Fakulta rybářství
a ochrany vod
Faculty of Fisheries
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Jihočeská univerzita
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Optimization of reproduction and gamete quality in percid fish

Optimalizace reprodukce a kvality gamet u okounovitých ryb

Jiří Křišťan

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CONTENT

CHAPTER 1

GENERAL INTRODUCTION

1.1. INTRODUCTION

Pikeperch (*Sander lucioperca*) and Eurasian perch (*Perca fluviatilis*) are commercially valuable species in European aquaculture (Kestemont and Mélard, 2000; Policar et al., 2008a; Fontaine, 2009). Both species have appetizing meat without "Y" bones (Dil, 2008; Watson, 2008), an acceptable growth rate to market size when reared intensively (Hilge and Steffens, 1996; Wang et al., 2009), and are favoured for recreational fishing (Bninska and Wolos, 2001; Brožová, 2005; Ženíšková and Gall, 2011). The species have been bred extensively since the 16th century in the Czech Republic in ponds where they are commonly a constituent of poly-culture fish stocks (Adámek et al., 2010, 2012). Pikeperch and Eurasian perch prey upon small omnivorous and benthivorous fishes (Lappalainen et al., 2003), thus reducing their populations and increasing food availabe to carp, keeping carp production in ponds stable (Mittelbach and Person, 1998; Adámek et al., 2010).

Currently, most market-size pikeperch come from natural waters (lakes, rivers, ponds or lagoons) with relatively few being produced under intensive or indoor conditions (Kucharczyck et al., 2007). In Europe, 9 078 tonnes of pikeperch and 22 906 tonnes of perch are produced annually in natural waters, especially lakes (Fig. 1) with 499 and 182 tonnes, respectively, from aquaculture (FAO, 2012a). Unfortunately, the current harvest of pikeperch from lakes is decreasing dramatically (Ruuhijärvi, 1995). During the past 20 years, the pikeperch harvested from open waters has decreased by more than 5 000 tonnes (Fig. 1). The production of perch is comparatively stable.

It is expected that in coming years the market will be increasingly supplied with perch and pikeperch through intensive aquaculture (Kestemont and Dabrowski, 1996; Martins et al., 2010; Zhang et al., 2011). Therefore, new technologies and methods for the production and reproduction of percids under controlled conditions of intensive aquaculture are being investigated.

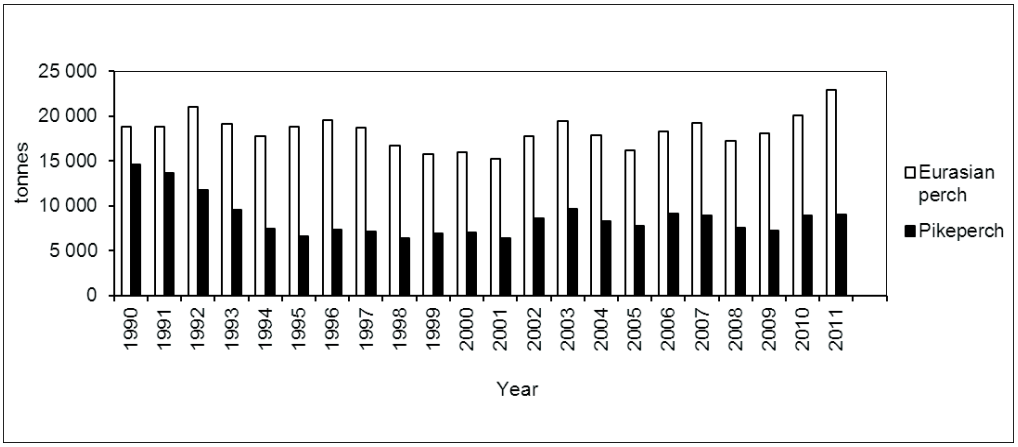

Figure 1. *The capture-production in Eurasian perch and pikeperch during the past twenty years (FAO, 2012b).*

This thesis presents results from a broad array of investigations into varying

aspects of broodstock management with respect to reproduction: anaesthetics, hormone induction, elimination of egg stickiness, and the ultrastructure and quality of sperm.

