.2 ± 6.2% found by Kristan et al. (2013). Incubation time was 89.9 °D in Group A and 90.4 °D in Group B. Schlumberger and Proteau (1996) reported that hatching in pikeperch usually occurs at 65–110 °D at water temperature 14–15°C.

To conclude, we found that hormonal treatment of both sexes in semi-artificial reproduction of pikeperch was useful and had important effects on the final production of larvae and effectiveness of the entire reproduction process.

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CHAPTER 4

ALCALASE TREATMENT FOR ELIMINATION OF STICKINESS IN PIKEPERCH (*SANDER LUCIOPERCA* L.) EGGS UNDER CONTROLLED CONDITIONS

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Alcalase treatment for elimination of stickiness in pikeperch (*Sander lucioperca* L.) eggs under controlled conditions

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Abstract

Elimination of egg stickiness is an important factor in artificial reproduction of pikeperch (*Sander lucioperca* L.). This study was conducted to evaluate the effectiveness of Alcalase enzyme to remove the adhesive layer of pikeperch eggs. The eggs were treated with Alcalase at 0.5, 1.0, 1.5, 2.0 and 5.0 mL L^{-1} or a milk/talc solution 2 min post insemination. Duration of exposure was 2 min in Alcalase and 60 min in milk/talc. The highest, albeit not significant, hatching rate (85.4%) was found with 1.5 mL L^{-1} of Alcalase, but hatching rates were similar in 0.5, 1.0 and 2.0 mL L^{-1}. Hatching rates were significantly lower groups treated with 5.0 mL L^{-1} Alcalase enzyme (56.4%) compared to groups treated with milk powder and talc (61.3%). Nominally complete removal of adhesiveness was observed in 1.5 and 2 mL L^{-1}. All Alcalase treatments led to significantly lower incubation duration compared with the traditional milk/talc treatment. The application of Alcalase successfully eliminated pikeperch egg stickiness in less time than with traditional milk/clay/talc methods.

Keywords: proteolytic enzyme, egg stickiness, incubation time, pikeperch

Introduction

Pikeperch (*Sander lucioperca* L.) is a promising species for inland European aquaculture (Fontaine 2009; Kristan, Alavi, Stejskal & Policar 2013; Policar, Stejskal, Křišťan, Podhorec, Švinger & Bláha 2013). Increased production of fish that are economically valuable is possible, thanks to the application of developments in broodstock management and egg incubation (Donaldson 1996). Elimination of egg stickiness is one of the most important factors for incubation of eggs in hatchery jars (e.g. Zug jars), which increase hatching rate and larval production (Linhart, Gela, Flajšhans, Duda, Rodina & Novák 2000; Gela, Linhart, Flajshans & Rodina 2003; Siddique, Psenicka, Cosson, Dzyuba, Rodina, Golpour & Linhart 2014).

The first attempts to remove adhesive layer from eggs were made with a suspensions of mud, starch or charcoal (Sakowicz 1928; Demska-Zakes, Zakes & Roszuk 2005). The traditional methods for elimination of fish egg stickiness involve stirring the fertilized eggs into Woynarovich solution (salt and urea) (Woynarovich & Woynarovich 1980), milk powder (Khan, Gupta, Reddy & Sahoo 1986), milk (Billard, Cosson, Perchec & Linhart 1995), talc or clay suspensions (Gela *et al.* 2003; Mizuno, Sasaki, Omoto & Imada 2004) or tannic acid (Dumas & Brand 1972; Colesante & Youmans 1983; Demska-Zakes *et al.* 2005; Zarski, Krejszeff, Kucharczyk, Palinska-Zarska, Targonska, Kupren, Fontaine & Kestemont 2015). Currently, three methods for removal of egg adhesive are used in pikeperch aquaculture. Adhesive can be removed from the eggs by bathing in a milk/talc solution

(100 g of talc to 10 L water + 100 g milk powder to 1 L water, 1:1), and stirring for 60 min (Kristan & Blecha, personal communication, 2015). Another method involves a talc/sodium chloride solution (100 g NaCL + 25 g talc + 10 L water) for 45–60 min (Schlumpberger & Schmid 1980). The third possibility is the use of tannic acid (500–1000 mg L^{-1}) as is routinely employed in Poland (Demska-Zakes *et al.* 2005; Kucharczyk, Kestemont & Mamcarz 2007; Zakes 2007; Kujawa, Kucharczyk & Mamcarz 2010; Zarski, Kucharczyk, Targonska, Palinska, Kupren, Fontaine & Kestemont 2012; Zarski *et al.* 2015). Unfortunately, these described methods were pointed out as an ineffective and/or time consuming. The low effectiveness and/or time consuming led to testing new chemical substance. One of these is application of proteolytic enzyme.

The first documented use of the proteolytic enzyme was reported in European catfish *Silurus glanis* (Horvath 1980; Proteau, Schlumberger & Albiges 1994), walleye *Sander vitreus* (Krise, Bulkowski-Cummings, Shellman, Kraus & Gould 1986; Krise 1988) and pikeperch (Zakes, Demska-Zakes, Roszuk & Kowalska 2006). Currently, proteolytic enzyme-Alcalase is commonly used for elimination of egg stickiness in tench *Tinca tinca* (Linhart *et al.* 2000; Gela *et al.* 2003; Linhart, Gela, Flajšhans & Rodina 2003; Linhart, Rodina, Gela, Flajšhans & Kocour 2003), European catfish (Linhart, Billard, Kouřil & Hamáčková 1997; Linhart, Stech, Svarc, Rodina, Audebert, Grecu & Billard 2002; Linhart, Rodina, *et al.* 2003; Linhart, Gela, Rodina & Kocour 2004) and common carp *Cyprinus carpio* (Linhart, Rodina, Gela, Kocour & Rodriguez 2003; Linhart, Rodina, *et al.* 2003). It is routinely used in hatcheries in the Czech Republic and in France (Linhart *et al.* 2002; Linhart, Rodina *et al.* 2003). Alcalase is a proprietary product manufactured from a proteolytic enzyme obtained by fermentation of a strain of *Bacillus licheniformis*. It is stable and is active at high temperatures and alkalinities, and is used in laundry detergents and various industrial processes.

The primary aim of this study was to assess the efficacy of Alcalase enzyme (Merck EC 3.4.21.14) in removing adhesiveness from eggs of pikeperch, and to identify the optimum treatment concentration, and compare exposure time with the traditional methods.

Materials and methods

The experiment was performed at a hatchery of the University of South Bohemia, Faculty of Fisheries and Protection of Waters (USB FFPW), Czech Republic.

Fish handling and gamete collection

Broodstock of pikeperch were obtained from the fish farm Rybářství Třeboň and kept in a storage pond at USB FFPW during March. In mid-April, following transfer from the pond to the hatchery, mature pikeperch were sorted by sex, and 10 males and 10 females were placed in separate 1 m^3 tanks of a recirculating aquaculture system. Broodfish were anesthetized with clove oil (Kristan, Stara, Turek, Policar & Velisek 2012) and given a single intramuscular injection of hCG (500 IU kg^{-1}) and stripped as described by (Kristan *et al.* 2013).

Experimental procedure

Eggs from five successfully stripped females were mixed and divided into six batches of 5 g (~5000 eggs) and inseminated with the mixed sperm from five males, 200 μL for each batch of eggs. Each group of eggs was dispersed into 2.5 mL fresh hatchery water (pH 7.6) for activation. After 2 min of activation, the hatchery water was slowly removed and the treatments to eliminate the adhesiveness of eggs were administered.

Two methods with several variants (6 groups × 3 replicates = 18 treatmens) were compared in this study:

1 Milk powder and talc suspension: Each batch of inseminated eggs was mixed with 5 mL each of a milk powder solution [100 g to 1 L of hatchery water (Laktino, 26% fat, Protein.Mléko.Laktóza, Nový Bydžov, Czech Republic)] and a talc solution (magnesiumhydrosilicate – EINECS 238-877-9) [100 g to 10 L of hatchery water (M. Rada, Litomyšl, Czech Republic)]. The eggs were stirred 60 min, rinsed in hatchery water, and transferred to incubators for fertilization test and Zug jars for hatchery production.
2 Five groups were treated with Alcalase® enzyme (*Bacillus licheniformis*, Merck EC 3.4.21.14, Darmstadt, Germany) added to 1% NaCl solution at 20°C. The concentrations of enzymes were 0.5, 1.0, 1.5, 2.0 and 5.0 mL

filled up to 1 L of NaCl solution. Five mL of each de-sticking solution was added to fertilized eggs, which were mechanically stirred for 2 min, rinsed in hatchery water and transferred to incubators and Zug jars.

Batches of 150–182 eggs (~three drops) per replicate were placed into 200 cm^2 incubator cages connected to the recirculation system and supplied with water at 16°C and 8.5 mg L^{-1} O_2. For the verification of elimination of adhesive (not stickiness to Zug jars), the remaining eggs were transferred to 0.5 L Zug jars supplied with flow-through water at a rate 2.5 L min^{-1}, 7–9 mg L^{-1} O_2, 15°C. Dead eggs and hatched larvae in incubators were counted and counts were used for calculation of fertilization and hatching rate. Duration of incubation to hatching was recorded in degree days.

Statistical analysis

Statistical analysis was carried out using Statistica software 9.0 for Windows (StatSoft, Prag, Czech Republic). Data were first tested for normality (Kolmogorov–Smirnov test) and homoscedasticity of variance (Bartlett's test). If those conditions were satisfied, a LSD multiple comparison test was applied to identify treatments that were significantly different. Probability values ($P < 0.05$) were considered as significant.

Results

Fertilization and hatching rate

Adhesive elimination was successful for all groups of eggs. Neither destruction of egg envelopes nor larval malformations were observed. The 100% unsticking success (all eggs not stickiness to incubator) was observed in 1.5 and 2 mL L^{-1} (Table 1). The fertilization rate (88.2–91.2%) was not significantly different among groups (Table 1). The highest hatching rate (85.4%) was observed in the group exposed to 1.5 mL L^{-1} of Alcalase, but hatching rates were similar at 0.5, 1.0 and 2.0 mL L^{-1} concentrations of Alcalase (Table 1). Hatching rates were significantly lower in the 5.0 mL L^{-1} Alcalase (56.4%) and milk/talc (61.3%) treatments than in the remaining Alcalase treated groups. The hatching rate of over 80% obtained in the groups with Alcalase 1.5 and 2.0 mL L^{-1} indicates the high efficacy of this method and exposure level.

Incubation time in degree days

The incubation time ranged from 110 to 126° D (Fig. 1) in all tested groups. All Alcalase treatments resulted in significantly decreased incubation time compared with the traditional milk/talc treatment. The longest incubation time was in milk/talc treatment (126° D) and the shortest incubation time (110° D) with 5.0 mL L^{-1} Alcalase.

Discussion

In most cultured species, for example, tench, common carp, pike and pikeperch, hatching rate is an important criterion to evaluate the efficacy of artificial reproduction. In the hatchery, the success of artificial reproduction partially depends on the incubation and elimination of egg stickiness (Demska-Zakes *et al.* 2005; Siddique *et al.* 2014; Zarski *et al.* 2015). The stickiness of eggs is not problematic for fertilization, but during incubation, eggs become attached to one another and increase fungal diseases. Concurrently, the gas exchange decrease

Table 1 Fertilization rate, hatching rate, egg mortality and unsticking success in pikeperch eggs under treatment to remove adhesive. Data are shown as mean ± SD. Values within the column with different superscripts are significantly different ($P < 0.05$)

Treatment	Enzyme L^{-1} of dilution solution (mL L^{-1})	Number of stocked eggs	Number of dead eggs	Fertilization rate (%)	Hatching rate (%)	Unsticking success (%)
Alcalase	0.5	159.5 ± 32.3	34.5 ± 14.3	91.2 ± 6.3	79.9 ± 6.0^{c}	60
	1.0	159.5 ± 1.2	38 ± 6.5	89.6 ± 2.9	76.1 ± 5.2^{bc}	90
	1.5	182 ± 23.7	25.5 ± 2.0	91.1 ± 4.2	85.4 ± 3.7^{c}	100
	2.0	150 ± 51.4	24 ± 4.1	90.3 ± 1.8	82.3 ± 4.1^{c}	100
	5.0	167 ± 66.1	68.5 ± 21.6	88.2 ± 0.9	56.4 ± 5.3^{a}	100
Milk/talc		169 ± 13.1	66 ± 9.8	90.0 ± 2.3	61.3 ± 3.4^{ab}	100

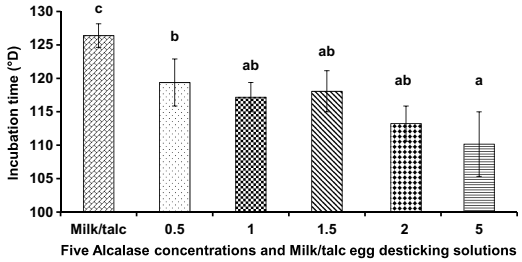

Figure 1 Incubation time in degrees days (°D) for pikeperch eggs treated for elimination of egg stickiness. All values are means ± SD. Values within the column with different superscripts are significantly different ($P < 0.05$).

between the eggs (Siddique *et al.* 2014). The pikeperch eggs and majority of fish oocytes are surrounded with the external proteinaceous layer called the egg envelope or chorion (Cherr & Clark 1982; Robles, Cabrita, de Paz & Herraez 2007), which are the main source of egg stickiness. The chemical treatments digest the chorion layer and remove the adhesiveness. The most effective treatment for elimination of pikeperch eggs stickiness is application of tannin acid or proteolytic enzymes.

In application of tannin acid, the highest hatching rate 95% with concentration 750 mg of tannin acid L^{-1} was observed by Zarski *et al.* (2015) for 1 min in 30 min after activation. Colesante and Youmans (1983) observed 65% hatching of walleye (*Sander vitreus*) eggs when treated with a solution of 250 mg tannic acid L^{-1} H_2O for 4 min. Demska-Zakes *et al.* (2005) reported that the application of low concentrations of tannic acid solution for a short exposure time is not effective and observed hatching rates of 43–51% following treatment with concentrations of 500 and 1000 mg L^{-1} for 5 min. These results are in accordance with the study by Krise *et al.* (1986) and Waltemyer (1975), who also indicated that tannic acid has a detrimental effect during incubation. Mizuno *et al.* (2004) as well showed that the traditional method of kaolin treatment resulted in higher hatching rates and lower mortality during incubation compared with tannic acid-treated eggs in shishamo smelt *Spirinchus lanceolatus*.

In case of proteolytic enzymes, the preliminary study on protease effect was described by (Zakes *et al.* 2006). In this study, the 0.05, 0.1 and 0.5% of protease solution was tested at 2, 5 and 10 min after activation of eggs. The highest hatching rate (80%) was achieved with 0.5% of protease in 2 min post activation. On the other hand, Krise *et al.* (1986) reported a hatching rate of approximately 80% with 0.01% protease with 8–10 U of enzyme activity per mg of protein for 30 min in walleye. Zakes *et al.* (2006) also showed significantly higher hatching rate with protease compared with tannin acid. This study also shows that the protease penetrated the chorin structure. However, this study do not described type and preparation of protease solution.

Despite this, there have been no published studies on effect of Alcalase enzymes on pikeperch egg stickiness. The five concentration of Alcalase treatment was compared for this reason in current study. The observations made under this study indicated that the 0.5–2.0 mL L^{-1} of Alcalase for only 2 min may increase pikeperch egg hatching success (to 80–85%) compared with control (traditional milk and talc solution). In this study, the hatching rate was not similar to Zarski *et al.* (2015), who achieved hatching rate around 95%. However, the lower hatching rate can by due to poorer quality of eggs. On the other hand, the application of Alcalase allows significantly reduced time for eggs rinsing and incubation in comparison with tannin acid. Simultaneously, the use of Alcalase for elimination of egg stickiness in pikeperch can facilitate the application of physical shock for chromosome manipulation as was described in tench (Flajšhans, Linhart & Kvasnička 1993; Gela, Kocour, Flajšhans, Linhart & Rodina 2010). The Alcalase treatment was also successfully used in tench (Linhart, Gela *et al.* 2003) and in European catfish (Linhart *et al.* 2004). These studies produced similar results (hatching rate 80–87%) but with higher concentrations (5–20 mL L^{-1}) of Alcalase. Linhart *et al.* (2000) described that the eggs of tench are damaged by concentration of 20 mL L^{-1}. The obtained results indicate that the 5 mL L^{-1} of alcalase treatment probably damages pikeperch eggs and also shortens time of incubation.

In conclusions, the application of Alcalase enzyme can be successfully used for elimination of

pikeperch egg stickiness, and requires considerably less time than other methods. This provides the potential for employing recently developed methods of intensive pikeperch production such as triploidization.

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CHAPTER 5

POST-OVULATORY OOCYTE AGEING IN PIKEPERCH (*SANDER LUCIOPERCA* L.) AND ITS EFFECT ON EGG VIABILITY RATES AND THE OCCURRENCE OF LARVAL MALFORMATIONS AND PLOIDY ANOMALIES

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Post-Ovulatory Oocyte Ageing in Pikeperch (*Sander lucioperca* L.) and its Effect on Egg Viability Rates and the Occurrence of Larval Malformations and Ploidy Anomalies

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Abstract

The effect of post-ovulatory oocyte ageing on egg quality was assessed in pikeperch *Sander lucioperca* to identify the reliable and the best post-ovulatory stripping time. Ovulated eggs were retained in the ovarian cavity for 0-3, 3-9, 6-12 and 12-18 hours post-ovulation (HPO) prior to fertilization. Fertilization, hatching and embryo mortality rates as well as the occurrence of larval malformations and ploidy anomalies were considered as indices for egg quality. The results indicated that the fertilization and hatching rates remained nearly constant, at approximately 80%, for the eggs retained in the ovarian cavity between 0-18 hours after ovulation. Post-ovulatory oocyte ageing did not affect the embryo mortality or the occurrence of larval malformations at least during the 12-hour experimental period. However, the incidence of triploidization in the larvae increased over time from 1.6% at 0-3 HPO to 5% in more aged oocytes at 6-18 HPO. Egg quality retention for at least 12 hours after ovulation appears to be helpful for pikeperch breeding programs by providing synchronous artificial egg insemination and thereby easing hatchery management.

Keywords: Pikeperch, ovulation, oocyte ageing, egg quality.

Introduction

In fish, matured eggs are released from follicle cells into the ovarian or body cavity during ovulation while they are in metaphase of the second meiotic division stage (Bobe and Labbe, 2010) and remain there until the stimulation of spawning by environmental stimulates or hand-stripping. Under farming conditions, environmental and social stimuli are absent (Aegerter and Jalabert, 2004) and stressors provide conditions that can inhibit reproduction (Schreck, 2010). Thus, oocytes usually remain in the body cavity until they are manually stripped by fish farmers (Aegerter and Jalabert, 2004). Delayed spawning in nature, delayed stripping in capture and even delayed fertilization after egg stripping can result in excessive oocyte ageing and finally over-ripening phenomenon. In the artificial propagation of many cultured fishes accordingly, the females are examined for ovulation time to time to avoid the ageing of ovulated eggs. Oocyte ageing has been reported to be the most important factor affecting egg quality of several fish species after ovulation (e.g., McEvoy, 1984; Rime *et al*., 2004; Policar *et al*., 2010). Through over-ripening, major morphological, physiological, biochemical, histological, cellular and molecular changes occur inside the eggs and ovarian fluid that negatively affect the egg fertilizing ability and larval developmental stages (e.g., Nomura *et al*., 1974; Craik and Harvey, 1984; Formacion *et al*., 1993; Lahnsteiner, 2000; Aegerter *et al*., 2005). The time period during which the eggs remain viable after ovulation differs from species to species and is largely dependent on temperature (e.g., Piper *et al*., 1982; Espinach *et al*., 1984; Harvey and Kelly, 1984; Formacion *et al*., 1993; Legendre *et al*., 2000; Rizzo *et al*., 2003; Samarin *et al*., 2008; Bahrekazemi *et al*., 2010).