1.2. PERCID BROODSTOCK MANAGEMENT SYSTEMS

Broodstock gamete management is a key factor in achieving optimal production of larvae on fish farms (Billard et al., 1995). Currently, two main methods of broodstock management are used in percid aquaculture. The first is traditional production based on pond rearing for all age categories. The reproduction of pond-cultured broodstock is primarily conducted under controlled conditions in hatcheries (Stejskal et al., 2010; Policar et al., 2011). An alternative is the rearing of broodstock in recirculating aquaculture systems with the maturation cycles taking place indoors (Migaud, 2006; Philipsen, 2008). Each of these methods has advantages and disadvantages. Many studies have found broodstock nutrition to have an effect on the quality of reproduction of farmed fish (Henrotte et al., 2010). Comparison of reproductive characteristics of farmed and wild broodstock of Eurasian perch is presented in Chapter 2.

1.3. ANAESTHESIA OF PERCIDS

Anaesthesia is routinely used in aquaculture to immobilize animals for transport, handling, sorting, and tagging, and for procedures related to artificial reproduction (Cooke et al., 2004; King et al., 2005; Velíšek et al., 2009, 2011). In general, wild percid broodstock do not tolerate vigorous manipulation, and minimal careful handling during the spawning period is recommended. It is therefore sometimes necessary to use an anaesthetic to reduce stress during handling (Policar et al., 2008a).

Currently, the most commonly used anaesthetics in percid aquaculture are tricaine methane sulphonate (MS222) (Zarski et al., 2012), 2-phenoxyethanol (Demska-Zakes et al., 2005; King et al., 2005), clove oil (Hamackova et al., 2001; Policar et al., 2008a), and Propiscin (Kazun and Siwicki, 2001; Zakes and Demska-Zakes, 2005).

The effect of anaesthetics on blood biochemical profiles and stress responses have been published for perch (*Perca fluviatilis*) (Hamackova et al., 2001, Velíšek et al., 2009) and black sea bass (*Centropristis striata*) (King et al., 2005). However, no data on blood biochemical and haematological profiles in pikeperch (*Sander lucioperca*) anaesthetized with Propiscin, 2-phenoxyethanol, clove oil, or MS222 are available. The first reports of haematological and biochemical profiles in plasma of pikeperch anaesthetized with Propiscin, 2-phenoxyethanol, clove oil, and MS222 are given in Chapter 3.

1.4. ARTIFICIAL REPRODUCTION OF PERCIDS

In percids, as in most commercially important farmed finfish, hormone stimulation is frequently applied to synchronize ovulation of females and/or stimulate release of semen in males. Ovulation in pikeperch and Eurasian perch can be obtained without artificial hormone stimulation (Demska-Zakes and Zakes, 2002; Műller-Bellecke and

Zienert, 2008; Ronyai and Lengyel, 2010). However, the application of hormone treatments significantly affects ovulation rate and synchronization of ovulation in both species (Kucharczyk et al., 2007).

Recently, artificially controlled spawning of perch, particularly with induced ovulation, has been investigated (Dabrowski et al., 1994; Kouril et al., 1997; Kucharczyk et al., 1998; Policar et al., 2008b), and an out-of-season spawning protocol is now available (Migaud et al., 2002, 2004, 2006; Fontaine et al., 2006). Many current studies address the synchronization of ovulation in perch and pikeperch (Zarski et al., 2011a,b, 2012). However, studies of methods of artificial reproduction, with emphasis on broodstock reproductive physiology, to define those optimal for achievement of stable mass production of pikeperch larvae and fry are still needed. Chapter 4 compares artificial and semi-artificial methods of perch reproduction and evaluates efficacy of each method.

1.4.1. Synchronization of ovulation in pikeperch

In principle, especially for pikeperch, it is possible to apply procedures similar to those used in other fish species based on injection of carp gonadotropin (Ronyai, 2007), synthetic analogues of GnRH, human chorionic gonadotropin (Kucharczyk et al., 2008), or LHRH-a (Schlumberger and Proteau, 1996). In artificial reproduction of pikeperch, hormone-induced spawning usually necessitates accurate determination of sexual maturity of females (Zarski et al., 2012). Comparison of artificial reproduction using human chorionic gonadotropin (hCG) and mammalian GnRH analogues is presented in Chapter 5.

1.4.2. Sperm quality, morphology, and fertilization success in pikeperch

The basic factor determining fertilization success in hatchery production is the availability of good quality gametes (Bromage et al., 1995). Under controlled conditions, sperm volume, density, and percent motility are the main parameters considered in semen quality assessment (Cosson, 2008a,b; Alavi et al., 2008). Spermatozoa velocity, ATP content, and morphological parameters determine quality of spermatozoa (Alavi and Cosson, 2006). Several factors affect sperm motility, including temperature, pH, osmolality, and ion content, as well dilution ratio of sperm in immobilization medium or activation medium (Billard, 1995; Alavi and Cosson, 2005, 2006). Chapter 6 presents results of investigations of sperm morphology and optimal activation media for fertilization in pikeperch.

1.4.3. Elimination of egg stickiness in pikeperch

For freshwater fishes (e.g. tench, carp, catfish, ide, pikeperch), the effectiveness of artificial incubation is measured by the number of larvae obtained. The chorion which can be found on the eggs of most fish species makes it possible for eggs to adhere to different types of substrates (Riehl and Patzner, 1998). Success of artificial incubation is influenced by the method used for the elimination of this egg stickiness.

In aquaculture, many methods exist for removing the adhesiveness of fish eggs, with traditional procedures having been developed many years ago (Sakowicz, 1928; Woynarovich and Woynarovich, 1980; Khan et al., 1986; Proteau et al., 1994). The most commonly used methods are mechanical separation of individual eggs, physical scouring with clean water or abrasives (clay, starch, charcoal, or bentonite), chemical treatment with urea in a salt solution, tannic acid solutions, or enzymes (Woynarovich and Woynarovich, 1980, Khan et al., 1986, Demska-Zakes et al., 2005).

In recent years the use of the enzyme Alcalase for egg de-sticking has become more popular. Trials on tench by Linhart et al. (2000) and later on carp and catfish (Linhart et al., 2003a,b, 2004) have been undertaken. However, de-sticking of pikeperch eggs has not been studied in detail, and only a few reports have been published (Schlumpberger and Schmidt, 1980; Demska-Zakes et al., 2005). Chapter 7 provides new data on technologies for eliminating egg stickiness in pikeperch.

1.5. AIMS AND OBJECTIVES

The overall goal of this thesis was to identify and define factors potentially improving reproduction and culture of percid fish. Major objectives were to:

1) compare and evaluate broodstock reproductive parameters and mortalities in farmed and wild Eurasian perch,

2) investigate the application of clove oil, Propiscin, and 2-phenoxyethanol as alternative anaesthetics to tricaine methane sulphonate (MS222) in pikeperch,

3) compare and evaluate semi-artificial and artificial reproduction of Eurasian perch,

4) investigate two hormone preparations for induction of ovulation in pikeperch,

5) investigate the activation of sperm motility in pikeperch,

6) improve the elimination of eggs adhesiveness in pikeperch.

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CHAPTER 2

COMPARISON OF REPRODUCTION CHARACTERISTICS AND BROODSTOCK MORTALITY IN FARMED AND WILD EURASIAN PERCH (*PERCA FLUVIATILIS* L.) FEMALES DURING SPAWNING SEASON UNDER CONTROLLED CONDITIONS

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Comparison of Reproduction Characteristics and Broodstock Mortality in Farmed and Wild Eurasian Perch (*Perca fluviatilis* L.) Females During Spawning Season Under Controlled Conditions

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Abstract

Reproduction characteristics and broodstock mortality were compared in farmed perch (FP) and wild perch (WP) under controlled conditions. Thirty-six farmed females (FF) (92.3%) and 30 wild females (WF) (76.9%) spawned during the reproductive season. Absolute fecundity in FF was 18 660±6 809 eggs per female compared to 31,081±3,276 eggs per female in WF. Similar differences were observed in relative fecundity (FF 112,470±13,370 eggs per kg body weight and WF 137,054±18,513 eggs per kg body weight). No significant differences in fertilization rate (FR), larval resistance (LR) and larval total length (LTL) were found (FR - 91.9±6.9% and 90.5±6%; LR - 51.1±25.2% and 53.5±26.2%; LTL - 5.88±0.55 mm and 5.82±0.51 mm in FP and WP, respectively). The highest differences were found in hatching rates of 27.9±9.3% in FP and 62.0±16.0% in WP. No mortalities were observed during the reproductive season, but post-spawning mortality among WP was 92.5±2.5% of females compared with 21.5±12.5% in FP.