Pikeperch *Sander lucioperca* has recently been considered to be involved in European aquaculture with the purpose of diversification in freshwater aquaculture (Zarski *et al*., 2012*a*). In the artificial reproduction of pikeperch, brood females are checked for their oocyte maturation stage after anaesthetization. This is done by sampling of eggs using a catheter and clearing them into a solution (e.g. Serra's solution). Then fish are divided into the separate groups depending on their egg maturity stage. Females with the eggs at stages III to V are induced for ovulation using different hormones. The time interval between hormone injection and the occurrence of ovulation varies between a few hours to

4.5 days depending on the egg maturational stage, water temperature, hormone type and its dosage (Kucharczyk *et al*., 2007). One of the most important problems in the artificial breeding of this fish species is that the females may release the eggs after ovulation and therefore the eggs cannot be easily obtained by stripping for artificial insemination. Indeed, egg deposits in the tank are very frequent in artificial spawning (Zarski *et al*., 2012b). On the other hand, in most pikeperch hatcheries, it is believed that the eggs will be over-ripened and lose their fertilizing ability soon after ovulation. Thus, in the artificial propagation of this fish species, brood fish are examined for ovulation using short time intervals. This provides stress for the fish and is also a time-, labour- and cost-intensive process. Successful *in vivo* retention of eggs using a suture on the female genital papilla not only provides the synchronous fertilisation of brood fishes but is also worthwhile when a completely mature male brood fish is unavailable for fertilisation. A better understanding and refinement of such egg retention methods is strongly desirable to maximize the efficacy of mass production of this fish species. The present study was performed to identify the reliable and the best post-ovulatory stripping time in pikeperch when females were retained at 15°C. The effect of delayed spawning caused by suturing female genital papilla on fertilization, hatching and embryo mortality rates as well as the occurrence of larval malformations and ploidy anomalies were assessed for the eggs fertilized between 0-18 hours after ovulation.

Materials and Methods

Fish

Seven pairs of pikeperch brood fish, females weighing 1543±225 g (mean±SEM) and males weighing 1392±184 g, were selected for the experiment. The fish were captured from the pond near Nove Hrady village and then transferred to the South Bohemian Research Centre of Aquaculture and Biodiversity of Hydrocenoses, Vodnany, Czech Republic, from April-May 2013. Pond-cultured brood fish adapted to the controlled conditions were then transferred to indoor holding tanks (600 L capacity) supplied with water from a recirculating system. The storage temperature was gradually increased to 15°C, which is the required temperature for pikeperch spawning.

Egg Retention in the Ovarian Cavity and Spawning

After one week of adaptation, non-ovulated females that were expected to ovulate in a few days (regarding to the soft and swollen belly) as well as males were treated with a single muscular injection of Human Chorionic Gonadotropin HCG (Chorulon preparation) (500 IU/kg). Subsequently, the genital papilla of females were sutured. Each pair of injected fish was then placed into a separate tank, which was previously covered at the bottom with an artificial grass-made spawning nest and kept at 15±0.5°C until spawning. Sperm motility was assessed before introducing the males into the spawning tanks. Females were examined for ovulation every 3 hours starting at 24 hours after injection. The state of ripeness was judged by gentle palpation of the abdomen without cutting the suture but only loosening it a little. Fish in which high number of eggs could be easily removed by applying gentle pressure on the abdomen were considered to have already ovulated. The fish were not fed during the entire experiment. For injection and to examine whether ovulation occurred, the fish were anaesthetized with a 0.03 ml/L clove oil water bath to minimize stress and to make them easier to handle (Kristan *et al*., 2012). When ovulation occurred, the eggs were retained inside the fish body for 0-3, 3-9, 6-12 and 12-18 hours post-ovulation (HPO) prior to fertilization by adjusting the time of suture cutting. Unexpectedly, some of the fishes didn't release the eggs immediately after removing the sutures. However, the exact time during which eggs were retained inside the fish body between ovulation and spawning was calculated for each fish by checking the nests. The occurrence of spawning was determined by observing the nests every 3 hours after ovulation.

Incubation and Examination of Egg Developmental Success

After the detection of spawning and its confirmation by egg sampling, 3 batches of spawned eggs were collected from each tank by cutting small parts of the substrate and placed into separate small rectangular-shaped incubators (0.25 L capacity) at 15 °C until determining the considered egg quality parameters. The number of eggs which were placed into each incubator was counted while transferring them into the incubators. During this period, the water in the incubators was changed every 6 hours. The ratios of the number of live embryos and hatched larvae to the number of initially fertilized eggs (fertilization and hatching rates, respectively) were used as indices of egg viability. The Fertilization rate was determined the day after spawning because after this time, it was easily possible to distinguish between the live and dead embryos. For this purpose, a stereo microscope was used. The hatching percentage was examined by counting the number of hatched larvae 7 days after fertilization. The embryo mortality rate was also used as an index of egg quality. The latter was measured as the percentage of embryos died between the measurement of fertilization rate and hatching stage in the total number of fertilized eggs. Malformed larvae (spinal cord torsion, yolk sac, eye deformations, etc.) were quantified using a

stereomicroscope to determine the incidence of malformation rates.

Measurement of Ploidy Levels

The ploidy level of each larvae specimen was verified as the relative DNA content using flow cytometry (Table 1) (Partec CyFlow Cube 8, Partec GmbH, Münster, Germany). The larvae were randomly sampled and processed according to the protocol of Lecommandeur *et al*. (1994) using a CyStain DNA 2-Step Kit (Partec GmbH, Germany) containing 4′,6-diamidino-2-phenylindol (DAPI; excitation/emission maximum 358/461 nm) for nuclear DNA staining (Otto, 1994). Larvae from the control group (i.e., from eggs fertilized at 0-3 HPO) were used as a diploid standard. The samples were analysed individually with a flow rate of 0.4 $\mu l.s^{-1}$.

Statistical Analysis

The normality of the data was ascertained using SPSS software version 18. Differences between the means of the groups for each measured parameter were evaluated using ANOVA, followed by Duncan's multiple range test. $P<0.05$ was considered to be significant.

Results

The fertilization and hatching rates for the eggs spawned and fertilized immediately after ovulation (0–3 HPO) were 85.9±2.2% and 79.6±2.1% (mean±SEM), respectively. The values remained nearly constant, at approximately 80%, for the eggs retained in the ovarian cavity between 0-18 hours after ovulation (Figure 1).

Post-ovulatory oocyte ageing did not significantly affect the embryo mortality as well as the occurrence of the larval malformations during the 18-hour experimental period (Figure 2). The lowest embryo mortality (1.7±1%) was observed for the eggs spawned and fertilized at 3-9 hours after ovulation, while no larval malformation and ploidy anomalies were detected in this group. Most of the measured larvae specimens from different HPOs were diploids (channel number or centre of the distribution graph: around 50) (Table 1). However, the incidence of ploidy anomalies with potentially triploidization (channel number: around 75) in the larvae increased over time from 1.6% at 0-3 HPO to 5% in more aged oocytes at 6-18 HPO.

Discussion

In this study, the egg viability rates did not show any marked reduction during the experimental period of 0-18 HPO. The time period during which the eggs remain viable inside the fish body after ovulation, which guarantees egg fertility, has been reported for a variety of species (Table 2). The results of the present study indicated that the *in vivo* retention of pikeperch

Table 1. Hours post-ovulation, number of specimens, their relative DNA contents (the mean channel number) and coefficients of variation, c.v. (%), verified using a flow cytometer

HPO	No. of fish analysed, Ploidy level	Channel no.	c.v. (%)
0-3	59 (2n)1 (3n)	48.4±3.4[a]78.5	8.6±5.63.5
3-9	39 (2n)0 (3n)	47.5±3.4[a]	8.3±6.1
6-12	19 (2n)1 (3n)	46.2±3.3[b]67.6	6.2±1.64
12-18	19 (2n)1 (3n)	46.7±1.2[b]70.5	3.9±23.9

Approximately 4000 cells were analysed for each measurement. Data with the same alphabetic superscript are not significantly different.

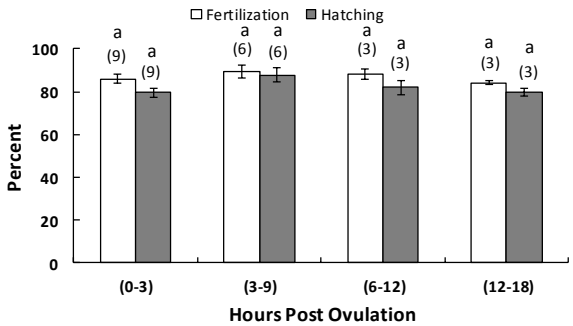

Figure 1. Effects of post-ovulatory oocyte ageing (Hours Post Ovulation-HPO) on the fertilization and hatching rates, which are shown as the mean±SEM. The number of batches for which the data were measured is shown in parentheses. For each measured parameter, the means sharing a common alphabetical symbol do not significantly differ.

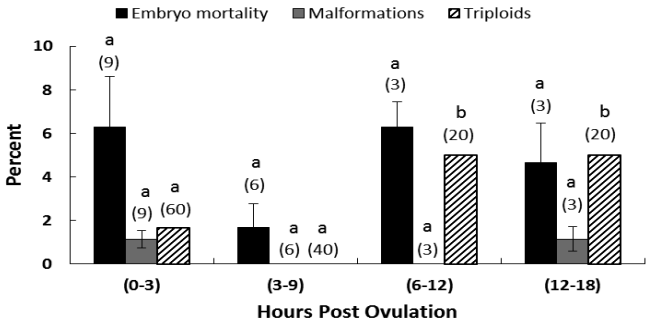

Figure 2. Effects of post-ovulatory oocyte ageing (Hours Post Ovulation-HPO) on embryo mortality, larval malformation and triploid percentages, which are shown as the mean ± SEM. The number of batches for which the data were measured is shown in parentheses. For each measured parameter, means sharing a common alphabetical symbol do not significantly differ.

Table 2. Successful egg *in vivo* storage time in different fish species

Species	Successful egg *in vivo* storage time	Temperature (°C)	Reference
Striped bass (*Morone saxatilis*)	30 minutes	-	Piper *et al.*, 1982
Tilapia (*Sarotherodon mossambicus*)	1.5 hours	18–20	Harvey and Kelley, 1984
Asian catfish (*Pangasius hypophthalmus*)	2 hours	28–29	Legendre *et al.*, 2000
Curimata (*Prochilodus marggravii*)	2 hours	18 and 26	Rizzo *et al.*, 2003
Senegalese sole (*Solea senegalensis*)	3 hours	16	Rasines *et al.*, 2012
South American catfish (*Rhamdia sapo*)	9 hours	20	Espinach *et al.*, 1984
	5 hours	24	
Goldfish (*Carassius auratus*)	12 hours	-	Formacion *et al.*, 1993
Kutum (*Rutilus frisii kutum*)	72-96 hours		Samarin *et al.*, 2011*a*
	60-72 hours	11	
Rainbow trout (*Oncorhynchus mykiss*)	5-15 days	14	Sakai *et al.* 1975; Bry, 1981; Springate *et al.*, 1984;
		8-17	Lahnsteiner, 2000; Azuma *et al.* 2003; Bonnet *et al.*, 2003; Aegerter and Jalabert, 2004; Samarin *et al.*, 2011
Caspian brown trout (*Salmo trutta caspius*)	30-40 days		Bahrekazemi *et al.*, 2010

eggs for at least 12 HPO does not have any adverse effect on the egg viability rates. Thus, to identify the time interval between ovulation and loss of egg quality in pikeperch, the egg retention needs to be studied for a longer time period after ovulation.

Although do not differ significantly the highest egg viability rates and the lowest embryo mortality, larval malformation and ploidy anomalies were observed for the eggs fertilized 3-9 hours after ovulation. Such an initial increase in the egg viability rates after ovulation has been documented in some studies (Sakai *et al*., 1975; Bry, 1981; Springate *et al*., 1984; Mylonas *et al*., 1992; Linhart and Billard, 1995: Aegerter and Jalabert, 2004; Samarin *et al*. 2008; Rasines *et al*., 2012), while it has not been found in other reports (e.g., Espinach *et al*., 1984; Lahnsteiner, 2000; Rizzo *et al*., 2003; Samarin *et al*., 2011). We found the same trend in common carp (*Cyprinus carpio*) and pike (*Esox lucius*) post-ovulatory oocytes as well (unpublished data). The lack of the initial increasing trend in these studies might be attributed to a masking effect due to the relatively longer time intervals between successive strippings used. It is likely that this initial increasing trend can be observed only when the time intervals between successive strippings are sufficiently short regarding to the fish species. A slight asynchrony between the processes of meiotic maturation and ovulation has been reported to be the most likely reason for this trend (Mylonas *et al*., 1992). The reason for observing this increasing trend in egg quality after ovulation is interesting for future studies.

Samarin *et al*. (2011) found an increase in embryo mortality rates with oocyte ageing in kutum, and concluded that the mortality of embryos can be indicative of over-ripening progression caused by the storage period. Oocyte ageing has also been reported to cause several malformations in the larvae of

different fish species (Table 3). Increasing eyed-egg mortality and malformation rates can be interpreted as post-ovulatory oocyte ageing is accompanied by biochemical changes inside the eggs (Craik and Harvey, 1984; Lahnsteiner, 2000) as well as by the leakage of a number of oocyte components, such as protein fragments, into the ovarian fluid (Rime *et al*., 2004). These components are most likely required not only for survival to the hatching stage but also for the development of the embryo into normal larvae, as the yolk nutritionally supports the embryo. However, in the present study, we did not find any changes in the embryo mortality rates as well as in larval malformations during 0-18 HPO. This might be attributed to the time duration of the experiment, i.e., longer time intervals between ovulation and spawning might result in increases in these values, as any significant decrease in egg viability rates was also not detected during the experimental period.

The incidence of ploidy anomalies larvae increased with elapsed time after ovulation, from 1.6% at 0-3 HPO to 5% at 6-18 HPO. Reports in European catfish (Varkonyi *et al*., 1998), salmonids (Yamazaki *et al*., 1989; Aegerter and Jalabert, 2004; Aegerter *et al*., 2005) and tench (*Tinca tinca*) (Flajshans *et al*., 2007) also indicated increases in the occurrence of ploidy anomaly larvae in more aged oocytes. In European catfish, 3-20% of the eggs fertilized 6 hours post-ovulation produced larvae with errors in chromosome distribution during fertilization such as aneuploidy, triploidy and tetraploidy, while no chromosomal abnormality was found in the larvae fertilized immediately after ovulation (Varkonyi *et al*., 1998). The incidence of triploid larvae increased with the post-ovulatory ageing time in rainbow trout with higher frequency and earlier at 17°C compared to 12°C (Aegerter and Jalabert, 2004). In tench, the incidence of triploid larvae significantly increased after 5 hours of *in vitro* storage at 24°C, as well as after 3 hours of *in vitro* storage at 21.9 and 17°C, while during *in vivo* storage, a significant triploid larval yield appeared after 5 hours of storage at 21.9°C only (Flajshans *et al*., 2007). Our recent experiments indicated that the rate of larval triploidization increased with oocyte ageing in pike, while all measured larvae from different HPOs were diploids in common carp (unpublished data). Changes in the oocyte cytoskeletal organization during ageing have been reported to be associated with a failure of second polar body extrusion and finally the occurrence of ploidy anomalies in larvae (Aegerter and Jalabert, 2004; Flajshans *et al*., 2007). Complete loss of egg viability rates in this study would be more clearly accompanied by increasing ploidy anomalies. Since the malformed larvae may include a number of ploidy anomalies (Varkonyi *et al*., 1998; Aegerter and Jalabert, 2004), measurement of the larval ploidy levels should be performed during the initial days of hatching while malformed larvae are still alive, as performed in the present study.

Conclusions

Based on the results obtained in this study, unfertilized eggs of pikeperch could retain their viability up to 80% at least 12 hours after ovulation when they are stored in the parental fish body at 15°C. Thus, the reliable time interval between successive fish examined for ovulation can be considered for up to 12 hours. The long egg quality retention time appears to be helpful for pikeperch breeding programs by providing synchronous artificial egg insemination using a suture on the female genital papilla and eventually easing hatchery management.

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Table 3. Larval malformation rates caused by *in vivo* egg storage in different fish species

Species	Malformation (%)	Storage time (*in vivo*)	Temperature (°C)	Reference
African catfish (*Heterobranchus longifilis*)	20	2 hours	30	Legendre and Oteme, 1995
European catfish (*Silurus glanis*)	50	6 hours	22-24	Linhart and Billard, 1995
Asian catfish (*Pangasius Hypophthalmus*)	100	5 hours	28	Legendre *et al*. 2000
Curimata (*Prochilodus marggravii*)	60	2 hours	26	Rizzo *et al*., 2003
Rainbow trout (*Oncorhynchus mykiss*)	50	16 days	12	Bonnet *et al*., 2007

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CHAPTER 6

TRIPLOIDISATION OF PIKEPERCH (*SANDER LUCIOPERCA*), FIRST SUCCESS

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Triploidisation of pikeperch (*Sander lucioperca*), first success

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ABSTRACT

The use of triploid fish in intensive aquaculture can lead to higher filet yield, faster growth and higher profit. To induce triploidy in pikeperch, fertilized eggs incubated at 15 °C were immersed in a water bath at shock temperatures (ST) of 29 °C (Exp. 1) and 31 °C (Exp. 2) at shock initiation times (TI) of 1, 3, 5, 7, and 10 min after egg and spermatozoa activation for 20 and 40 min. Ploidy levels of hatched larvae were determined by flow cytometry. Shock temperature of 29 °C led to low hatching rates (0–11.5%) and low rates of triploidy (0–75%). With ST of 31 °C, 100% triploidy was obtained with TI of 1 min and duration of 20 and 40 min and at TI of 5 min for 20 min. A shock temperature of 31 °C was associated with 0.9–74.1% malformed larvae compared to 0–3.3% at ST of 29 °C. The most important finding of this study is that heat shock treatment can produce all-triploid pikeperch population. However, further research is required to determine a method of production of all-triploid pikeperch population at higher hatching rates.

1. Introduction

The pikeperch (*Sander lucioperca*) is a valued freshwater fish in European aquaculture and there is a prediction that the production of marketable pikeperch originated from intensive culture will grow up due to excellent flesh quality, high growth rate, good market value (Uysal and Aksoylar, 2005; Wuertz et al., 2012; Kristan et al., 2013), suitability to intensive culturing (Wang et al., 2009; Szczepkowski et al., 2011), and especially decreasing numbers of fish in wild populations and catches which have been reduced by over 50% during the past 30–40 years (Policar et al., 2013). In the past, pikeperch was extensively farmed in ponds or lakes (Hilge and Steffens, 1996) and in last few decades, it is successfully reared under complete recirculation aquaculture system (RAS) conditions (Molnar et al., 2004) or in combination of pond and RAS as well (Policar et al., 2014).

The use of triploid fish in intensive aquaculture can lead to higher filet yield, faster growth and higher profit (Piferrer et al., 2009). Triploid refers to individuals with somatic cells containing three sets of chromosomes (Legatt and Iwama, 2003). Differences from the standard ploidy level can occur naturally (Aegerter and Jalabert, 2004; Samarin et al., 2015) or be artificially induced (Chourrout, 1980). Triploidisation can be induced in finfish via blocking of the second polar body extrusion by shocking of the eggs short time after fertilisation (Malison et al., 1993) and via reproducing of 4n and 2n individuals (Piferrer et al., 2009). Retention of the second polar body and production of triploid fish has been successful in percids including yellow perch *Perca flavescens* (Malison et al., 1993), Eurasian perch *Perca fluviatilis* (Rougeot et al., 2003), walleye *Sander vitreus* (Malison and Garcia-Abiado, 1996), and saugeye (female walleye *S. vitreus* × male sauger *Sander canadensis*) (Garcia-Abiado et al., 2001). However, to our knowledge, there is currently no available information on induction of triploidy in pikeperch. The objective of this study was to assess the effect of heat shock on hatching rate, malformation of larvae, and ploidy level in pikeperch.

2. Material and methods

2.1. Broodfish and egg and sperm collection

Fifteen male and twenty female pikeperch were collected (in April) from the ponds of Rybarstvi Nove Hrady Ltd., Czech Republic, transferred to the South Bohemian Research Centre of Aquaculture and Biodiversity of Hydrocenoses in Vodnany, Czech Republic, and held in 600 L tanks equipped with a recirculating water system (15.1 ± 0.2 °C). The method of Zarski et al. (2012) was used to assess oocyte maturity stage and establish optimal time for hormone injection (in this study, oocyte stage 4). Both sexes of pikeperch broodfish were stimulated with 500 IU of hCG per kg (Kristan et al., 2013). The genital papilla of females was stitched closed to avoid spontaneous ovulation. Beginning 48 h post-injection, fish were examined for ovulation by gentle palpation of the abdomen at intervals of 3 h.

2.2. Heat shock treatment conditions and egg sampling

When ovulation occurred, eggs from five fish for Exp. 1 and five different fish for Exp. 2 were stripped and pooled at a similar ratio. Eggs were fertilized with pooled semen from five males. The spermatozoon

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Table 1
Hatching rate (HR), larvae malformation (Malf.), and percent of triploidy after 20 min of heat shock of 29 °C at different times of initiation (TI) of treatment in pikeperch. Different letters indicate significant difference ($P < 0.05$).

TI, min post fertilisation	1	3	5	7	10	Control group	*P*-value
HR, %	0c	1.3 ± 0.2c	3.1 ± 2.5c	8 ± 1.3b	11.5 ± 1b	59.1 ± 3a	<0.000*
Malf., %		0a	3.3 ± 5.7a	0a	3.3 ± 5.7a	2.1 ± 2.4a	<0.046
Triploid, %		11 ± 19.5a	33.3 ± 23.6a	16.6 ± 11.5a	25.3 ± 14.4a	0b	<0.024

to egg ratio was >100.000:1 (Kristan et al., 2012). Eggs were activated with hatchery water at 15.2 °C to induce fertilisation. At shock initiation times (TI) of 1, 3, 5, 7, and 10 min following egg and spermatozoon activation, three 2 mL batches of eggs (n = 1361 ± 70) were immersed separately in a water bath at the shock temperature (ST) of 29 °C (Exp. 1) or 31 °C (Exp. 2) for 20 or 40 min; a total of 60 samples. After the shock treatment, 300 randomly chosen eggs from each repetition of each experimental treatment were transferred to hatchery water at 15.4 ± 0.3 °C. Three 2 mL batches of fertilized eggs (for each experiment) with no treatment in three repetitions incubated under similar conditions were used as control. Eggs were incubated and larvae hatched in 250 mL plastic incubators. Water in incubators was changed twice daily. Hatching rate (HR) was calculated as the number of hatched larvae relative to the initial number of eggs in the incubator. Hatched larvae and malformed larvae were counted at the end of hatching.

2.3. Assessment of ploidy level

To assess the ploidy level and proportions of diploid and triploid larvae, 30 larvae were randomly taken from each experimental treatment (10 from each repetition) or all the larvae if there were fewer than 10 hatched individuals in repetition. The ploidy level of each individual larvae was verified as the relative DNA content by using of the flow cytometry (Partec CyFlow Cube 8, Partec GmbH, Münster, Germany). All the tested larvae were randomly sampled and processed according to Lecommandeur et al. (1994) using a CyStain DNA 2-Step Kit (Partec GmbH, Germany) containing 4′,6-diamidino-2-phenylindol (DAPI; excitation/emission maximum 358/461 nm) for nuclear DNA staining (Otto, 1994). Larvae from the control group (no heat shock treatment) were used as a diploid standard. The samples were analyzed individually with a flow rate of 0.4 $\mu L \cdot s^{-1}$ (Samarin et al., 2015).

2.4. Statistical analysis

The data were analyzed using the appropriate analysis of variance (One-way ANOVA with Tukey's post-test) with $P < 0.05$ as the level of significance for all tests. Data expressed as percents were evaluated using arcsin transformation.

3. Results

Shock temperature of 29 °C, used in Exp. 1, led to low hatching (0–11.5%) and triploidisation (0–75%). The optimal TI and shock duration in Exp. 1 for producing triploid larvae was TI of 10 min for 40 min (Tables 1 and 2). Higher yield of triploids was obtained in Exp. 2, with the water bath at 31 °C. Three groups produced 100% triploid larvae: TI of 1 min for 20 and 40 min and TI at 5 min for 20 min. Other evaluated TIs also induced high rates of triploid larvae but not 100% (Tables 3 and 4). The heat shock treatment applied for 20 min at ST of 31 °C was accompanied with high occurrence of malformed larvae (Table 3).

4. Discussion

Results of present study showed that heat shock treatment can be used for effective triploidy induction in pikeperch. Heat shock treatment at 31 °C initiated at 1 min and extending for 20 and 40 min and initiated at 5 min for 20 min duration can induce 100% triploidy. Garcia-Abiado et al. (2002) produced 86.7 ± 9.4% triploid saugeye using shock temperature of 31 °C for 15 min duration at TI of 5 min. Malison et al. (2001) reported that heat shock did not produce a complete triploid population in walleye and recommended hydrostatic pressure shock at 8000 psi applied at TI of 4 min for a duration of 30 min. In contrary, the optimal triploidisation procedure for Eurasian Perch is a heat shock of 30 °C ongoing for 25 min, induced 5 min after fertilisation at 16–17 °C which results in 100% triploids (Rougeot et al., 2003). In Yellow Perch, a heat shock of 28 °C ongoing for 25 min and initiated 5 min after fertilisation at 11 °C, results in 100% triploids (Malison et al., 1993).

The effectiveness of the heat-shock-based triploidisation method is determined by the time of initiation of the shock (Ihssen et al., 1990; Levanduski et al., 1990; Felip et al., 1999, 2001) coupled with water temperature used for gamete activation (Piferrer et al., 2009). In Exp. 2 of present study, later shock initiation resulted in lower numbers of triploid individuals, but, in Exp. 1, at a lower temperature, no relationship between TI and rate of triploidisation was observed. It clearly shows that lower shock temperature (29 °C) was not effective enough to induce triploidy regardless to time of initiation. On the other hand, higher shock temperature induced higher triploidy level and the results show that later shock initiations (7 and 10 min) were ineffective to induce the triploidy in 100% rate. Higher duration of shock treatment negatively affected the hatching rates in Exp. 1 and 2, in accordance with statements of several authors who have described a negative effect of long duration treatment in rainbow trout (Chourrout, 1984), grass carp *Ctenopharyngodon idella* (Cassani and Caton, 1985) and Eurasian perch (Rougeot et al., 2003). In present study, there was found quit high variance among the results in each tested group. Some authors (Johnstone, 1985; Malison et al., 1993) refer that the variance in results obtained in each tested (experimental) group could be caused by and associated with factors such as egg quality (mixed eggs from several females) and differing susceptibility of eggs of different origins (in case of wild broodstock originated from different areas) to heat shock.

Heat shock treatment is promising to induce 100% triploidy in pikeperch, but it leads to low fertilisation and hatching rates. Successful triploidisation in walleye with hydrostatic pressure shock (Malison et al., 2001) suggests that this method also may be worthy of investigation in pikeperch.

Table 2
Hatching rate (HR), larva malformation (Malf), and percent of triploidy after 40 min of heat shock 29 °C at different times of initiation (TI) treatment in pikeperch. Different letters indicate significant difference ($P < 0.05$).

TI, min post fertilisation	1	3	5	7	10	Control group	*P*-value
HR, %	0b	0b	0b	0b	1.3 ± 1.8b	59.1 ± 3a	<0.000*
Malf., %					0a	2.1 ± 2.4a	<0019
Triploid, %					75 ± 0a	0b	<0.000*

Table 3
Hatching rate (HR), larva malformation (Malf), and percent of triploidy after 20 min of heat shock 31 °C at different times of initiation (TI) treatment in pikeperch. Different letters indicate significant difference ($P < 0.05$).

TI, min post fertilisation	1	3	5	7	10	Control group	*P*-value
HR, %	2.3 ± 2c	11.8 ± 8.8b	7.8 ± 10.1b	15.3 ± 5.1b	20.0 ± 5.2b	61.6 ± 7.3a	<0.000*
Malf., %	74.1 ± 0a	9.6 ± 9c	35.5 ± 33.5b	7 ± 2.5c	5.2 ± 1.4c	1 ± 0.8d	<0.000*
Triploid, %	100 ± 0a	95.8 ± 7.2a	100 ± 0a	86.3 ± 15.1a	53.3 ± 5.7b	0c	<0.000*

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Table 4
Hatching rate (HR), larva malformation (Malf), and percent of triploidy after 40 min of heat shock 31 °C at different times of initiation (TI) treatment in pikeperch. Different letters indicate significant difference ($P < 0.05$).

TI, min post fertilisation	1	3	5	7	10	Control group	*P*-value
HR, %	3.1 ± 5.4c	7.1 ± 1.8bc	8.5 ± 4.3bc	13.2 ± 1.9bc	18.3 ± 7.6b	61.6 ± 7.3a	<0.000*
Malf., %	0a	0a	0a	0a	0.6 ± 0.5a	1 ± 0.8a	<0.045
Triploid, %	100 ± 0a	91 ± 7.7a	88.8 ± 19.2a	90 ± 0a	41.8 ± 19.1b	0c	<0.000*

CHAPTER 7

EFFECTS OF WATER SURFACE TREATMENTS ON SURVIVAL, SWIM BLADDER INFLATION AND GROWTH IN PIKEPERCH *SANDER LUCIOPERCA* L. LARVAE

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EFFECTS OF WATER SURFACE TREATMENTS ON SURVIVAL, SWIM BLADDER INFLATION AND GROWTH IN PIKEPERCH *SANDER LUCIOPERCA* L. LARVAE

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ABSTRACT

Four treatments were used to assess the influence of water surface conditions on survival, swim bladder inflation and growth rate of pikeperch larvae. Larvae (n=~204 000) were divided among 16 tanks (density 85 individuals/L), each employing one of four water surface treatments in four repetitions: fan (Group FAN), air bubbles (Group DIF), spray (Group SPR), or no treatment (Group CON). Larvae were fed *ad libitum* twice daily with brine shrimp *Artemia salina*; all were fed the same amount from day 5 post hatching (dph) to 16 dph. The highest survival was in Group FAN (62.1%) compared to the other treatments Group DIF – 9.4%, Group SPR – 20.5%, and Group CON – 26.7%); Group FAN also had the highest growth with significantly higher total length (TL=9.20±0.79 mm) at the end of the rearing. Larvae in Group DIF were 6.95±0.67 mm and were significantly smaller than larvae in Group SPR (TL=8.27±0.97mm) and Group CON (TL=7.89±0.78 mm). Specific growth rate (SGR) was similar in Groups FAN (35.98%/day), SPR (37.37%/day), and CON (34.37%/day) but significantly lower in Group DIF (25.67%/day). No significant differences were found in swim bladder inflation (SBI) among Group FAN (58.2%), Group SPR (61.8%), and Group CON (50.2%) but SBI for Group DIF was only 15.2%. The air bubble treatment did not enhance swim bladder inflation nor growth compare to the other groups. However the fan system had overall positive effects on survival of pikeperch larvae compare to the other tested water surface treatments.

Keywords: *larviculture; gas bladder; intensive rearing; brine shrimp*

1. Introduction

Pikeperch *Sander lucioperca* L. have long been reared in ponds and lakes (Hilge and Steffens, 1996; Steffens et al., 1996) and in the past decade have been successfully cultured in combination pond and recirculating aquaculture systems (RAS) (Policar et al., 2013), as well as exclusively in recirculating aquaculture systems (Molnar et al., 2004; Szkudlarek and Zakes, 2007). Current research on pikeperch primarily focuses on methods of intensive aquaculture in RAS (Lund et al., 2012, Zakes, 2012). However, improvements are needed in RAS rearing of pikeperch larvae and juveniles, issues that need to be resolved are optimal composition of artificial feed (Zakes, 2012), cannibalism (Szczepkowski et al., 2011), and failure of swim bladder inflation (SBI) (Demska-Zakes et al., 2003; Szkudlarek and Zakęś, 2007). Failure to inflate the swim bladder (SB) has been regarded as one of the major obstacles for intensive aquaculture of striped bass *Morone saxatilis* Walbaum (Martin-Robichaud and Peterson, 1998), sea bream *Sparus auratus* L. (Chatain, 1994), and walleye *Stizostedion vitreus* Mitchill (Summerfelt, 1996), as well as pikeperch (Demska-Zakes et al., 2003).

Pikeperch is a physoclistous fish (Craig, 2000). SBI is required before the pneumatic duct closes. Initial filling is accomplished by breaching the water surface and swallowing an air bubble, then passing it from the digestive tract into the swim bladder (SB) via a pneumatic duct (Chatain and Ounais-Gushemann, 1990; Rieger and Summerfelt, 1998). Development of the SB in walleye is similar to pikeperch (Demska-Zakes et al., 2003). Depending on temperature, these species inflate the SB beginning at 5 or 6 days post-hatching (dph). Walleye must inflate the SB by 13 dph (Barrows et al., 1988; Marty et al., 1995) and pikeperch by 11–12 dph (Demska-Zakes et al., 2003). The SB fails to inflate during the intensive larviculture because of high viscosity of the water surface caused by oils from feed (Chatain and Ounais-Gushemann, 1990), pollutants on the water surface, which are a potential source of infection (Marty et al., 1995), sub-optimal water temperature (Trotter et al., 2003b) and the other abiotic factors such as light intensity, photoperiod, salinity and turbidity (Weppe and Joassard, 1986; Ronzani Cerqueira and Chatain, 1991; Barnabé and Guissi, 1993; Rieger and Summerfelt, 1997) or even tank colour (Martin-Robichaud and Peterson, 1998). Light intensity has a strong influence on development and behaviour in teleost larvae (Boeuf and Le Bail, 1999), and a relationship between light intensity and function of the SB have been studied (Kitajima et al., 1993; Martin-Robichaud and Peterson, 1998; Uotani et al., 2000). Photoperiod and the associated diel vertical migrations can lead to changes in SB volume in physostomous larvae, or while the pneumatic duct is open in physoclists (Kitajima et al., 1985; Kitajima et al., 1993). It has problems with food capturing also been observed that onset of darkness can provide a stimulus for initial SBI (Trotter et al., 2003a). In addition, biotic factors such as progeny quality and nutrition, have been reported to enhance SBI (Harel et al., 1992; Kitajima et al., 1994; Bailey and Doroshov, 1995; Tandler et al., 1995). Non-inflation of the SB results in high larval mortality (Demska-Zakes et al., 2003) which are related to spinal deformities (lordosis) (Kindschi and Barrows, 1993), lower growth rate (Chatain, 1986) because of problems with food capture and high energy cost of swimming and buoyancy control (Summerfelt, 1996). Also, increased cannibalism is associated with non-inflation of the SB due to limited mobility of larvae with un-inflated SB (Kindschi and MacConnell, 1999; Szczepkowski et al., 2011). A delay in swim bladder inflation is permanent (Kitajima et al., 1981).

Disruption of the water surface enhances the initial SB inflation in walleye spraying water at the surface is the most commonly used method (Barrows et al., 1993; Moore et al., 1994; Bristow and Summerfelt, 1994; Bristow et al., 1996; Summerfelt, 1996). In pikeperch intensive culture, usually no water surface treatment (Kowalska et al., 2006) or spraying (Szkudlarek and Zakes, 2007) are used. The aim of this study was to determine a water surface treatment that would optimize survival, growth, and SBI of cultured larvae during 13 days (4–16 dph) of intensive rearing of pikeperch larvae in a RAS.

2. Materials and methods

Pikeperch larvae were obtained via hormone stimulation and controlled reproduction of fourteen pikeperch broodfish. All the broodfish were hormonally stimulated with human chorionic gonadotropin hCG) (Chorulon, Intervet International B.V.) which was injected into the dorsal muscle at 500 IU kg. Experimental Fish Culture Facility of the Faculty of Fisheries and Protection of Waters at the University of South Bohemia in Vodnany.

2.1. Experimental groups, different water surface treatments and experimental conditions

A total of 204 000 4-d post-hatch (dph) larvae (total length (TL)=5.036±0.419mm) were transferred to 16 black plastic tanks (2.5x0.5x0.2 m; usable volume 150 L and water depth of 130 mm) with a water supply from a closed recirculation system with four treatment groups and four repetition for each group.

The water surface r of three groups was manipulated in the following ways:

Group FAN, a fan (Rohnson R-814) located 500 mm from the end of the tanks was directed at an acute angle onto and across the water surface a single fan served two tanks. Floating impurities were blown to the edge where they were removed (skimmed with bowl) three times a day. Group DIF (diffuser), three 450 mm aeration stones (Juan Lin 20) connected inline were placed in the centre of the bottom of each tank. Group SPR (spray), 1.7 m of 10 mm pipe with thirty 1 mm perforations was connected to the water inflow and placed 100 mm above the water surface in the middle of each experimental tank. Group CON (control) had no water surface treatment, remaining calm.

Each tank was stocked with 85 larvae per litre (12 750 individuals/tank). Water flow was 2.4 L/min, illumination intensity 100 lux, and light regime 13L:11D. The water temperature (WT) and dissolved oxygen (DO) were measured twice daily with an oximeter (OxyGuard) throughout the experiment; average WT = 16.9±0.5 °C and DO = 7.9±0.3 mg O_2/L. Larvae were fed *ad libitum* from 5 dph, twice daily (at 7 am and 13 pm) with brine shrimp *Artemia salina* (INVE HE/EG 270 000 and 260 000 nauplii/g) the same quantity in each treatment tank. Artemia were hatched following the protocol of Sorgeloos et al. (2001). The rearing tanks were cleaned twice daily to remove excrements and uneaten food. Fish were reared to 16 dph (13 days).

2.2. Observations and measurements

Ten larvae were removed from each tank every day and anaesthetised with MS 222 (0.1 g/L) (Grozea et al., 2010) to determine the status of the SB according to Chantain (1986). Every second day, 10 larvae from each tank were measured to the nearest 0.01 mm stereomicroscopically (Nikon SMZ745T) using Quick PHOTO MICRO 3. On the first day exogenous feeding, 6 dph and the final day of the experiment, 16 dph, 40 larvae from each tank were weighed (Kern, ABT220-5DM) to the nearest 0.1 mg to assess specific growth rate (SGR). At the conclusion of the experiment, larvae were counted using the volume method: larvae in the tank were concentrated into 10 L of water, and five samples of 1 L of water with larvae were taken. The total number of larvae was calculated as ten times the mean count in the samples. To assess the percent of larvae with inflated SB, three samples of 100 larvae were randomly taken from each tank at the end of the experiment, and the numbers of larvae with and without inflated SB were counted. Cannibalism was assessed by counting the larvae with black material in their intestines from the consumed fish. The intestinal content of non-cannibalistic larvae had an orange colour from the brine shrimp. These differences were visible with the naked eye.

The data are presented as mean ± standard deviation. Statistical analysis was based on one-way Analysis of Variance (ANOVA, Statistica 12, StatSoft, Inc.) including F-statistic. Significant differences between groups were estimated using Tukey's post-hoc test. The level of significance was set at $P<0.05$.