Keywords: Wild perch, farmed perch, fecundity, fertilization rate, hatching rate, mortality.

Introduction

The Eurasian perch (*Perca fluviatilis* L.) is a freshwater fish species highly valued in Europe (Fontaine, 2009). At present, the largest perch producing countries in Europe, where wild perch are mainly caught from local lakes (Öberg, 2008), are Finland, Russia, and Estonia (Watson, 2008). Unfortunately, the perch fisheries are unstable in both quality and quantity of fish produced (Fontaine, 2004) and is in drastic decline due to the decrease of wild stocks and overfishing (Öberg, 2008, FAO, 2009). Consequently, intensive perch culture has been developed in the past two decades, and perch has become an important species in European aquaculture, especially in Ireland, Switzerland, France, Denmark, and Sweden (Fontaine, 2004; Watson, 2008).

The perch market has a local character, but, in spite of this, the current production of market-size perch in Europe is unable to satisfy demand, which is mainly centred in the Alpine region (Watson, 2008; Setälä *et al*., 2008).

Successful control of reproduction and mass reproduction of perch broodstock are basic prerequisites for profitable intensive perch production (Fontaine *et al*., 2008). Current reproduction of perch relies mainly on wild broodstock from ponds and other natural waters, captured immediately prior to spawning (Kucharczyk *et al*., 1998; Kouril and Hamackova, 1999; Policar *et al*., 2008a). However, an optimal protocol for the induction of reproduction in farmed broodstock, including out-of-season spawning, has been developed under controlled conditions (Migaud *et al*., 2002, 2004, 2006; Fontaine *et al*., 2008).

Many studies have found broodstock nutrition to have an effect on the quality of reproduction of farmed fish, including chemical composition of eggs, fertilization and hatching rates, and larval survival (Bell *et al*., 1997; Izquierdo *et al*., 2001; Henrotte *et al*., 2008; Wang *et al*., 2009; Henrotte *et al*., 2010). An effect of n-3 highly unsaturated fatty acids (HUFAs), especially eicosapentaenoic acid (20:5 n-3), docosahexaenoic acid (22:6 n-3) and arachidonic acid (20:4 n-6), on the reproduction of perch in captivity has been reported (Henrotte *et al*., 2010).

Maintaining perch broodstock in captivity requires specialized artificial rearing conditions (light and temperature regimes) and the creation of special artificial diets (Henrotte *et al*., 2010), which require high initial investment and operating costs to provide continuous production of eggs year-round (Fontaine *et al*., 2008; Toner and Schram, 2008). On the other

hand, wild and farmed perch broodstock partially fed on forage fish can be an annual source of eggs and larvae for later use in intensive culture. With annual spawning, requirements for investment and operating cost are much lower (Policar *et al*., 2008a; Kestemont *et al*., 2008a, Stejskal *et al*., 2009).

This study compared the gonadosomatic index, individual reproduction characteristics (female fecundity, spawning rate in female, fertilization rate, hatching rate, larval resistance to the osmotic shock and size of larvae), and post spawning mortality in wild and farmed perch broodstock fed on a combination of commercial salmon food and forage fish during the annual spawning season.

Materials and Methods

Farmed Perch Broodstock management

This study used both farmed and wild populations. Farmed perch broodstock was obtained from a study by Stejskal *et al*. (2009). Perch were reared under controlled conditions in a recirculating aquaculture system (RAS) at the University of South Bohemia, Faculty of Fisheries and Protection of Waters (USB FFPW). In total, 102 three-year-old perch (total length TL = 220.0±20.9 mm and weight W = 146.5±43.9 g) were kept under a natural photoperiod and water temperature regime, in flow-through systems supplied with water from the River Blanice, at USB FFPW from September 2008 to February 2009. The water temperature and light regime is summarized in Table 1.