3. Results

The highest larval survival was in Group FAN (62.1%). The other groups had significantly lower survival (9.4–26.7%) (Table 1). Through 8 dph, there were no differences in the length among groups, but at 10 dph, the larvae in Group FAN were significantly larger than those in other groups (Table 1, Fig. 1) and the growth trajectory of larvae in Group FAN was uniformly linear (Fig.1). Larval weight was significantly higher in Group SPR, followed by Groups FAN, CON, and DIF (Table 1). There were no significant differences among Groups FAN, SPR, and CON in SGR but the significantly lowest SGR was found in Group DIF (Table 1). Fewer than 1% of the larvae were cannibalistic in each group. The cannibalistic fish were much larger than non-cannibalistic individuals (Table 1), but total length and weight of cannibals were not significantly differ among groups. No head or jaw deformities were observed. The first SB inflation was observed at 12 dph in Group FAN (Table 1). Group DIF (the bubble treatment) had the significantly lowest number of larvae with inflated SB at the end of experiment. There were no significant differences among the other three groups (Table 1). Most of the dead larvae were with non-inflated swim bladder (Group FAN 96.4±1.3%; Group DIF 97.1±0.9%; Group SPR 95.6±2.2%; Group CON 96.6±1.8%) and there were no differences among the groups.

Table 1. *Survival, first observation of SB inflation (FOSB), percentage of SB inflation (SBI), TL of larvae at 16 dph, W of larvae at 16 dph, SGR, rate of cannibals in each group (RC) and TL and W of cannibal assessed at the end of experiment in pikeperch (Sander lucioperca L.) larvae. Different letters indicate significant difference (P<0.05).*

Groups	Surv [%]	FOSB [dph]	SBI [%]	TL [mm]	W [mg]	SGR [%.day^{-1}]	RC [%]	TL of cannib [mm]	W of cannib [mg]
FAN	62.1±14.7^{a}	12	58.2±8.7^{a}	9.2±0.7^{a}	33.9±1.1^{b}	35.9±1.4^{a}	0.6±0.2^{a}	10.9±0.4^{a}	80.2±1.9^{b}
DIF	9.4±11.6^{b}	14	15.2±18.1^{b}	6.9±0.6^{c}	12.2±0.4^{c}	25.6±2.3^{b}	0.5±0.4^{a}	10.4±0.5^{a}	73.2±1.6^{c}
SPR	20.5±12.8^{b}	13	61.8±15.8^{a}	8.2±0.9^{b}	39.7±1.0^{a}	37.3±2.5^{a}	0.7±0.4^{a}	10.8±0.5^{a}	87.8±1.5^{a}
CON	26.7±18.9^{b}	13	50.2±18.4^{a}	7.8±0.7^{b}	28.6±0.9^{b}	34.3±0.6^{a}	0.5±0.1^{a}	10.4±0.4^{a}	79.9±0.5^{b}
F-stat	9.50		22.12	53.92	61.48	15.31	31.04	13.57	7.02

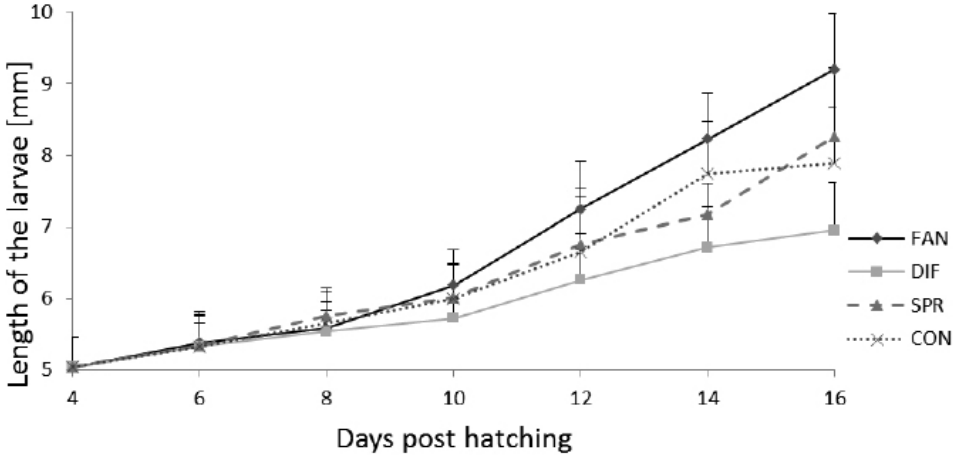

Figure 1. *Course of growth in pikeperch (Sander lucioperca L.) larvae according to water surface treatment (FAN – fan, DIF – diffuser, SPR – spray, CON – no treatment, control).*

4. Discussion

The most important result of our study was the differences in survival of larvae and growth among the experimental groups. The highest survival at 16 dph was in Group FAN (62.1±14.7%), the fan treatment. Similar pikeperch larvae survival was reported by Kowalska et al. (2006) (57.3%, 13 dph), with no water surface treatment. Only 26.7±18.9% survived in Group CON witch had also no water surface treatment and just 9.4% survived in the air bubble treatment. In case of larvae growth, we recorded. The best larval growth was in Group FAN with a 35.98% per day SGR and a final average TL of 9.2±0.76 mm. Kowalska et al. (2006) reported TL 13.5 mm and SGR 16.5%.day^{-1} (19 dph) in pikeperch larvae reared at 20 °C, density of 80 individuals/L, fed with brine shrimp three times daily. Smaller larvae in our experiment may have been due to lower water temperature (Ott et al., 2012). We chose a low water temperature for rearing because fish are poikilothermic organisms (Hardewig et al., 2004), and we thought that slower development of organs (*i.e.* swim bladder) might improve SB inflation. The difference in survival and growth of larvae in Group FAN compared to Groups DIF and SPR might have been a result of the additional water movement produced by water surface spray (Group SPR) and air bubbles (Group DIF). The larvae in these treatment conditions probably used additional energy from induced swimming and movement. This higher turbulence in the water might have caused higher mortality in Groups DIF and SPR and the lower density might have affected growth of the survivors in a density-dependent manner. The high mortality in Group CON might have been caused by water surface contaminants as a potential source of infection (Marty et al., 1995; Demska-Zakes et al., 2003). In this group, pollutants were spread over the water surface, which could result in infection during SBI. We also observed some impurities on the water surface in tanks of Group FAN, but these were blown to edge of the tank opposite by the fan and it was easy to remove the debris (described in MM). This may have reduced potential contact of the larvae with pollutants and decrease their mortality.

Two major factors may influence SBI in intensive larvae culture. The first is an oil film on the water, which interferes with larvae penetrating the surface to reach the air (Chatain and Ounais-Gushemann, 1990). A second factor in successful pikeperch larvae SBI may be waves on the water surface. Since pikeperch larvae are pelagic (Lammens, 1999), their natural environment would include moving water at the surface. In comparison, the water surface in an intensive larvae culture system is usually motionless. To disrupt a possible oil film on the water surface and produce waves, we used spraying (Barrows et al., 1993), fan and air bubbles. Kowalska et al. (2006) observed 52% of pikeperch larvae with inflated SB in a system (WT=20 °C, light regime 24 h light, illumination intensity 40 lx) with no water surface treatment, which is similar to our results for Group CON (50.2±18.4%), in which no surface treatment was applied. The second function of fan (next to the production of waves) was to blow and concentrate the impurities in a small part of the water surface area and provide the larvae a clean water surface which is essential for successful SBI. These results (no difference among the groups) show that the waves or water surface movement is not essential factor of SBI in pikeperch larvae but the most important factor is presence or absence of impurities on the water surface.

To conclude this study, we can say that air bubbles, Group DIF treatment, and the sub-surface turbulence might have negatively influenced survival, SBI, and growth in pikeperch larvae, while the fan had a major positive impact on larvae survival and growth which was linear and without any fluctuations compare to the other tested groups. The application of the fan in intensive aquaculture of pikeperch larvae is a simple, effective, and inexpensive method to improve the effectiveness of larviculture and increase its profits.

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CHAPTER 8

ADAPTATION OF INTENSIVELY REARED PIKEPERCH (*SANDER LUCIOPERCA*) JUVENILES TO POND CULTURE AND SUBSEQUENT RE-ADAPTATION TO A RECIRCULATION AQUACULTURE SYSTEM

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PROOF RESEARCH PAPER

Adaptation of Intensively Reared Pikeperch (Sander Lucioperca) Juveniles to Pond Culture and Subsequent Re-Adaptation to a Recirculation Aquaculture System

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Abstract

A combination of intensive rearing and pond culture was evaluated on its benefits for pikeperch year-round production. Fifteen hundred intensively reared juveniles (group IRJ) and 1500 pond-reared juvenile pikeperch as a control (group C) were divided into three batches and stocked into three ponds for 178-day long culture. Survival of fish in group IRJ was significantly higher (65.2 ± 15.9%) compared to group C (47.3±1.6%) ($P<0.05$). SGR (group IRJ = 0.018±0.012%.d-1; group C = 0.014±0.027%.d-1) and Fulton coefficient (FC for group IRJ = 1.02±0.13; FC for group C = 0.95±0.57) did not differ at the end of pond culture which included winter and spring seasons. After harvesting, fish from group IRJ were transferred to a recirculation aquaculture system and after a short adaptation period, they were fed with pellet feed. The duration of following intensive rearing in the RAS lasted 46 days. SGR was 0.732±0.441%.d-1, FCR = 2.1±1.2, FC = 1.20±0.09 and survival rate was 98.4±0.9% at the end of the 46-day rearing period. No difficulties were observed in adaptation of intensively cultured juveniles to pond conditions. During the study, it was obtained a high survival rate and excellent ability of pikeperch juveniles to consume dry feed after their re-adaptation to recirculation aquaculture system.

Keywords: Intensive culture, pellet feed, pond rearing, survival.

Introduction

Pikeperch (*Sander lucioperca*) is one of the most attractive freshwater fish for European aquaculture (Blecha *et al*., 2015; Kristan *et al*., in press) and its diversification (Samarin *et al.,* 2015) due to flesh palatability and attractiveness to anglers (Schulz *et al*., 2007; Kristan *et al*., 2013). The demand for pikeperch is increasing (Wang *et al*., 2009), while the wild populations in Europe are decreasing due to overfishing (Dil, 2008; Muller-Belecke and Zienert 2008). The main role of pikeperch in open waters is to regulate the populations of small cyprinids (Peterka *et al*., 2003). In the past, pikeperch were farmed in ponds or lakes (Hilge and Steffens, 1996), but in the last few decades they have been successfully reared in combination of pond and recirculation aquaculture systems (RAS) (Zakes and Demska-Zakes, 1998; Policar *et al*., 2013). It is possible to rear the pikeperch under complete RAS conditions as well (Molnar *et al*., 2004).

The combination of pond and RAS rearing is based on 6-8 weeks of extensive pond rearing followed by harvesting and converting the pikeperch juveniles with TL 35–55 mm to an artificial diet and RAS conditions (Ruuhijarvi and Hyvarinen, 1996; Zakes and Demska-Zakes, 1996; Zakes, 1997). Mentioned culture technic is well described in Policar *et al*. (2014). According to their experience, after hormonally stimulated semi-artificial spawning, pikeperch larvae are stocked to small (maximum 1.5 ha) ponds which were wintered and fertilised before. When the pikeperch juveniles achieve the total length of 30-50 mm or rapid decrease of zooplankton abundance is observed, the ponds are drained and harvested and the juveniles are moved to RAS for the second part of the culture. To convert the juveniles from live to pellet feed a technic called co-feeding (mixture of live and pellet feed) is applied. During 10-12 days, the live feed (frozen bloodworm) is mixed with pellet feed. The rate of live feed is being decreased in time and in contrary, rate of pellet feed goes up for 25% every two days until the fish intake the pellet feed only. This rearing method provides a stable production of high-quality pikeperch juveniles for subsequent aquaculture in countries with large pond area such as Czech Republic, Hungary, Germany, etc. (Policar *et al*., 2014).

The primary objective of our study was to assess

the capacity of intensively cultured pikeperch juveniles to adapt to pond culture and then to re-adapt to RAS conditions. This approach was tested as an alternative method which can provide year-round pikeperch production.

Materials and Methods

Fish Groups and Characteristics of Pond Culture

Intensively reared juveniles used in this study were obtained from Experimental Fish Facility of the Faculty of Fisheries and Protection of Waters (FFPW). Before this study, they were reared in RAS (for 120 days) with water temperature (WT) = 23.6 ± 1.3°C, oxygen saturation (OS) = 104 ± 8 %, light regime 12 hours light and 12 hours dark, and fed with pelleted feed (BioMar, INICIO Plus 2 mm) during the entire light period of the day. To acclimate the fish for pond rearing, WT in rearing tanks was decreased (about 1°C per day) to pond WT (8.2 °C), fourteen days before stocking. Pond reared fish (stocked into ponds in May after semiartificial spawning) were captured from the ponds of Rybarstvi Nove Hrady Ltd. and then transferred to the FFPW.

In total, 1500 intensively reared (group IRJ) juveniles (TL=133.3±7.2 mm; W=23.8±3.2 g) and 1500 pond-reared (group C) juveniles (TL=122.4±6.6 mm; W=21.1±2.8 g) were used in this study. Fish were marked with a ventral fin clip (group C left fin, group IRJ right fin) and separated into three batches, each comprising 500 pond-reared juveniles and 500 intensively reared juveniles. Batches were stocked into separate 400-m^2 earth ponds (three ponds, 1000 fish in each pond) for 178-day culture, from 19th October until 5th May. To support the fish during the pond rearing and provide them some supplemental nutrition, prey fish *Pseudorasbora parva* (TL 15-46 mm) in total biomass of 50 kg was added to each experimental pond. *Pseudorasbora parva* is not a native fish species but it forms naturalized populations in the Czech Republic (Lusk *et al*., 2011) and it is commonly used as prey fish in Czech pond fishery. Water temperature in ponds was measured continually using a data logger (Minikin T, Czech Republic). Average WT was 2.5±1.6 °C during the trial. Ponds were drained on 5 May and fish of both groups (C and IRJ) were identified and counted for calculation of the survival rate. A sample of 35 fish from each group and pond was measured and weighed to assess the total length and average weight, respectively. Specific growth rate (SGR) and Fulton coefficient (FC) were calculated according to Policar *et al*. (2011a; 2011b) at the harvesting day.

Re-adaptation to RAS Conditions

Intensively cultured fish (W= 24.6±8.1 g; TL= 151.7±13.3 mm) from all three ponds were separated into six groups of 70 fish and transferred to six 180-l circular tanks of a RAS in FFPW. A warm-up period from ambient pond temperature took three days (continual increasing of WT). Juveniles were treated with NaCl bath (3 $kg.m^{-3}$, duration of bath 30 minutes) at the first day of warm-up period.

After the warm-up period, fish were not fed for 24 hours, but they were subsequently fed *ad-libitum* for 48 hours with a 1:1 mixture of frozen bloodworm larvae (*Chironomus* sp.) and artificial pellet feed (BioMar, INICIO plus 2 mm). From the following day, all fish were fed only the commercial pelleted feed at 1% of fish biomass (Zakes *et al*., 2004), adjusted every seventh day according to the current biomass of each experimental tank.

Belt feeders were used for continual feeding during the light period of a 12L : 12D light regime and illumination intensity of 40 lux at the water surface. Fish were reared under RAS conditions for 46 days. WT and OS were measured twice daily with an oximeter (OxyGuard). Average WT was 22.8±1.4°C and OS = 109±7%.

At the end of the rearing period, fish were counted for determination of survival rate, measured and weighed for calculation of SGR, FC (Policar *et al*., 2011a; 2011b) and FCR (Fiogbe and Kestemont, 2003).

The program Statistic 10, one way Anova analys with Tuckey *post-hoc* test were used for comparison of the survival rate, SGR and FC of group IRJ with group C after the pond culture. The level of significance was set at $P<0.05$.

Results

Adaptation and Culture under Pond Conditions

Survival rate of intensively cultured fish was significantly higher than that of group C. However, there were observed no significant differences in SGR and FC between group IRJ and group C (Table 1). More than 90% of the prey fish (91-100%) had been consumed in all ponds at the end of pond culture.

Re-adaptation and Following Culture of Intensively Cultured Fish in RAS

No IRJ fish died during the 3-day adaptation period after re-transfer to the RAS. The intensively cultured fish harvested from ponds were re-adapted without any complications. Survival of IRJ fish

Table 1. Survival, specific growth rate (SGR) and Fulton's coefficient (FC) assessed after 178-day pond culture. Different letters indicate significant difference ($P<0.05$).

Experimental Groups	Survival [%]	SGR [$\%.d^{-1}$]	FC
IRJ group	65.2±15.9^a	0.018±0.012^a	1.02±0.13^a
Group C	47.3±1.6^b	0.014±0.027^a	0.95±0.57^a

during the 46-day intensive culture was 98.4±0.9% and SGR was at 0.732±0.441%.d^{-1}. FC of IRJ fish was 1.20±0.09 and FCR of fed feed was 2.1±1.2.

Discussion

The primary aim of intensive aquaculture is to produce high-quality marketable fish throughout the year. Out-of-season spawning can lead to production of several batches of larvae and fry ensuring stable year-round production of marketable fish (Zakes and Szczepkowski, 2004). With the system assessed in this study, based on a combination of pond and RAS culture, production can be staggered over the course of the year with relatively low additional effort and production cost.

One of the most important results of our study was no complications and non-problematic adaptation of intensively cultured juveniles to pond conditions. The survival rate of group IRJ was higher (65.2 ± 15.9%) than that of control fish (47.3 ± 1.6%) during the pond culture phase. Rennert *et al*. (2005) carried out a similar experiment where they also compared the survival of juvenile pikeperch after wintering. They compared a group of pikeperch juveniles (31.6 g) that had been fed pellet feed with a group of fish (13.8 g) which had been fed only live food. These groups were stocked into ponds in the fall and were harvested in the spring after a winter period of 176 days. Survival after wintering was the same in both groups at 83.3%. Survival was higher than groups in our study, but Rennert *et al*. (2005) used a much lower fish density. They stocked only 24 fish (12 fish from each group) into ponds with average area of 667 m^2 compared to 1000 fish per 400 m^2 of pond area in our study. This different fish density in both studies could affect the survival rate.

According to presented results and those of Rennert *et al*. (2005), intensively reared pikeperch juveniles can be stocked in open waters instead of one-summer-old pond-produced pikeperch juveniles. Production of pikeperch juveniles in intensive aquaculture is more stable and effective method compared to pond culture which suffers from annual fluctuations of production (Policar *et al*., 2014).

The second most important outcome of presented study was the ease of which the intensively reared fish converted from natural feed to an artificial diet after pond culture. This demonstrated that methods of year-round production of pikeperch might not only be limited to out-of-season spawning which is currently the most common practice for year-round production of pikeperch fry, juveniles and subsequently, marketable fish. Combination of pond and RAS culture can also be employed to produce several size categories of pikeperch with the same age within one natural production cycle, which is easier to be manage and cheaper than out-of-season spawning techniques. Pikeperch growth under pond culture is slower than RAS (WT in ponds is usually lower than in RAS and fish growth rate is highly affected by WT), therefore, fish of the same age can be available in several different size. One disadvantage of this rearing technique could be the potential for transferring parasites from the ponds to the RAS system. If the farmers follow the basic rules of hygiene, quarantine or antiparasitic treatments, the problems with parasites occurrence should not appear (Policar *et al*., 2014). This type of controlled-combined pikeperch culture can be suitable especially for fish production in countries with pronounced winter season and large areas of production ponds such as: the Czech Republic, Hungary, Germany, etc.

To conclude this study, we can say that presented kind of pikeperch culture could be used as an alternative to out-of-season spawning to produce several size categories within natural production cycle and help the farmers to supply the market with marketable fish all year round. High winter survival of intensively cultured fish is also promising for restocking of pikeperch juveniles to fishing grounds.

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CHAPTER 9

ADAPTATION AND CULTURE OF PIKEPERCH (*SANDER LUCIOPERCA*) JUVENILES IN RECIRCULATING AQUACULTURE SYSTEM (RAS)

Policar, T., Kristan, J., Blecha, M., Vanis, J., 2014. Adaptation and culture of juvenile pikeperch (*Sander lucioperca*) to recirculation aquaculture system (RAS). FFPW USB Vodňany, Methods, 141. 46 pp.

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Adaptation and Culture of Pikeperch (*Sander lucioperca* L.) Juveniles in Recirculating Aquaculture System (RAS)

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Optimalization of rearing aspects in pond and intensive aquaculture
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Development and optimalization of intensive culture methods in pikeperch (Sander lucioperca) and Eurasian perch (Perca fluviatilis) in the Czech Republic
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CONTENT

1. INTRODUCTION

Pikeperch, *Sander lucioperca* (L.) (Fig. 1) is currently one of the most promising fish species reared in European aquaculture (Müller-Belecke and Zienert, 2008; Policar et al., 2013b). This species is very popular with consumers (Dil, 2008) and sport anglers (Pivnicka and Rybar, 2001) thanks to its high-quality meat (Uysal and Aksoylar, 2005). Pikeperch grows fast under optimal conditions and it is possible to culture it in high densities using pellet feed (Policar et al., 2013b). The year-round pikeperch production is quantitatively and qualitatively erratic (Dil, 2008; Müller-Belecke and Zienert, 2008). On the basis of the above-mentioned facts, it is possible to predict an increase in production of pikeperch in European intensive aquaculture (Policar et al., 2013b).

Currently, about 85–90% of European production of marketable pikeperch comes from Russian, Kazakhstan and Estonian lakes. European capture fisheries of marketable pikeperch ranges between 9,000 to 15,000 tons (FAO, 2012b). However, this level of production has been decreasing. In the 1970's, the annual production from capture fisheries in Easter European lakes was about 40,000 tons of marketable pikeperch (Dil, 2008; FAO, 2012a), but this production has been reduced by fifty percent during the last 30–40 years. The main reason for this trend is overfishing and poor fishery management (Dil, 2008; Müller-Belecke and Zienert, 2008).

High popularity with consumers and sport anglers as well as decreasing capture production have led to an insufficient market supply mainly in Western Europe (France, Germany, Austria, Denmark, Belgium and Switzerland) and to an increase in price. Current wholesale price in Western Europe of pikeperch (0.7–2 kg) ranges from EUR 5 to 8 per kilogram and from EUR 6 to 9 per kilogram of fish weighing 2–4 kg (Dil, 2008). Retail price varies from EUR 13 to 15 (Tamazouzt, 2013). In the Czech Republic, an average retail price of marketable pikeperch is about CZK 290–350 (Zvonař, personal communication) which is approximately EUR 11.5–14.

The above-mentioned problems (poorly supplied market, overfishing and lower capture production) of pikeperch market in Europe have forced farmers to focus more on intensive culture of this fish species (Policar et al., 2011, 2013b). Nowadays, pond and intensive pikeperch culture produce only 5–7% (500–1,000 tons) of total production in Europe (FAO, 2012b). In Central and Eastern Europe (Czech Republic, Hungary, Ukraine, Bulgaria, Romania, Poland, Germany), pikeperch is usually reared in pond polyculture. Annual production of marketable pikeperch ranges between 300–500 tons in the mentioned countries. Pikeperch is an additional species cultured in pond polyculture with

ADAPTATION AND CULTURE OF PIKEPERCH (*SANDER LUCIOPERCA* L.) JUVENILES IN RECIRCULATING AQUACULTURE SYSTEM (RAS)

Fig. 1. *Marketable pikeperch, Sander lucioperca (L.), body weight 1,500 g (photo T. Policar).*

the main cultured fish species such as common carp (*Cyprinus carpio* L.), tench (*Tinca tinca* L.), grass carp (*Ctenopharyngodon idella* Valenciennes) and silver carp (*Hypophthalmichthys molitrix* Valenciennes). The main role of pikeperch in this system is to eliminate small cyprinids such as: topmouth gudgeon (*Pseudorasbora parva* Temminck and Schegel) roach (*Rutilus rutilus* Rafinesque), rudd (*Scardinius erythrophthalmus* Bonaparte) and bream (*Abramis brama* L.) (Wedekind, 2008; Adamek et al., 2012; Kratochvil, 2012).

Besides the traditional pond culture, pikeperch has been reared in recirculating aquaculture systems (RAS) in Western Europe in the last fifteen years (Policar et al., 2013b). Pikeperch culture in the RAS has used domesticated fish (Fontaine, 2009), out-of-season spawning (Zakes and Szczepkowski, 2004; Rónyai, 2007; Müller-Belecke and Zienert, 2008), pellet feed (Wang et al., 2009) and high fish densities (30–50 $kg.m^{-3}$; Wedekind, 2008). In the last several years, the intensive pikeperch aquaculture has been developed mainly in Denmark, Holland, Finland, France, Czech Republic, Austria, Germany, Romania, Bulgaria and Croatia (Van Mechelen, 2008; Philipsen, 2008; Policar et al., 2011, 2013b). At present, about 30 companies

are producing marketable pikeperch under the RAS conditions (Aquapri and Lyksvad Fish Farm, Denmark; van Slooten Aquacultuur; Excellence fish; Lont en s van Barren and Viskweekcentrum, all based in Holland; Kidus; Savon-Taimen; Imatra Kala ja Kaviari, all based in Finland; LucasPerch and Asialor based in France (Fig. 2); Fish Farm Bohemia based in the Czech Republic; Fishzucht Pottenbrunn based in Austria; Osnabruck Pikeperch farm based in Germany; Sterlet-Timisoara farm based in Romania; Eko-Hidro-90 Ltd. based in Bulgaria; Aqua Campus based in Croatia). The current top RAS producer of pikeperch is a Danish company, Aquapri (www.aquapri.dk) which has been dealing with pikeperch since 2006. This company produced about one million pikeperch juveniles (with body weight of 1 g) and 100 tons of marketable fish (1.2–2 kg) in 2012 (Overton, personal communication, 2012). Only one company in the Czech Republic has been producing pikeperch in the RAS since 2011 (Junek, personal communication, 2011).

Fig. 2. *Production facility of company Asialor in France for production of marketable pikeperch, Sander lucioperca (L.) built in 2010 (photo T. Policar).*

One group of European pikeperch farmers works with a "closed production cycle" of pikeperch; it includes all fish stages (larvae, juveniles, marketable fish, broodstock). Broodstock are held under regulated environmental conditions

(light and water temperature regime) to stimulate the gametogenesis. The main aim of this environmental stimulation is to achieve out-of-season spawning and subsequently, to produce larvae, juveniles and marketable fish year-round (Philipsen, 2008). The second group of European pikeperch farmers uses an open production cycle, which means that the farmers do not have broodstock; they purchase 8–10 g juveniles (van Slooten, personal communication, 2013).

Rearing of pikeperch in the closed production cycle is a very complicated and technologically demanding process, requiring high standards of zoohygiene, high water quality, proper nutrition (Zakes et al., 2006) and size-balanced population (Szczepkowski et al., 2011). Further, environmental and hormone stimulation of broodstock are required for spawning (Rónyai, 2007; Müller-Belecke and Zinert, 2008; Hemerlink et al., 2013; Kristan et al., 2013), plus egg incubation (Musil and Kouril, 2006; Policar et al., 2011) and nursing of larvae must be optimized to produce juveniles and marketable fish (Zakes et al., 2004, 2006; Kestemont et al., 2007; Wang et al., 2009; Lund and Steenfeldt, 2011). The entire rearing process is very challenging and expensive (Schram, 2008). Apart from high production costs, the complicated technological system (closed production cycle) also often results in low oocyte quality and, of course, decreased fertilisation (50–60%) and hatching rates (30–40%). It also is possible to have low quality larvae with a high frequency of body deformations. All these problems can be related to poor nutrition of broodstock in the closed production cycle (Policar et al., 2011).

For these reasons, since 2009 our team of scientists has been developing a process that combines pond culture (Fig. 3) and the RAS system (Fig. 4) for pikeperch production. This approach uses advantages of both pond culture and the RAS. Pond culture is used for broodstock, larvae and juvenile (up to the total length of 35–50 mm) production, providing optimal nutrition for all reared categories. It has a positive effect on broodstock reproduction, larvae viability and production of high quality juveniles. This technological process is not as expensive as production of pikeperch larvae in RAS (Policar et al., 2011). Intensive aquaculture is integrated when pond-cultured pikeperch juveniles are adapted to the RAS with subsequent intensive rearing of juveniles to marketable-size fish under intensive conditions (Policar et al., 2013a,b). This optimized intensive culture provides pikeperch with the best conditions for rapid growth and guarantees high efficiency of the whole rearing cycle (Zakes et al., 2006). According to our experience, it is advisable to apply this rearing technique (combination of pond and RAS culture) especially in countries with a high number of small ponds such as: the Czech Republic, Hungary and Germany.

Fig. 3. *Suitable pond for pikeperch, Sander lucioperca (L.) summer-fry production (photo T. Policar).*

Fig. 4. *Suitable recirculating aquaculture system used for intensive culture of older pikeperch, Sander lucioperca (L.) in Asialor Ltd. company, France (photo T. Policar).*

ADAPTATION AND CULTURE OF PIKEPERCH (*SANDER LUCIOPERCA* L.) JUVENILES IN RECIRCULATING AQUACULTURE SYSTEM (RAS)

2. AIM OF THE TECHNOLOGY

The primary objective of this publication is to describe the details of an optimized technological process which includes: 1) introduction of pond-cultured pikeperch juveniles (TL = 40–50 mm) into the RAS, 2) food and space adaptation of pond-cultured fish to the RAS, and 3) subsequent intensive culture of adapted fish in the RAS up to the body weight of 8 to 50 g. The next tasks were to observe and take care of health condition of fish before the stocking to RAS and during their intensive culture by using therapeutic and preventive treatments and to perform a regular sorting or size-grading so as to keep comparable-size fish in the culture system. An important component of the developmental process was to make a financial analysis to calculate the final product price i.e. an 8 g pikeperch juveniles. The last step was to make a market research and search for possible prospective buyers.

Fig. 5. *Recirculating aquaculture system of FFPW USB used for adaptation and intensive culture of juvenile pikeperch, Sander lucioperca (L.) (photo T. Policar).*

3. VERIFICATION OF THE TECHNOLOGY

The technological process was verified in 2012 at two Czech fish farms (Rybářství Nové Hrady, Ltd. and Fish Farm Bohemia, Ltd.) and in the research facilities (Faculty of Fisheries and Protection of Waters, University of South Bohemia – FFPW, USB) (Fig. 5). This technological process followed the methodology by Policar et al. (2011). Background for this technological process was provided by Stejskal et al. (2010) in the description of the combination of pond and RAS production system for marketable Eurasian perch (*Perca fluviatilis* L.).

4. DESCRIPTION OF THE TECHNOLOGY

4.1. Stocking of pond-cultured pikeperch juveniles to the RAS

4.1.1. Technological process

The Bejkovna pond (surface area of 1.33 ha, GPS 48º48´12´´N; 14º48´53´´ E) was harvested on June 6 and the production was 86,000 pikeperch juveniles (TL = 41 ± 0.3 mm; W = 0.45 ± 0.05 g) (Fig. 6). Fish were graded and uniform-size were stocked into the RAS.

At the beginning of the harvesting, a sample of fish was taken and sent to a veterinary office for analysis using the methodology of Citek et al. (1997). Fish were infected with *Ichthyophtirius multifiliis*. Consequently, at harvest fish were treated during transport with a therapeutic bath (detailed description in section 4.4.) The health condition was inspected again before stocking into the RAS and any infection was not obserevd. Ideally, fish should be quarantined to include a common recirculating system without bio-filtration with a period of two or three days. This system should be used for observation and treatment of fish before their stocking to the RAS.

Juveniles sorted by size and without ectoparasites were put into RAS, which included 18 square tanks (size of 1 x 1 x 1 m) with water volume of 700 l. The RAS included a mechanical drum filter (IN-EKO Tisnov Ltd.), a retention tank (2,000L), three fluid-biofilters (4,000 l each), a ozonizer with a UV lamp (Ozon UV-C redox) and oxygenation column (Kovo Net Ltd.). The entire volume of the RAS was 28,100 litres.

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Fig. 6. *Juvenile fish (summer-fry) pikeperch, Sander lucioperca (L.) stocked into RAS (photo T. Policar).*

All the fish were transferred from the transport tanks (WT = 17.7 ± 0.3 °C; oxygen saturation = 104 ± 23% and pH = 6.8) directly to the RAS. In total, 84,000 pikeperch juveniles were divided into 14 tanks. The 4 remaining tanks (total = 18 tanks) were subsequently used for rearing faster growing fish or cannibals (see 4.2.1.). The juvenile density was 8.6 individuals per litre and the fish biomass was 3.85 $kg.m^{-3}$. The water quality parameters at the beginning of acclimation were as follows: WT=19 °C; oxygen saturation = 90%; pH = 7.3; NO_2 = 0.15$mg.l^{-1}$; NO_3 = 58$mg.l^{-1}$; NH_4 = 0.16$mg.l^{-1}$; CHOD – chemical oxygen demand = 28$mg.l^{-1}$. The water inflow in each tank was 20 $l.min^{-1}$. An antifungal bath in NaCl with the concentration of 3 $g.l^{-1}$ (for details about effects of antifungal bath see section 4.4.) was applied in each tank. After that, space and feed adaptation began. The entire procedure of acclimating fish to new conditions, including the conversion from live to pellet feed is called weaning. Fish were initially fed bloodworm larvae (*Chironomus* sp.) and gradually a mixture of bloodworm and pellet feed (BioMar, Inicio Plus 1.1 mm) were introduced. The details of the weaning procedure are depicted in Fig. 7; water temperature was increased to 23 °C during the first 24 hours and a special photoperiod 15L/9D and light regime 100 lux were established.

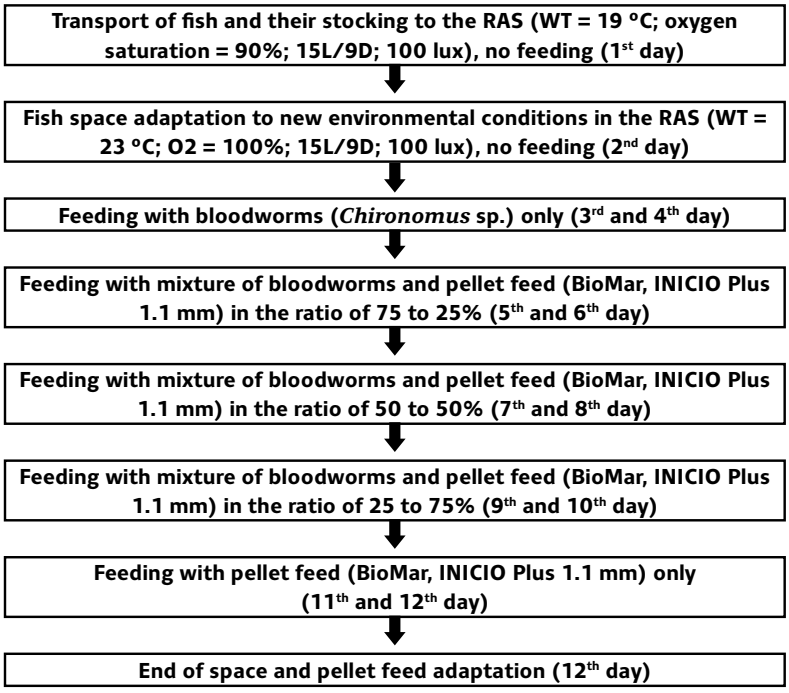

Fig. 7. *Scheme of space and feed adaptation of juvenile pikeperch, Sander lucioperca (L.) in 12 days.*

No food was given during the first and second day so as to encourage subsequent feeding activity. During the feeding transition, the amount of bloodworms was decreased and the ratio of the pellet feed was increased every two days. Nutritional content of bloodworm and pellet feed are described in Tab. 1.

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Tab. 1. *Nutritional content of bloodworm and 1.1 mm pellets BioMar, Inicio Plus during the weaning.*

Parameter	Bloodworm	Pellet feed
Size [mm]	9.13	1.1
Proteins[a]	65	55
Fat[a]	1	20
Digestible energy[c]	15.1	20
Fatty acids[b]		
12:0	0.1	0.2
14:0	3.2	5.2
15:0	2.2	0.4
16:0	20.3	16.4
17:0	1.6	0.2
18:0	6.6	2.5
20:0	0.7	0.2
22:0	0	0
14:1	1.5	0.1
16:0 (n-7)	13.9	7.8
17:1	2.1	0
18:1 (n-9)	15.1	12.9
18:1 (n-7)	4.6	1.7
20:1 (n-9)	0	3.8
22:1	0	6.2
24:1	0	0.4
18:2 (n-6)	13.6	22.6
18:3 (n-6)	0.6	0
20:4 (n-6)	2.4	0.3
18:3 (n-3)	3.4	2.4
18:4 (n-3)	0.4	2.4
20:5 (n-3)	7.7	7.3
22:5 (n-3)	0	0.6
22:9 (n-3)	0	6.6
Σ SFA	34.7	25
Σ MUFA	37.2	33
Σ PUFA	28.1	542.1
n-3	11.5	19.3
n-6	16.6	22.8
n-9/n-3	1.4	1.2

[a] percentage in dry matter, [b] percentage from fatty acids, [c] MJ/kg in dry matter

The daily feeding ratio (DFR) was *ad libitum* during the entire weaning and fish were fed every 30 minutes by hand from 6:30 till 21:30. The production parameters were:

Feed Conversion Ratio (FCR) = TAF/(FB - IB)

TAF total amount of feeding, FB final fish biomass and IB initial fish biomass (Stejskal et al., 2009a)

Survival (S, %) = NSF/NFB * 100

NSF means number of surviving fish and NFB means number of fish at the beginning.

Cannibalism (C, %) = (NFB - NDF - NSF)/NFB * 100

NDF means number of dead fish

Specific Growth Rate (SGR, %/day) = 100 * t^{-1}ln (FW - IW)

t means number of days, FW means final body weight and IW means initial body weight

Fultons Coefficient (FC) = 100 * TL^3/FW

TL means total length of fish (Policar et al., 2011, 2013b).

Body weight and total length of 10 fish (in each tank) were measured at the beginning and at the end of the weaning (Fig. 8). A common measuring tape with 1 mm increments and scale (Mettler, AE 200) with an accuracy 0.01 g were used. Fish were not anaesthetized during measurement.

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Fig. 8. *Manipulation of pikeperch, Sander lucioperca (L.) during intensive culture (photo T. Policar).*

Dead fish, uneaten feed, excrements and other sediment were removed from the tanks twice daily (at 8 a.m. and 14 p.m.). The dead fish were counted to assess mortality in each rearing period (NDF). Tank walls were brush-cleaned every 3 days (Fig. 9).

Fig. 9. *Cleaning of the tanks during intensive culture of pikeperch, Sander lucioperca (L.) (photo T. Policar).*

4.1.2. Results

After stocking, fish swam in shoals close to the tank bottom, then gradually during the 12-day adaptation, they began to swim in the upper part of water column. During the initial period of weaning, the juveniles fed somewhat reluctantly, but later they were more aggressive. After 5–10 days the fish rapidly reacted to the feed. Some of the fish attacked (by biting head and fins) each other during feeding but this behaviour did not lead to increased fish mortality. The mortality of reared pikeperch juveniles was low (a few individuals daily) at the beginning of the weaning. Later, several hundreds of fish died each day (from day 5 to 9 of the weaning). The fish that died were weak and did not eat. After day 10 of the weaning, mortality decreased and all surviving fish fed well. The results and production parameters assessed after weaning are presented in Tab. 2. The most important data from the weaning period were survival (S = 78 ± 5.5%), cannibalism (C = 5 ± 2%) and percentage of fish converted to pellet feed (97%). Cannibalism was low due to size grading before fish stocking to RAS. Very high Feed Conversion Ratio

(FCR = 4.7 ± 0.5 $g.g^{-1}$) was calculated after adaptation period. High value of FCR after this period was mainly caused by: 1) using of feed which had a higher water content (frozen bloodworms), 2) higher amount of uneaten feed and 3) higher fish mortality. Fish that had adapted to the intensive culture conditions were in good condition (FC = 0.67 ± 0.1).