Thirty-four fish were held in each of three 480 l fibreglass tanks (1000 x 800 x 600 mm) and fed daily on a mix of commercial salmon diet (Stejskal al. 2009) and forage fish according to Wang *et al*. (2009). Forage cyprinids *Pseudorasbora parva* (TL = 40-60 mm; W = 0.3-0.4 g), reared at 20°C; with a 16L:8D photoperiod and fed on a dry salmon diet(Stejskal al. 2009), were fed three days a week to perch at a rate of two forage fish each. The dry salmon diet was used on the other four days at a rate of 1% of perch total biomass per tank. The dry diet was distributed by automatic beltfeeders during daylight hours. The ration was adjusted daily according to the quantity of uneaten feed removed from the tanks.

At the beginning of March 2009, all fish were moved to a controlled environment and placed in three identical tanks in the RAS at USB FFPW. The groups of fish were not mixed and the same feeding regime was maintained. The water temperature was raised by 0.3°C increments per day from 1 March (5.5°C) to 1 April (14.5°C) and kept constant thereafter. A constant light regime (11.5L: 12.5D) was used during this phase of the study.

Wild Perch Broodstock Management

The second group of perch comprised wild perch reared for three years in three experimental ponds (each 0.06 ha). The first two years, culture conditions were similar to studies of Policar *et al*. (2009) and Kestemont *et al*. (2008a). In April 2008, a mixed culture of perch and forage cyprinids *Pseudorasbora parva*, at a biomass rate of 1:5, was stocked in the experimental ponds and held for the winter season of 2008-2009 until March 2009. No additional feeding or manipulation of temperature or lighting was done. All ponds were harvested on 1 March 2009. A total of 102 of the three-year-old wild perch (TL = 174.1±21.9 mm, W = 75.5±31.3 g) were moved to the controlled environment as described for the cultivated fish and placed randomly in three similar tanks in the RAS at USB FFPW. Thirty-four fish with a sex ratio of 1:1 were held in each tank. Perch were fed with *Pseudorasbora parva* obtained from harvested ponds at a rate of two per perch.day^{-1}. The temperature and light regime was the same as for the cultivated perch. Table 1 summarizes average temperature and light regimes for the grow-out season through March 2009.

Morpho-Anatomical Parameters in Perch Broodstocks

On 8 April (7 days after the temperature in the tanks reached its final value of 14.5°C), 8 males and 8 females from each tank (24 females and males from each group) were killed. Total length and standard length (TL, SL±1 mm) was measured and weight (W±0.1 g) of all individuals was recorded. Gonads, liver, and viscera including mesenteric fat were removed and weighed (± 0.01 g). The gonadosomatic (GSI), hepatosomatic (HSI), and viscerosomatic (VSI) indices (%) were calculated as follows:

GSI = (GW/BW) x 100

HSI = (LW/BW) x 100

Table 1 Average water temperature and light regime during out-growing period until spawning period in farmed and wild perch broodstock

Group	Factor	Sep. 08	Oct. 08	Nov. 08	Dec. 08	Jan. 09	Feb. 09	Mar 09	Apr.09
Farmed	Temperature (°C)	14.2±0.4	8.4±0.5	6.2±0.6	3.0.±0.4	2.6±0.3	2.5±0.4	10.0±2.7	14.5±0.3
	Light (h)	12.5±0.5	10.5±0.3	9.3±0.3	8.2±0.3	8.3±0.3	9.3±0.3	11.5±0.0	11.5±0.0
Wild	Temperature (°C)	13.9±1.2	8.0±2.2	5.9±1.9	2.5±1.3	1.8±1.4	1.6±1.1	10.0±2.7	14.5±0.3
	Light (h)	12.5±0.5	10.5±0.3	9.3±0.3	8.2±0.3	8.3±0.3	9.3±0.3	11.5±0.0	11.5±0.0

Data are shown as mean ± S.D

VSI = (VW/BW) x 100

Where GW is the weight of gonads (g), LW is the weight of the liver (g), VW is the weight of the viscera (g) and BW is the body weight (g).