Tab. 2. *Production parameters such as body weight, total length, SGR, survival, cannibalism, FCR and FC in pikeperch, Sander lucioperca (L.) juveniles assessed after the 12-day-long weaning.*

IW (g)	FW (g)	ITL (mm)	FTL (mm)	SGR (%/g)	S (%)	C (%)	FCR	FC
0.45 ± 0.05	0.70 ± 0.1	41 ± 0.3	47 ± 0.6	3.8 ± 0.2	78 ± 5.5	5 ± 2	4.7 ± 0.5	0.67 ± 0.1

IW – initial body weight; FW – final body weight; ITL – initial total length; FTL – final total length

4.1.3. Conclusion and recommendations for farmers

It is possible to efficiently convert pond-reared pikeperch juveniles to pellet feed with a high survival. The most important points of a successful weaning are:

a) careful and professional pond harvest and transport of fish to the RAS;
b) stocking of healthy size-graded fish;
c) optimal rearing conditions (high water quality);
d) fish can be stocked into the RAS with a 2–3 °C higher WT than in transport tanks.

4.2. Intensive culture of fully adapted pikeperch juveniles with a different average body weight 8; 25 and 50 g under the RAS conditions

4.2.1. Technological process

The following culture of pikeperch juveniles under the complete RAS (Fig. 10) conditions was divided into 3 phases [1st phase up to 8 g (Fig. 11); 2nd phase up to 25 g; 3rd phase up to 50 g (Fig. 12)] using different durations of culture phases, fish density and biomass (Tab. 3). Fish were prophylactically treated immediately after stocking into the tanks or handling during sampling and sorting, when a 3 $g.l^{-1}$ bath of NaCl was used. Average water quality parameters were: WT = 24.7 ± 2.7 °C; oxygen saturation = 101 ± 5%; pH = 7 ± 0.3; NO_2 = 0.24 ± 0.07 $mg.l^{-1}$; NO_3 = 65 ± 8.07 $mg.l^{-1}$; NH_4 = 0.18 ± 0.0207 $mg.l^{-1}$; CHOD = 30 ± 5.5 $mg.l^{-1}$. Light regime was established at 15L/9D and water inflow in each tank was about 25 $l.min^{-1}$.

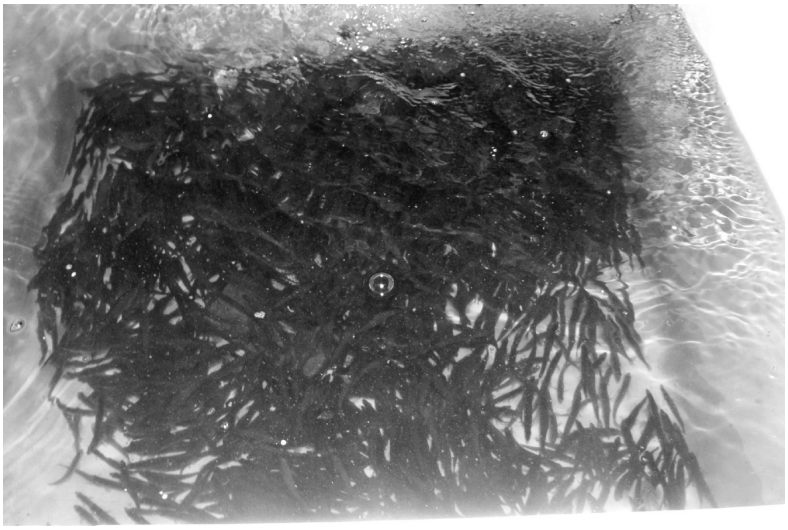

Fig. 10. *Juvenile pikeperch, Sander lucioperca (L.) in rearing tank during intensive culture (photo T. Policar).*

ADAPTATION AND CULTURE OF PIKEPERCH (*SANDER LUCIOPERCA* L.) JUVENILES IN RECIRCULATING AQUACULTURE SYSTEM (RAS)

Fig. 11. *Juvenile pikeperch, Sander lucioperca (L.) cultured up to average weight of 8 g (photo M. Blecha).*

Fig. 12. *Juvenile pikeperch, Sander lucioperca (L.) with average weight of 50 g at the end of intensive culture (photo T. Policar).*

Tab. 3. *Duration of each pikeperch Sander Lucioperca (L.), rearing period, average fish biomass and density at the beginning and at the end of each rearing period in 700-L tanks (average ± S.D.)*

Rearing period	Length of rearing (days)	Initial fish biomass (g)	Initial fish density (n)	Final fish biomass (g)	Final fish density (n)
I. period (0.7–8.2 g)	65	3,283 ± 320	4,680	34,038 ± 1,680	4,151 ± 205
II. period (8.2–25.1 g)	40	9,840 ± 580	1,245	30,145 ± 875	1,201 ± 35
III. period (25.1–50.6 g)	45	19,200 ± 400	800	38,658 ± 365	764 ± 7

A very important procedure during each rearing phase was to size grade the fish (1st phase every 10 days; 2nd and 3rd phase every 21 days). The sorting permitted removal of cannibals (Fig. 13) (more information about this issue see section 4.3.). All cannibals were cultured in 4 separated tanks.

Fig. 13. *Comparison of the size of cannibalistic (above) and non-cannibalistic (below) pikeperch, Sander lucioperca (L.) individuals (photo T. Policar).*

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The first feed was given approximately 5 hours after sorting. Fish were fed with BioMar Inicio Plus and Efico Sigma 570 pelleted feed. The pellet size, kind of feed, DFR and the feeding technique are listed in Tab. 4. The pellet feed was applied by hand (every 30 minutes during the day-light period) and by belt feeders (during the entire day-light period). Hand feeding was used to achieve better distribution of the pellets among all fish in the tanks. This procedure led to a more uniform size-balanced fish population in tanks. At the end of each rearing phase, the production parameters were counted. It was conducted in the same manner and according to the same formula as in section 4.1.1.

Tab. 4. *Daily feeding ratio (DFR), kind of pellet, pellet size and feeding techniques applied during the rearing phases of pikeperch intensive culture to different body weights.*

Rearing phase	DFR (%)	Kind of feed	Pellet size (mm)	Feeding technique
1st phase (0.7–8.2 g)	12–15	Inicio Plus	1.5 and 2	by hand : belt feeder 1 : 1
2nd phase (8.2–25.1 g)	5–7.5	Inicio Plus	2	by hand : belt feeder 1 : 1
3rd phase (25.1–50.6 g)	1.5–3	Inicio Plus Efico Sigma 570	2 and 3	belt feeder

4.2.2. Results

Production parameters at the end of each rearing phase are described in Tab. 5. The pikeperch juveniles achieved a very good specific growth rate in the first phase (3.9%.day^{-1}) and in the second phase (2.8%.day^{-1}), but a somewhat lower SGR was observed after the third phase (1.7%.day^{-1}). It was caused by bigger size of the fish and unstable water temperature during this phase. The good SGR positively affected absolute increment of the fish biomass in the tanks which ranged between 19.5–30.8 kg. High survival (88.7–96.5%) during each rearing phase was also observed. Survival was the lowest during the first rearing phase, primarily because of a high cannibalism of 7.5%. On the other hand, cannibalism was lower during the second and third phases (1.5–2.5%). To explain that, smaller pikeperch juveniles (up to 5–8 g) are able to prey on the same size individuals but bigger juveniles (10 g and bigger) are not able to do so. Fast growth of the cultured pikeperch juveniles was associated with a very good feed conversion rate (FCR), only 0.93 during the first phase, but higher during the second (1.5) and third phases (1.7) was caused by an unstable

water temperature regime and therefore lower feeding activity of the cultured pikeperch juveniles. Generally, condition values (FC = 0.82–0.89) were good during all phases. At the end of each phase, very high content of perivisceral fat was observed in fish. This finding showed unbalanced nutritional composition of the used feed. Therefore, it is possible to say that the BioMar feed which was used in this study was not optimal for intensive pikeperch juvenile culture.

Tab. 5. *Production parameters such as body weight, total length, SGR, survival, cannibalism, FCR and FC in pikeperch (Sander lucioperca) juveniles assessed after each rearing phase.*

Rearing phase	IW (g)	FW (g)	ITL (mm)	FTL (mm)	SGR (%/ day)	S (%)	C (%)	FCR	FC
1st phase	0.7 ± 0.1	8.2 ± 0.6	47 ± 0.6	97.3 ± 10.1	3.9 ± 0.3	88.7 ± 5.3	7.5 ± 2.5	0.93 ± 0.15	0.89 ± 0.15
2nd phase	8.2 ± 0.6	25.1 ± 5.6	97.3 ± 10.1	145 ± 10	2.8 ± 0.3	96.5 ± 3	2.5 ± 0.75	1.5 ± 0.1	0.82 ± 0.2
3rd phase	25.1 ± 2.7	50.6 ± 12.2	145 ± 10	182.5 ± 12.3	1.7 ± 0.2	95.5 ± 1	1.5 ± 0.5	1.7 ± 0.25	0.83 ± 0.18

In total, 58,114 fish were produced at the end of the first phase (initial stock comprised 65,520 ind.), during the second phase it was 4,804 fish (initial stock 4,980 ind.) and in the third phase the production amounted to 3,056 fish (initial stock 3,200 ind.). The pikeperch juveniles from the third phase were used for the study which focused on survival of intensively cultured pikeperch juveniles under the pond conditions during the winter time (Choteborsky, 2013).

4.2.3. Conclusion and recommendations for farmers

Rearing of pond-cultured pikeperch juveniles readily adapted to conditions of intensive aquaculture, demonstrating its feasibility. It is important to provide fish with the following rearing conditions: water temperature 23–24 °C, oxygen saturation about 100%, pH 7, CHSK approximately 30 $mg.l^{-1}$, minimum values of nitrites, nitrates and ammonium. The initial stocking density should be at the level of 6.7 $ind.l^{-1}$. An appropriate pellet feed represents a very important factor with respect to intensive culture of pikeperch juveniles. According to our experience, the pellet feed by BioMar is satisfactory but not optimal. Next, the pellet feed should be served both by hand and belt feeders during the first two phases. This combined method leads to better distribution of pellets to all fish

in a tank and results to a size-balanced population. Daily cleaning of tanks is important to maintain a healthy environment.

4.3. Sorting of fish

4.3.1. Technological process

Regular sorting represents a very important procedural step in intensive culture of pikeperch juveniles. The purpose of sorting is to separate faster growing and cannibalistic individuals (Fig. 14) from other juveniles and thereby eliminate losses caused by predation of cannibals.

Fig. 14. *Cannibalistic pikeperch, Sander lucioperca (L.) individual which was separated during sorting of fish in intensive aquaculture rearing (photo M. Blecha).*

Basic information about the size grading or sorting was mentioned in section 4.2.1. The first sorting was done after pond harvest and before stocking juveniles into RAS. Subsequent sorting was conducted after weaning. Even after the population was size-balanced before the weaning, growth disparity produced several cannibals in each tank during weaning. Therefore, size sorting was applied again. During the first phase, sorting was done every 10 days and afterwards (from the TL = 100 mm; W = 8–9 g), subsequently, it were carried out at 21-day intervals.

Fish were not anaesthetized during grading, hand sorters function more effectively when fish are active (Fig. 15). A 20-min salt bath of 3 $g.l^{-1}$ was administered immediately after sorting and stocking of fish into the tanks. Salt treatment has a positive effect on health of percid fish cultured in RAS (Kestemont et al., 2008).

Fig. 15. *Sorting of pikeperch, Sander lucioperca (L.) juveniles with hand sorter during intensive culture (photo T. Policar).*

4.3.2. Results

Size-grading with hand sorters requires some skill, but it is more rapid than individually measuring. Fish should not be fed the day before the sorting. According to our experience, it is advisable to start the sorting in the morning because the process is time-consuming. Three persons are required to sort several tens of thousands of fish (sorting carried out within work described by this publication comprised 58,000–86,000 fish). One worker catches the fish, cleans the tanks and then re-stocks the fish back to the tanks. Two workers sort and record data (information concerning fish biomass, number of fish

in tanks, average weight of fish). This working group is able to sort 58,000–86,000 fish in 6 to 8 hours. Because the sorting takes quite a long time and it is not possible to feed fish during the process, it is advisable to divide the process in two days, or to use more people to shorten the process. The main disadvantage of the alternative is that it requires additional experienced staff. Another possibility is to use an automatic sorting machine (Fig. 16). This machine is relatively expensive and can increase the cost so as to be unprofitable, especially in small RAS (annual production of 50,000 juveniles).

Fig. 16. *Automatic sorter used in commercial intensive farms of Eurasian perch, Perca fluviatilis (L.) and pikeperch, Sander lucioperca (L.) in Lucas Perch Ltd. company, France (photo T. Policar)*

4.3.3. Conclusion and recommendations for farmers

Size sorting is a very important step in intensive culture of pikeperch juveniles. It facilitates the rearing of a size-balanced population, eliminates cannibals, increases survival of cultured fish and profit of the operation. Optimal frequencies of sorting are: fish with the body weight from 0.5 to 8 g every 10–12 days; fish with the body weight from 8 to 50 g every 21 days.

4.4. Control of fish health, preventive and therapeutic baths in intensive culture

4.4.1. Technological process

As already described in section 4.1., fish should be sampled for fish health analysis at the time of the pond harvesting. Depending on the diagnosis, appropriate treatment can be applied. During this test, *Ichthyophtirius multifiliis* was diagnosed. Therefore, the 8-hour-long treatment bath in 35.2% formaldehyde at the concentration of 15 $ml.m^{-3}$ was applied in the transport box. In the Czech Republic treatment with a formaldehyde bath must be prescribed by a veterinarian because formaldehyde is an unregistered medical substances. No maximum residue limit (MRL) for treated market fish has been defined for formaldehyde. The maximum residue limit determines the maximum amount of an active ingredient which can be allowed in edible tissue of a given fish species. In the absence of MRL, fish cannot be used for human consumption until the longest protection period – in this case 500-day degrees (Kolarova and Svobodova, 2009). It is also necessary to follow the safety rules with toxic substances because formaldehyde is a carcinogenic substance.

The control of fish health was made once a week during intensive culture of pikeperch juveniles and ectoparasite occurrence and health conditions were determined in at least 7 individuals from all used tanks. Special attention was given to condition of the skin and gills (Citek et al., 1997). When ectoparasites were found, possible treatment was discussed with a veterinary surgeon. In this particular case, a formaldehyde bath at the concentration of 0.015 $ml.l^{-1}$ was suggested.

According to our experience, low formaldehyde concentration (0.015 $ml.l^{-1}$) has no negative effect on the functioning of biological filters, even if it is applied 2–3 times a week. We tested the effect of formaldehyde on function of the biological filters in 2010 and 2011. We found no serious changes in ammonium and nitrites concentrations when quality of water was examined according to Stejskal et al. (2009b) and Kroupova et al. (2013) in different places of the RAS. The values of ammonium and nitrite concentrations were stable for 3–4 days after the formaldehyde application. These results are in agreement with the results of Yanong (2012) who claimed that formaldehyde bath did not affect the biological filtration function.

When a bacterial infection was found, a therapeutic bath in Chloramin T at the concentration of 0.02 $g.l^{-1}$ was applied in rearing tanks with fish for 20 minutes. Water inflow was stopped during the bath. Fish were transferred to other rearing tank and all water was drained from the RAS after the treatment.

This treatment was repeated every 4 days till the end of the occurrence of the bacterial infection.

A preventive antifungal bath of NaCl at the concentration of 3 $g.l^{-1}$ for 20 minutes was applied after every manipulation (sampling and sorting) and the water containing the salt solution was diluted in the entire RAS. The salt solution at the concentration of 3 $g.l^{-1}$ or lower has no negative effect on biological filtration and, on the contrary, increased concentration of sodium chlorine has a positive effect on fish health (Kestemont et al., 2008; Yanong, 2012).

4.4.2. Results

Slight infections with *Ichthyophtirius multifillis* and *Chilodonella cyprinid* were found twice during the entire culture. *Dactylogyrus sp.* was diagnosed once. Bacterial disease of gills and fins appeared one month after the weaning. Preventive and therapeutic baths were applied.

According to the veterinary surgeon's recommendation, a long-term therapeutic formaldehyde bath at the concentration of 0.015 $ml.l^{-1}$ was applied. This treatment was used only in exceptional cases because formaldehyde is an aggressive and carcinogenic chemical and its possible usage has to be discussed with and permitted by a veterinarian.

To eliminate and stop the bacterial infection of gills and fins, a short-term therapeutic bath in Chloramin-T at the concentration of 0.02 $g.l^{-1}$ for 20 minutes was applied. It is possible to use Chloramin T repeatedly after discussing the issue with a veterinarian.

Application of NaCl (concentration of 3 $g.l^{-1}$ for 20 minutes) baths as a preventive treatment against fungal infection has proved very useful. Salt preventive bath was applied after every manipulation (sorting and sampling) with fish and it was subsequently diluted in the whole RAS system without any problem.

4.4.3. Conclusion and recommendations for farmers

Preventive examinations of fish health represent the basis for successful and economically effective intensive culture of pikeperch in the RAS conditions. However, every therapeutic treatment should be discussed with a veterinarian. To make safer treatment for the whole tank stock, a tolerance test (application of the treatment only to a few individuals at first) should be conducted beforehand. All mentioned therapeutic treatments can be recommended as effective and safety protection of intensively cultured pikeperch juveniles in RAS.

4.5. Calculation of production costs and final price of a 8 g pikeperch juveniles completely adapted and cultured in RAS conditions

4.5.1. Technological process

Production costs of combined culture of pikeperch juveniles up to the body weight of 8 g were recorded in detail. It was impossible to calculate the market price of 25 and 50 g juveniles because the culture of these fish was performed in the same RAS as other experimental fish.

The calculation of the production costs was divided into four parts:

1. direct production costs of pond-reared juveniles;
2. direct production costs connected with the RAS culture;
3. indirect production costs connected with the RAS culture;
4. depreciation of tangible assets connected with the production of pikeperch juveniles in RAS.

4.5.2. Results

All the direct production costs (CZK 97,020) connected to production of pond-cultured juveniles involved:

- Pikeperch broodstock (CZK 10,500): 10 pairs: 30 kg x CZK 350 = CZK 10,500;
- Reproduction of broodstock (CZK 26,940): hormones = CZK 7,500; staff salary: 2 persons x 7 days x 3 hours x CZK 300 = CZK 12,600; water supply: 30 m^3 x CZK 61.2 = CZK 1,840; other costs: syringes, anaesthetics, bathtubs, artificial spawning nests, disinfection of fish and incubated eggs = CZK 5,000. Total = CZK 26,940;
- Pond preparation (CZK 6,560): 1,600 kg of compost = CZK 2,000; staff salary: 2 persons x 5 hours x CZK 300 = CZK 3,000; transport of material: 120 km x CZK 13 = CZK 1,560.
  Total = CZK 6,500;
- Stocking of larvae into ponds (CZK 4,560): transport of material: 120 km x CZK 13 = CZK 1,560; staff salary: 2 persons x 5 hours x CZK 300 = CZK 3,000. Total = CZK 4,560;
- Pond culture and juvenile harvesting (CZK 48,460): rent of 4 ha of ponds for 2 months: 4 x 2 x (7,000/12) = CZK 4,700; pond control and sampling: 5 x 2 persons x 5 hours x CZK 300 = CZK 15,000; pond harvesting: 4 x 2 persons x 8 hours x CZK 300 = CZK 19,200; material for pond harvesting = CZK 8,000. Total = CZK 48,460.

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The total production of pond-reared pikeperch juveniles amounted to 86,000 fish and total costs for this production represented CZK 97,020. The price of pond-reared juveniles was CZK 1.13 per fish. Only, one pond was used for juvenile production, but it is advisable to use more ponds (there are 4 in this model calculation). One of the disadvantages of pond culture is an unstable production due to several natural and unpredictable conditions and situations (flood, fish predators, oxygen deficits etc.). Therefore, we recommend the use of more ponds for stable annual production of pikeperch juveniles.

All the direct production costs (CZK 391,739) connected with RAS culture comprised:

- Staff salary (CZK 234,000): 2 persons x 6 hours x 65 days x CZK 300. Total = CZK 234,000;
- Feed (CZK 52,735): 995 kg of feed x CZK 53. Total = CZK 52,735;
- Pure oxygen used for oxygenation of water in the RAS (CZK 40,978): 4 x CZK 10,244. Total = CZK 40,978;
- Electrical energy (CZK 16,779): CZK 12,496 + CZK 283 + 2 x CZK 2,000. Total = CZK 16,779;
- Water supply used for filling and cleaning of used RAS (CZK 13,097): daily water exchange was 9% of the total RAS volume: 2.6 m^3 x 65 days = 169 m^3; cleaning of the system 5 m^3 weekly x 9 weeks = 45 m^3; (169 m^3 + 45 m^3) x 61.2 CZK/m^3. Total = CZK 13,097;
- Amortization of tangible assets (CZK 4,000): air pump CZK 2,000 + scales CZK 2,000. Total = CZK 4,000;
- Used material (CZK 30,150): a vet fee and therapeutic treatment CZK 5,500; nets CZK 3,000; aeration stones CZK 1,000; filtration medium CZK 12,000; netting for the mechanical drum CZK 7,500; buckets CZK 800; bathtubs CZK 1,000; anaesthetics CZK 350. Total = CZK 30,150.

Indirect production costs connected with the RAS culture (CZK 13,200): phone fee CZK 1,200; office supplies CZK 500; cleaning service CZK 2,500; accountant's salary 20 x CZK 450 = CZK 9,000. Total = CZK 13,200.

Depreciation of tangible assets connected with the production of pikeperch juveniles in RAS (CZK 70,000): depreciation of the building CZK 40,000; depreciation of the RAS CZK 30,000. Total = CZK 70,000.

The total production costs for the production of 58,000 fish amounted to CZK 571,959 (direct production costs for pond-reared juveniles 17%, direct production costs connected with the RAS culture 68.5%, indirect production costs connected with the RAS culture 2.3%, depreciation of tangible assets

connected with the production of pikeperch juveniles in the RAS culture 12.2%). Total production cost for cultured pikeperch juveniles with the body weight 8 g was calculated as CZK 9.9 per one fish in total production of 58,000 fish.

4.5.3. Conclusion and recommendations for farmers

The pikeperch culture implemented according to this methodology is characterised by rather high production costs (CZK 9.9 per fish), however, production is stable, and predictable and yields a high-quality product. The produced pikeperch juveniles are mainly intended for further rearing in RAS and not for stocking into open waters or production ponds. The main reason is the price of pikeperch juveniles. A current price of 5–15 g pond-reared pikeperch (used as a stocking material for open waters or ponds) ranges from CZK 3 to 7 in the Czech Republic. On this ground, pond farmers prefer pond-cultured pikeperch juveniles. However, the RAS farmers are willing to pay even more (CZK 15 to 20 or more per one fish) for quality stocking material. Therefore, described pikeperch production technology can be very effective and profitable for RAS fish farmers.

4.6. Possibilities of selling and next using of 8 g pikeperch juveniles

4.6.1. Technological process

The European pikeperch market was analysed in order to discover market demands, potential customers and current price of juvenile pikeperch with body weight 8 grams. Almost all European pikeperch RAS farmers were addressed and asked whether they would be interested in the produced fish (8 g pikeperch juveniles), how many fish they would be willing to buy and how much they would pay for them. After that, incomes and expenses were defined and possible profitability of the entire production was calculated.

It had be noted that no fish were sold and all of the fish were used for further experiments, research and education of bachelor's, master's and doctoral students in FFPW, USB.

4.6.2. Results

Positive reaction and interest of eight farmers from Belgium, France, Netherlands, Denmark, Bulgaria and the Czech Republic were received. Detailed information concerning the hypothetical price and amount of demanded fish is

presented in Tab. 6. Total demand for juvenile pikeperch amounted to 93,000 fish and we only produced 58,000 fish. The farmers offered a purchasing price for one 8 g pikeperch juvenile ranging from EUR 0.6 to 0.8 exclusive of VAT. If we sold all our produced fish for EUR 0.8 per fish, we would earn EUR 45,800 exclusive of VAT (approximately CZK 1,145,000 exclusive of VAT). After calculating all incomes and expenses, the hypothetic profit of this culture would be about CZK 573,041 (profit per 1 fish was CZK 9.9). This profit was achieved with two workers after using of combined culture with four 1ha ponds during 2 months period and the RAS with water volume of 28.1 m^3 for 65–77 days. We can mention that this type of aquaculture is able to get very good economic profit in fish farms at present.

Tab. 6. *List of companies interested in buying 8 g pikeperch juveniles including the demanded amount of fish and price.*

Company, country	Amount of demanded fish [ind.]	Price per fish [EUR]
Eko-Hidro-90, Bulgaria	20,000	0.8
Inagro vzw, Roselare, Belgium	3,000	0.8
Aquapri, Frederiksvaerk, Denmark	3,000	0.8
Van Slooten Aquacultuur, Urk, Holland	20,000	0.75
Asialor farm, Dieuze, France	20,000	0.7
Excellence fish, Horst, Holland	15,000	0.6
Maatschap Lont en Baaren, Hippolytushoef, Holland	6,000	0.6
Švarc-chov ryb na oteplené vodě, Velká Bystřice, CZE	6,000	0.6

4.6.3. Conclusion and recommendations for farmers

The results of the European market analysis showed high demand of RAS farmers for 8 g pikeperch juveniles and their willingness to pay quite a high price for one fish (EUR 0.6–0.8 per fish). According to our findings, the profit of 8 g pikeperch juvenile production could amount approximately to CZK 9.9 per fish if the stated technology was followed.

5. THE ECONOMIC BENEFITS OF THIS TECHNOLOGY

The financial analysis of the entire production cycle of 8 g pikeperch juveniles showed potential profitability of the introduced production method. If our technological process was followed, it would bring profit to farmers amounting approximately to CZK 9.9 per one produced 8 g pikeperch juvenile. It is not a problem to produce hundreds of thousands of pond-reared pikeperch juveniles in average Czech pond fish farm. If the intensive aquaculture will be developed in this farm, production of 8 g pikeperch juveniles might represent tens to hundreds of thousands of fish. This production (100,000–300,000 8 g juveniles) could yield profit of about CZK 990,000–2,970,000.

6. USE OF THE TECHNOLOGY IN THE PRODUCTION FIELD IN THE CZECH REPUBLIC

This verified technology of adaptation of pond-cultured pikeperch juveniles and their following intensive culture in RAS was and will be used especially in the Fish Farm Bohemia Ltd. The mentioned fish farm will produce tens of thousands of juvenile pikeperch due to application of this technology every year. The Fish Farm Bohemia Ltd. will use most of the produced pikeperch juveniles for its production of marketable fish and broodstock. The goal of the fish farm is to produce about 2–5 tons of marketable pikeperch in 2016–2018 and sell a small part of juvenile production to other regional fish farmers.

We suppose that this technological process could increase production of marketable pikeperch in the Czech Republic. It could lead to a better offer of pikeperch in the market as well as increased consumption of fish in the Czech Republic.

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CHAPTER 10

GENERAL DISCUSSION

Pikeperch culture

The history of the systematic pikeperch pond culture dates back to the 18th and 19th century in Central and Eastern Europe and it is linked to carp culture in earthen ponds where pikeperch was the only additional fish. Later, monoculture of pikeperch summer and fall fry were produced for stocking of open waters or RAS in ponds. With development of intensive methods of pikeperch culture, production of pond reared summer fry began to rise. It was found that the pond-reared pikeperch juveniles (TL=30–50mm) could convert from live food to pellet feed very easily if the basic rules were followed (Zakes and Demska-Zakes, 1998; Molnar et al., 2004; Altun et al., 2008; Policar et al., 2013, 2016). The success or failure of this method (combination of pond and RAS culture) strongly depends on several factors such as weather conditions during the pond culture, production capacity of a chosen pond, effectiveness of pond harvesting and, of source, a weaning method which is the key point of the entire procedure. As it was demonstrated in our work (Policar et al., 2016), rearing of pikeperch juveniles with the use of pond and intensive aquaculture is a unique method which combines two completely different environments. Compared to the complete RAS culture, it is possible to get excellent results and produce high quality, well-growing and priced juveniles. Another advantage of this method is the production of fish without body deformations and with fully developed and inflated swim bladders (Policar et al., 2016; Blecha et al., 2015b).

The inability to inflate the swim bladder is one of the main obstacles in intensive pikeperch larviculture which can lead to high mortality, occurrence of body deformations and cannibalism (Demska-Zakes et al., 2003; Szkudlarek and Zakes, 2007; Szczepkowski et al., 2011). The reason for the swim bladder non-inflation is the inability of pikeperch larvae to break the water surface and swallow an air bubble. It is caused by a layer of oil (originating from used feed) on the water surface. Usually, no treatment (Kowalska et al., 2006) or water surface spraying (Szkudlarek and Zakes, 2007) are used in intensive pikeperch larvae culture. In our study (Blecha et al., submitted), we applied several different treatments (fan, spraying, air bubbles) to weaken water surface tension. We found that there was no significant effect for any of the surface treatments on an increased success in the swim bladder inflation, except that the air bubble treatment negatively influenced the swim bladder inflation. The swim bladder inflation ranged between 50 and 61% (spray, fan and control group). In comparison with other studies, it was the same or lower than the authors presented (Kowalska et al., 2006; Szkudlarek and Zakes, 2007) but they assessed the swim bladder inflation success in older fish (18 and 19 dph) which could increase the rate of fish with inflated swim bladder. We know from our experience (Blecha, unpublished data) that increased mortality of fish without an inflated swim bladder occurs after 16 or 17 dph. However, there were significant effects of each water surface treatment on larvae survival. The larvae in the group where the water surface was treated with a fan had more than three times higher survival than in other groups. Our explanation is that the other water surface treatments affected also the movement of the water column in tanks which caused exhaustion of larvae and their death.

With the development of the pikeperch farming, establishment of new farms and increasing demand of customers for marketable pikeperch, a need of continual larvae and/or juvenile production increased. To provide several batches of larvae or juveniles per year, the out-of-season spawning must be applied (Zakes and Szczepkowski, 2004; Ronyai, 2007). In this case, broodstock are held under special photothermal regime to stimulate the oocyte and spermatogonia maturation. In addition to the out-of-season spawning, an alternative method could be used to produce several size categories of juveniles in one production cycle (Blecha

et al., 2016b). We tested and described a method which used a combination of RAS and pond culture which could also supply farmers with pikeperch continually. This method is based on stocking (rearing) of intensively reared pikeperch juveniles in ponds for a cold period of the year and subsequent readaptation and culture of this kind of fish in RAS again. Pikeperch grew slower when cultured under the pond conditions compared to the exclusively reared RAS ones. This feature can be used for production of several different sizes of pikeperch because WT in ponds is usually lower than in RAS and the fish growth rate is highly affected by WT. Therefore, fish of the same age can be available in several different sizes when they are cultured under different water temperatures. As we presented in this study, there were no problems with the backward adaptation of these fish to RAS conditions and pellet feed. It was also found that intensively reared pikeperch displayed higher survival in cold period of the year than the exclusively pond (wild) reared juveniles. This feature could be employed for stocking of intensively reared pikeperch juveniles to open waters.

Reproduction of pikeperch

Everywhere in aquaculture, every culture system, farm production and/or scientific study are strongly dependent on former successful reproduction (artificial, nest spawning, seasonal, out-of-season). Of course, pikeperch is not an exception. Careful and correct pre-spawning stimulation of broodstock is crucial and in case of out-of-season spawning even more. Many authors described how to stimulate pikeperch for the out-of-season spawning, which factors are important to deal with and what dose of hormonal treatment to use to get the best results (Zakes and Szczepkowski, 2004; Ronyai, 2007; Zakes, 2007; Zakes and Demska-Zakes, 2009). In our study (Blecha et al., 2015a), we focused on the effect of varying cold water periods, simulating different lengths of winter time before spawning (out-of-season and seasonal) on quality and quantity of pikeperch spermatozoa. We found that there was no significant effect of duration of the cold water period (WT below 5 °C) on the spermatozoa quality. The only parameter which was highly influenced by the length of the cold water period was the ability of males to produce sperm. Lower number of males hormonally treated and sampled after the longest cold water period released (produced) sperm in this study. Thus a greater number of males is needed for successful spawning after longer cold water period to ensure sufficient amounts of quality sperm.

It is well known that hormonally non-stimulated pikeperch females which are held in captivity usually do not ovulate and, therefore, hormonal treatment is needed (Kucharczyk et al., 2007; Ronyai, 2007). Zakes and Demska-Zakes (2009) presented in their review that in the case of the nest spawning of pikeperch, hormonal treatment is usually performed on females and rarely on males. Also, most Czech farmers do not use hormonal treatment of pikeperch males before the nest spawning. However, in the spring of 2013, we tested whether hormonal treatment of both sexes in the nest spawning might improve the outcome. In the study (Blecha et al., 2016c), we compared pikeperch with both hormonally treated sexes and with treated females only and found a large difference between these two groups. The main variance was found in fertilisation and hatching rates and in spermatozoa quality and quantity parameters. Groups where both sexes were hormonally treated had many improved reproduction aspects and the final production of larvae per one pair of broodstock was more than twice that of the group with only treated females. In contrast to the former practice, we found that hormonal treatment of both sexes in the nest spawning of pikeperch should be used to get better efficiency in the pikeperch nest spawning.

Estimation of the exact ovulation time can be quite difficult in the pikeperch artificial reproduction (Ronyai, 2007) because the latency time can range from few hours to several

days (Kucharczyk et al., 2007). Despite the fact that the ovulation beginning is checked in short time intervals, females often release eggs into the tank without any chance to fertilise them because both sexes are kept separately (Zarski et al., 2012). To avoid or prevent spontaneous ovulation, the urogenital papilla can be sutured. This procedure can prevent the egg loss but it can also lead to egg quality deterioration which is recognised as one of the most important factors affecting the egg quality (McEvoy, 1984; Rime et al., 2004; Policar et al., 2010). In the past, many studies were carried out to investigate the successful *in vivo* egg storage in different fish species (Harvey and Kelley, 1984; Formacion et al., 1993; Legendre et al., 2000; Samarin et al., 2008). The length of the *in vivo* storage is strongly dependent on fish species and water temperature and it varies from minutes (*Morone saxatilis*, Piper et al., 1982) to days (*Salmo trutta caspius*, Bahrekazemi et al., 2010). In our study (Samarin et al., 2015), we focused on investigation of the post ovulatory oocyte ageing effect on pikeperch egg viability, larvae deformations and ploidy anomalies during different *in vivo* incubation. It was found that fertilisation and hatching rates were nearly constant (approximately 80%) between 0–18 hours after ovulation. The embryo mortality and the occurrence of larval malformations were not affected by the oocyte ageing for at least 12 hours post ovulation; however, the rate of naturally induced triploid individuals increased over time.

Egg stickiness (adhesiveness) has been a problem for applying the artificial reproduction with many fish species. For example, common carp have been cultured in ponds for several thousand years. However, the artificial reproduction has been applied since the 1950s when the methods of elimination of egg stickiness were found (Billard et al., 1995). The same situation can be described with fish species such as tench, European catfish (*Silurus glanis*) or different sturgeon species (Horvath, 1980; Linhart et al., 2000; Siddique et al., 2014). The methods used for elimination of egg stickiness are divided into two basic groups: mechanical and enzymatic. The most used enzymatic solution is alcalase which can be described as a product of fermentation of a strain of *Bacillus licheniformis*. Compared to the mechanical methods (milk/talc, clay...), the use of alcalase is much less time consuming because the duration of the alcalase treatment usually takes only 2 minutes (Linhart el al., 2003). In our study (Kristan et al., 2015), we tested the efficiency of the alcalase treatment on the process of egg adhesiveness elimination and the reproduction characteristics such as fertilisation and hatching rates in pikeperch eggs. We found that the alcalase treatment was effective against the adhesiveness of pikeperch eggs and compared to the traditional method (milk and talc solution), the alcalase treatment at the concentration of 1.5–2 $ml.l^{-1}$ provided a shorter application time and higher values of fertilisation and hatching rates.

Ploidy manipulation in pikeperch

Polyploidy induction has played an important role in aquaculture and fish farming for the production of triploid and tetraploid fish (Rougeot et al., 2003). Triploidy can result naturally when overripe eggs are fertilised (Pifferer et al., 2009; Samarin et al., 2015) or artificially induced by using specific shock treatments (Chourrout, 1980; Teskeredzic et al., 1993; Pifferer et al., 2009). There are several reasons for production of triploid fish such as partial or complete sterility, faster growth, higher fillet yield and flesh quality (Flajshans et al., 1993; Cal et al., 2006, Piferrer et al., 2009). Artificially induced triploidy has been induced in many freshwater and marine fish species and shellfish (Pifferer et al., 2009), but there is no information about triploidisation in pikeperch. In our study (Blecha et al., 2016a), we presented data from the very first attempt to induce triploidy in pikeperch by using the heat-shock treatment. We found that the heat shock at a water temperature of 31 °C (shock treatment initiated 1 and 5 minutes post activation for 20 minutes and shock treatment

initiated 1 minute post activation for 40 minutes) induced 100% triploidy in pikeperch. The side effect of the heat shock treatment was low fertilization (0–55.1%) and hatching rates (0–20.0%) of the treated eggs. Further research should investigate optimisation of shock treatment to induce complete triploidy population of pikeperch with higher fertilisation and hatching rates. A possible variant could be the use of alcalase treatment before the heat shock treatment. Alcalase could make the egg membrane thinner and it could help embryos to hatch. Second possibility is the use of a hydrostatic pressure shock as a friendlier tool for retention of a second polar body and triploidy induction.

CONCLUSIONS

This thesis includes eight publications describing several specific methods which can enhance the reproduction and rearing success of pikeperch. These can be used to improve pikeperch culture.

The following conclusions were obtained:

1) The length of the cold water period (122; 149 and 223 days) had no influence on the spermatozoa quality, but it significantly influenced the ability of males to produce sperm in the group of fish tested after the longest cold water period. Therefore, it is suggested that a greater number of males is needed for the artificial reproduction if the cold water period is longer (delayed out-of-season spawning) to ensure sufficient amounts of quality sperm.
2) The hormonal treatment of both sexes in the nest spawning of pikeperch was useful and had significant effects on the final production of larvae and effectiveness of the entire reproduction process.
3) Alcalase enzyme (concentration of 1.5–2 $ml.l^{-1}$) was effective and provided a shorter treatment for eliminating egg adhesiveness in the pikeperch artificial reproduction.
4) Suturing female genital papilla can be used to avoid egg loss from unexpected ovulation during artificial spawning; pikeperch eggs retained their viability and high fertility up to 80% for at least 12 hours after the ovulation.
5) A heat shock of 31 °C (shock treatment initiated 1 and 5 minutes post activation for 20 minutes and shock treatment initiated 1 minute post activation for 40 minutes) was successful for induction of all-triploid pikeperch population.
6) Adaptation of pond-cultured pikeperch juveniles to RAS was verified in large scale production conditions and can be considered as a very effective method to obtain and produce high quality stocking material for further intensive culture.
7) It was found that the intensively reared pikeperch juveniles cultured in a pond before and adapted to RAS conditions and pellet feed were able to adapt to pond conditions, survive in a pond during the cold period of the year for 178 days and had no problems with readaptation to RAS conditions and pellet feed. The combination of RAS-pond-RAS culture could be used as an alternative to the out-of-season spawning to produce several size categories within a natural production cycle and help farmers to supply the market with marketable fish all year round. A high winter survival of intensively cultured fish is also promising for restocking of pikeperch juveniles to fishing grounds.