Conditions for the Reproductive Season

The 26 remaining perch broodstock: 13 males and 13 females from each tank (78 fish, 39 males and 39 females from each group) were used for comparison of reproductive activity and reproductive characteristics. All remaining perch were measured and weighed (TL and standard length SL±1 mm and W±1 g) on 8 April. Females and males were identified based on characteristics of the dissected perch (mainly according shape of the abdomen). All females were marked with PIT tags and their biometric data and PIT number were recorded. The broodstock was fed on *Pseudorasbora parva* at a rate of two fish (TL = 40-60 mm) per perch.day^{-1}. The fish were kept at a constant water temperature and light regime (Table 1). Eight dry branches of *Sambucus nigra* (length = 800 mm) were placed in each tank to provide a spawning substrate according to Policar *et al*. (2008a). The presence of this spawning substrate stimulated reproduction. No hormonal or other stimulation of perch broodstock was used to induce reproductive activity.

Female Reproductive Activity

The reproductive season was divided into three periods:
1- Early (spawning from 8 April to 15 April),
2- Middle (spawning from 16 April to 23 April), and
3- Late (spawning from 24 April to 1 May).

When the first female began laying egg ribbons, the reproductive activity of both perch broodstock groups was checked at regular 6 h intervals, and egg ribbons were removed from the tanks. The percent of spawned females in each period was recorded and compared between groups, and the total number of spawned females was recorded.

Number of Eggs in Egg Ribbon and Fecundity

After removal, the egg ribbon was associated with a spawned female (empty abdomen) which was caught and removed from the tank. The PIT number was identified with a scanner (I MAX plus, Virbac France SAS). The number of eggs in 1 ml of egg ribbon was counted by the volume method established by Kouril and Hamackova (1999). Four samples of similar size were taken from different parts of each egg ribbon (volume of each sample was approximately 1 ml). The exact volume of the sample was measured with a graduated cylinder±0.05 ml (EV). The number of eggs was determined (EN) and the number of eggs per 1 ml (TEN) egg ribbon was calculated as follows: TEN = EV x EN. The remaining egg volume from each female was measured in a graduated cylinder±0.1 ml (RVS). This value was used to calculate absolute fecundity of each female (AF) using the formula: AF = RVS x TEN. Relative fecundity RF (number of eggs. kg^{-1} of body weight) of each female was calculated according the formula: RF = AF/ BW, where BW is body weight (in kg).

Fertilization and Hatching Rate

The four samples with the exact number of eggs of each spawning female recorded were used to assess the fertilization and hatching rate of that female. Egg samples were incubated in two tanks (2600 x 400 x 350 mm) integrated into a recirculation system (total water volume 700 l). Each sample of eggs was separately incubated in a small cage (150 x 100 x 100 mm) for aquarium fish. The water temperature (14.5±0.3°C) and water flow (0.5 l min^{-1}) in the tanks were kept constant. Twenty-four hours after stocking of eggs into the cages fertilization rate was determined as the percent fertilized eggs of the total number of eggs in each sample. After all larvae in the sample had hatched the hatching rate was determined as the percent of hatched larvae in the stocked eggs. Fertilization and hatching rates were evaluated and compared among the three periods of the reproductive season and between females of the groups.

Larval Total Length and Resistance to Osmotic Stress

Fifty newly hatched larvae from each female were measured under a stereomicroscope Olympus SZ 40 (Olympus, Japan) with a slide micrometer scale to the nearest 0.1 mm.

The resistance of newly hatched larvae to osmotic stress was determined through exposure to a solution of 2% saline, made from hatchery water at 14.5±0.3°C (Migaud *et al*., 2001). Thirty-three 24 h post-hatched larvae from each female were placed in 1 l of the saline solution. The trial was performed in triplicate (99 larvae from each female). The survival rate of larvae was determined after 120 min (Migaud *et al*., 2001).

The larval total length and resistance to osmotic stress was evaluated and compared among the three periods of the reproductive season and between wild and farmed fish.