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ENGLISH SUMMARY

Innovative methods in culture and reproduction of pikeperch (*Sander lucioperca*)

Miroslav Blecha

Hundreds of studies and experiments have been carried out to identify and understand such a stunning fish species as pikeperch. Many scientific teams and researchers have spent countless hours to describe the pikeperch culture, behaviour, biology, reproduction and genetics. It could seem that there is not anything else to investigate in pikeperch because everything has already been said. However, there are still lots of unanswered questions related to this species. This thesis tried to clarify at least a few of them.

The objective of the first study was to compare pikeperch spermatozoa quality and quantity parameters after varying periods of cold water treatment which simulated cold period of the year and led to seasonal or out-of-season sperm production. Three groups of seven pond-cultured pikeperch males were held under controlled conditions of different cold water periods (WT<5 °C) and natural light regime for different time (group A 122 days, group B 149 days, group C 223 days). Males of each group were injected with 500 IU of hCG per kg after each CWP and the final water temperature increasing up to 14–15 °C. The results of this study showed that the length of the cold water period had no influence on the spermatozoa quality, but influenced the ability of males to produce sperm. It is clear now that in case of a longer cold water period, a higher number of pikeperch males is needed to obtain a sufficient amount of quality sperm.

The main aim of the second study was to compare reproductive success and effect of male hormonal treatment on the nest spawning in pikeperch. Fourteen pairs of pikeperch broodstock were divided into two groups (seven pairs in each group). Seven females in Group A and both sexes in Group B were hormonally injected with 500 IU of hCG per kg. One day (24 hours) after the observed spawning, three times three hundred randomly selected eggs were removed from the spawning nests and transferred to 250 mL plastic incubators to assess fertilisation and hatching rates and sperm samples were collected from males (pairs where the spawning took place). It was found that the hormonal treatment of both sexes had a crucial effect on the result of the nest spawning success. The hormonal treatment of males could enhance the spermiation, spermatozoa quality and indirectly fertilization and the hatching rate which altogether contributed to a higher number of produced larvae.

A possible application of alcalase treatment for elimination of egg stickiness in pikeperch was tested in the third study. The eggs were treated with alcalase at the concentration of 0.5, 1.0, 1.5, 2.0 and 5.0 mL per L or a milk/talc solution 2 minutes post egg fertilization. The duration of the exposure was 2 min in alcalase and 60 min in milk/talc solution. Alcalase treatments led to a significantly lower incubation time compared to the traditional milk/talc treatment. The application of alcalase successfully eliminated pikeperch egg stickiness in less time compared to traditional milk/talc methods and could be recommended for a common practical use in the artificial reproduction of pikeperch.

The effect of post-ovulatory oocyte ageing on the egg quality in pikeperch was described in Chapter 5. Ovulated eggs were retained in the ovarian cavity for 0–3, 3–9, 6–12 and 12–18 hours post-ovulation prior to their fertilization. The fertilization, hatching and embryo mortality rates as well as the occurrence of larval malformations and ploidy anomalies were considered as indices for the egg quality during their ageing. The results indicated that the fertilization and hatching rates remained nearly constant, at approximately 80%, for the eggs retained in the ovarian cavity between 0–18 hours after ovulation. Post-ovulatory

oocyte ageing did not affect the embryo mortality or the occurrence of larval malformations at least during the 12-hour *in vivo* storage period. However, the incidence of spontaneous triploidisation in the larvae increased over time from 1.6% at 0–3 HPO to 5% in more aged oocytes at 6–18 HPO. According to these results, it is possible to fertilize the pikeperch *in vivo* storaged eggs till 18 hours post ovulation with high fertilization and hatching rates which can help to synchronization of pikeperch artificial reproduction.

The aim of the fifth study was to find a possible effect of the heat shock treatment on the triploidy induction in pikeperch. To induce triploidy in pikeperch, fertilized eggs incubated at 15 °C were immersed in a water bath at the shock temperatures of 29 °C and 31 °C at the shock initiation times of 1, 3, 5, 7, and 10 min after the egg fertilization for 20 and 40 min. Ploidy levels of hatched larvae were determined by the flow cytometry. Triploidy at the rate of 100% was induced with the use of the shock temperature of 31 °C, at the time of initiation (TI) of 1 min and the duration of the shock treatment for 20 and 40 min and at the TI of 5 min where the shock treatment was applied for 20 min. The most important finding of this study was that the heat shock treatment could produce all-triploid pikeperch population. However, further research is required to determine a method for producing all-triploid pikeperch population with higher hatching rates which were very low here.

The aim of the study, described in Chapter 7, was to determine a water surface treatment that would optimize survival, growth and swim bladder inflation of intensively cultured pikeperch larvae. Three water surface treatments such as fan, diffuser and spraying were tested in four repetitions for the mentioned effects on pikeperch larvae to day 16 post hatching. The diffuser treatment did not enhance the swim bladder inflation nor the growth and provided the worst results in comparison to the other groups. However, the fan system had overall crucial and positive effects on survival (62.1%) of pikeperch larvae compared to other tested water surface treatments (9.4–26.7%).

The objective of the seventh study was to discover whether the intensively reared pikeperch juveniles would have been able to adapt to the pond conditions, survive in a pond during the cold period of the year for 178 days and to readapt to RAS conditions and pellet feed again. The result of this study was that the survival of intensively reared juveniles was significantly higher than that of the exclusively pond-reared juveniles in a control group after the 178-day pond culture. Another result was a nonproblematic readaptation of intensively reared fish to RAS and pellet feed. It could be stated that the combination of RAS-pond-RAS culture could be used as an alternative to the out-of-season spawning to produce several size categories within a natural production cycle and help farmers to supply the market with marketable fish all year round. A high winter survival of intensively cultured fish is also promising for restocking of pikeperch juveniles to fishing grounds.

The sophisticated protocol of adaptation of pond-reared pikeperch summer fry to RAS conditions and pellet feed is described in the last study of the thesis. The aim of this methodology was to describe in detail each step within the mentioned production way from harvesting of the summer fry to production of completely RAS adapted pikeperch juveniles reaching 8 g of body weight.

CZECH SUMMARY

Inovativní metody v chovu a reprodukci candáta obecného (*Sander lucioperca*)

Miroslav Blecha

V minulosti již byly provedeny stovky různých studií a experimentů za účelem poznání a porozumění tak úžasné rybě, jakou candát bezesporu je. Mnoho výzkumných týmů a vědeckých pracovníků strávilo nespočet hodin nad tím, aby popsali chov, reprodukci, biologii, chování a genetiku candáta. Mohlo by se tak zdát, že již bylo vše řečeno a není zde nic, nad čím by se ještě dalo bádat a přemýšlet. Opak je ovšem pravdou. Stále je zde spousta otázek, na které neznáme odpovědi a tato dizertační práce se pokusila odpovědět alespoň na některé z nich.

Cílem první studie bylo porovnat kvalitu a kvantitu produkovaných spermií candáta v návaznosti na délku chladového období, které předcházelo odběru spermatu a mělo za úkol simulovat chladová období (teplota vody < 5 °C) před sezonním a mimosezonním výtěrem. Tři skupiny obsahující vždy po sedmi mlíčácích byly drženy v kontrolovaných podmínkách, za přirozeného světelného režimu, ale po různě dlouhé chladové období (skupina A 122 dnů, skupina B 149 dnů, skupina C 223 dnů). Všichni mlíčáci byli po uplynutí daného chladového období a období zvyšování teploty vody na 14–15 °C injikováni 500 IU hCG na kilogram a po uplynutí 72 hodin latence jim byly odebrány vzorky spermatu pro následnou analýzu a porovnání. Zjistilo se, že délka chladového období neměla vliv na kvalitu produkovaného spermatu, ale zásadně ovlivnila schopnost mlíčáků produkovat sperma. Ukázalo se, že nejdelší chladové období negativně ovlivnilo schopnost mlíčáků produkovat sperma. Z toho vyplývá, že pro zajištění dostatečného množství kvalitního spermatu od mlíčáků vystavených delšímu chladovému období je k výtěru nutné použít větší množství generačních ryb.

Cílem druhé studie bylo porovnat, výsledek a efektivitu hormonální stimulace mlíčáků při poloumělém výtěru candáta obecného. Celkem čtrnáct párů generačních ryb bylo rozděleno do dvou skupin po sedmi. Každý jednotlivý pár byl umístěn do samostatné nádrže, kde bylo na dně umělé výtěrové hnízdo. Ve skupině A byly hormonem injikovány pouze jikernačky a ve skupině B obě pohlaví. K hormonální stimulaci byl použit hormon hCG v dávce 500 IU na kilogram hmotnosti jikernačky. Jeden den (24 hodin) po pozorovaném výtěru byly z každého výtěrového hnízda odebrány 3 vzorky po 300 ks jiker, které byly umístěny do 250 ml inkubačních misek, kde byla stanovena oplozenost jiker a líhnivost larev. V ten samý moment (24 hodin po výtěru) byl od mlíčáků, pocházejících z nádrží, kde proběhl výtěr, odebrán vzorek spermatu pro následnou analýzu. Tímto experimentem bylo zjištěno, že hormonální stimulace obou pohlaví má zásadní vliv na výsledek poloumělého výtěru candáta a hormonální stimulace mlíčáků může napomoci ke zvýšené produkci spermatu a jeho vyšší kvalitě a nepřímo tak ovlivnit oplozenost a líhnivost larev, což zaručí větší množství produkovaných larev.

Ve třetí studii byla sledována možnost využití enzymu alkalázy k odstranění lepivosti u jiker candáta. K testování účinnosti enzymu alkalázy byla použita koncentrace 0,5; 1,0; 1,5; 2,0 a 5,0 ml alkalázy naředěné v jednom litru vody. Jako kontrola byla použita tradiční metoda odlepkování jiker ve směsi plnotučného mléka a talku. Odlepkovací procedura byla zahájena 2 minuty po aktivaci jiker a spermií a trvala 2 minuty v případě alkalázy a 60 minut v případě směsi mléka a talku. Odlepkování jiker alkalázou vedlo ke zkrácení inkubační doby a zvýšení líhnivosti larev v porovnání s použitím směsi mléka a talku. Roztok enzymu alkalázy je schopen bez problémů odlepkovat jikry candáta, navíc v mnohem kratším časovém horizontu než tradiční roztok mléka a talku, a je ho tedy možné doporučit pro praktické využití při umělé reprodukci candáta obecného.

V kapitole 5 této práce jsou uvedeny výsledky studie zabývající se vlivem přezrávání ovulovaných oocytů na kvalitu jiker candáta. Ovulované oocyty byly drženy v tělní dutině

jikernaček po dobu 0–3, 3–9, 6–12 a 12–18 hodin po ovulaci a následně uměle vytřeny a osemeněny. Kvalita jiker byla hodnocena na základě oplozenosti jiker, líhnivosti larev, výskytu deformit a mortality embryí a ploidních anomálií u čerstvě vykulených larev. Výsledky této studie ukázaly na fakt, že jikry candáta si udržovaly vysokou míru oplozenosti (přibližně na hodnotě 80 %) a líhnivosti až do doby 18 hodin po ovulaci. Přezrávání jiker nemělo taktéž vliv na mortalitu a výskyt deformit u embryí (do 12 hodin po ovulaci), ale ovlivnilo množství přirozeně indukovaných triploidních jedinců, kdy s prodlužující se dobou od ovulace se zvyšoval podíl triploidních jedinců ve vzorku (1,6 % v 0–3 hodiny po ovulaci, 5 % v 6–18 hodin po ovulaci). Na základě těchto výsledků tedy lze konstatovat, že je možné bez obav použít k reprodukci jikry candáta až do doby 18 hodin po ovulaci, což může zásadně přispět k synchronizaci umělé reprodukce candáta obecného.

Cílem páté studie bylo sledovat vliv vystavení jiker candáta teplotnímu šoku na indukci triploidie, která byla u jiker vytřených při teplotě vody 15 °C indukována šokem o teplotě 29 °C, respektive 31 °C. Jednotlivé šoky byly zahájeny 1, 3, 5, 7 a 10 minut po aktivaci pohlavních buněk a trvaly 20, respektive 40 minut. Bylo zjištěno, že teplotní šoky o teplotě 31 °C zahájené 1 minutu po aktivaci gamet a trvající 20 nebo 40 minut a šok začínající 5 minut po aktivaci a trvající 20 minut vedou k produkci kompletně triploidní populace candáta. Za nejdůležitější zjištění této studie lze považovat to, že teplotním šokem je možné získat kompletně triploidní populaci candáta. Je ale nutné provést další studie ke zvýšení procenta líhnivosti jiker, která byla v tomto případě velmi nízká.

V kapitole 7 je popsána studie zabývající se ovlivněním povrchového napětí vodní blanky v odchovné nádrži na přežití, růst a naplnění plynového měchýře u larev candáta. K tomuto účelu byla hladina vody v nádržích ofukována ventilátorem, ovlivněna pohybem a reakcí vzduchových bublin a zkrápěna několika desítkami jemných proudů vody. Celý pokus probíhal od 5. do 16. dne života larev v plně kontrolovaných podmínkách chovu. V porovnání s ostatními skupinami nemělo použití vzduchových bublin, jako prvku ovlivňujícího povrchové napětí vodní hladiny v nádrži, žádný vliv na úspěšnost naplnění plynového měchýře ani na růst larev, a navíc působilo velmi negativně na přežití chovaných jedinců. Oproti tomu v nádržích s ventilátorem (jakožto nástrojem ovlivňujícím povrchové napětí vodní hladiny) bylo zaznamenáno několikanásobně vyšší přežití (62,1 %) odchovávaných larev oproti ostatním skupinám (9,4–26,7 %).

Cílem další studie bylo zjistit, zda jsou juvenilní jedinci candáta, kteří jsou kompletně adaptováni na podmínky recirkulačních akvakulturních systémů (RAS), schopni adaptovat se na rybniční podmínky, přežít zimní období (178 dnů) a následně se opět přizpůsobit podmínkám a životu v RAS, včetně příjmu granulovaného krmiva. Výsledkem tohoto pozorování bylo, že intenzivně chovaní jedinci candáta neměli žádný problém s adaptací na rybniční podmínky a na konci rybničního odchovu u nich dokonce bylo zaznamenáno vyšší přežití než u kontrolních rybničně odchovaných jedinců. Následná zpětná adaptace těchto ryb (intenzivně chovaných ryb) na RAS byla opět bez nejmenších problémů. Dalo by se tedy říci, že je možné využít kombinace těchto dvou chovů jako alternativní metody doplňující mimosezonní výtěr zajišťující produkci několika velikostních kategorií candáta ročně. Vzhledem k vysokému přežití intenzivně chovaných juvenilních ryb v zimním období by se jich také dalo využít jako vhodného násadového materiálu pro rybářské revíry.

V kapitole 9 této práce je uvedena metodika popisující adaptaci rychleného plůdku candáta na podmínky intenzivní akvakultury. Cílem této práce bylo detailně popsat celý postup od výlovu rychleného plůdku až po produkci juvenilních ryb o velikosti 8 g s plnou adaptací na podmínky v RAS a příjem granulované krmné směsi.

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TRAINING AND SUPERVISION PLAN DURING STUDY

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Biostatistic	2013
Ichthyology	2014
Pond aquaculture	2014
Fish genetics	2014
English language	2015

Scientific seminars	**Year**
Seminar days of RIFCH and FFPW	2013 2014 2015 2016

International conferences	**Year**
Blecha, M., Mohagheghi, A.S., Kristan, J., Policar, T., 2013. Semi–artificial spawning of pikeperch (*Sander lucioperca*): Why is important to do the hormonal treatment of both sexes. Aquaculture Europe 2013, 9–12 August 2013, Trondheim, Norway (Oral presentation).	2013
Blecha, M., Kristan, J., Mohagheghi, A.S., Rodina, M., Policar, T., 2013. Comparison of the quality of pikeperch (*Sander lucioperca*) sperm in different spawning seasons. Diversification in Inland Finfish Aquaculture II., 24–26 September 2013, Vodnany, Czech Republic (Oral presentation).	2013
Blecha, M., Kristan, J., Policar, T., 2014. Adaptation of intensively reared pikeperch (*Sander lucioperca* (Linnaeus)) juveniles to pond culture and subsequent re–adaptation to a recirculation aquaculture system. Aquaculture Europe 2014, 14–17 October, Donostia San Sebastian, Spain (Oral presentation).	2014
Blecha, M., Svacina, P., Kristan, J., Lebeda, I., Flajshans, M., Policar, T., 2015. The first results of heat shock triploidisation in pikeperch (*Sander lucioperca*). 5th International Workshop on the Biology of Fish Gametes, 7–11 September 2015, Ancona, Italy (Poster presentation).	2015

Foreign stays during Ph.D. study at RIFCH and FFPW	**Year**
Jurgen Adriaen – College University KaHo Sint–Lieven, Hospitaalstraat 23, 9100 Sint–Niklaas; and Stefan Teerlinck – Maatschappij en leefmilieu aquacultuur Ieperseweg 87, 8800 Rumbeke–Beitem, Belgium (1 month)	2012
Daniel Zarski, Ph.D. – University of Warmia and Mazury, M. Oczapowskiego 5, 10–719 Olsztyn, Poland (5 weeks)	2013
Prof. Pascal Fontaine – University of Lorraine, BP 70239 54506 Vandoeuvre les Nancy Cedex, Nancy, France (1 month)	2014

CURRICULUM VITAE

PERSONAL INFORMATION

Surname: Blecha
First name: Miroslav
Title: M.Sc.
Born: 18th August, 1988
Nationality: Czech
Martial Status: Single

EDUCATION

2007–2010 B.Sc. – University of South Bohemia, Faculty of Fisheries and Protection of Waters, Ceske Budejovice, specialization fishery.

2010–2012 M.Sc. – University of South Bohemia, Faculty of Fisheries and Protection of Waters, České Budějovice, specialization Fishery.

PROFESSIONAL EXPERIENCE:

2012–present: Ph.D. student at the University of South Bohemia in České Budějovice (USB), Faculty of Fisheries and Protection of Waters (FFPW, www.frov.jcu.cz), Research Institute of Fish Culture and Hydrobiology (RIFCH), South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses (CENAKVA), Laboratory of Intensive Aquaculture, Vodňany, Czech Republic.

Ph.D. courses: Biostatistic, Fish genetics, Basics of Scientific Comunication, Pond aquaculture, Ichthyology, English language.
Specialization: Reproduction and culture of pikeperch
Knowledge of languages: Czech, English

FOREIGN STAYS DURING PH.D. STUDY AT FFPW:

M.Sc. Jurgen Adriaen – College University KaHo Sint-Lieven, Hospitaalstraat 23, 9100 Sint-Niklaas; and Stefan Teerlinck – Maatschappij en leefmilieu aquacultuur Ieperseweg 87, 8800 Rumbeke-Beitem, Belgium (1 month)

Daniel Zarski, Ph.D. – University of Warmia and Mazury, M. Oczapowskiego5, 10–719 Olsztyn, Poland (5 weeks)

Prof. Pascal Fontaine – University of Lorraine, BP 70239 54506 Vandoeuvre les Nancy Cedex, Nancy, France (1 month)