Broodstock Mortality

The mortality of the perch broodstock (males and females) was observed and compared in both groups during the reproductive season and the following 14 days (to 15 May). At the conclusion of the spawning season (2 May) all broodstock were

transferred to the six tanks of the flow-through system that were used for the culture of farmed perch broodstock during the winter period. The water was from the Blanice River (14.0±0.5°C). *Pseudorasbora parva* (TL = 40-60 mm) were used as feed for all broodstock at a daily rate of two per perch. Mortality during the reproductive season and the following 14 days was compared between groups.

Monitoring of Water Temperature and Quality

The water temperature and the concentration of dissolved oxygen were measured twice daily (08:00 and 16:00), and water pH, NH_3, NO_2^-, and NO_3^- was checked weekly in all ponds and tanks used. All values complied with accepted fish culture requirements.

Statistical Analysis

All determined parameters: size of perch broodstock, anatomical indices, number of eggs in 1 ml egg ribbons, absolute and relative female fecundity, fertilization and hatching rate, larval total length and their resistance to osmotic stress, and survival rate of perch broodstock were recorded and characterized by simple statistics given as means±standard deviation (SD). Two-way analysis of variance ANOVA ($P<0.05$) was used for comparison of all parameters. The non-parametric Kruskal–Wallis test was used to test for differences in number of eggs in 1 ml of egg ribbons, absolute and relative female fecundity, fertilization and hatching rate, larval resistance to osmotic stress, and survival rate of perch broodstock. Tukey's multiple comparison tests was used for comparison of size of perch broodstock, anatomical indices and larval total length between groups. Statistical assessment of all data was carried out with Statistica 7.0 (StatSoft, Inc., Czech Republic).

Results

Morpho-Anatomical Parameters in Perch Groups

Morpho-anatomical parameters of perch groups are shown Table 2. The highest differences were observed between farmed and wild perch in GSI and VSI.

Female Reproductive Activity

Thirty-six cultivated females (92.3%) and 30 wild females (76.9%) spawned. The first spawning in both groups was recorded on 8 April. The reproductive season extended 19 days in farmed perch and 24 days in wild perch. The highest spawning activity was recorded during the Middle Period in both groups. The lowest spawning activity was found in the Late Period. Table 3 summarises Early, Middle and Late period and are shown number and percentage of spawned farmed and wild perch.

Number of Eggs in Egg Ribbons and Female Fecundity

The mean number of eggs was 156±42 ml^{-1} in farmed perch and 169±38 ml^{-1} in wild perch, which were not significantly different.

Average values of fecundity (both absolute and relative) were significantly different between groups ($P<0.05$). The mean absolute fecundity of farmed fish was 18,660±6,809 eggs $female^{-1}$ compared to wild perch with 31,081±3,276 eggs $female^{-1}$. Similar differences were revealed in relative fecundity (farmed: 112,470±13,370 eggs kg^{-1} body weight and wild: 317,054±18,513 eggs kg^{-1} body weight).

Fertilization Rate

No significant difference in average values of

Table 2. Morpho-anatomical parameters of perch broodstock at the beginning of the reproductive season

Perch broodstock	TL (mm)	SL (mm)	W (g)	HSI (%)	GSI (%)	VSI (%)
Farmed females	$220.4±21.4^b$	$189.8±20.0^b$	$159.4±49.3^b$	$1.8±0.3^a$	$18.5±2.3^a$	$3.2±1.3^b$
Wild females	$188.8±16.4^a$	$168.0±14.8^a$	$98.9±22.9^a$	$2.8±0.3^b$	$26.9±4.4^b$	$1.9±0.3^a$
Farmed males	$220.4±20.8^b$	$191.2±18.6^b$	$133.5±34.0^b$	$1.9±0.5^a$	$5.3±0.9^a$	$6.0±2.6^b$
Wild males	$159.5±16.2^a$	$139.2±14.3^a$	$52.0±18.2^a$	$2.6±1.9^b$	$8.2±2.6^b$	$3.5±0.8^a$

Data are shown as mean ± S.D. Values within column with different superscripts are significantly different ($P < 0.05$)

Table 3. The spawning activity during whole reproductive season in wild and farmed Eurasian perch (*Perca fluviatilis*)

Spawning period	Nummber of spawned FF	Percentage of spawned FF	Nummber of spawned WF	Percentage of spawned WF
Early	12	33	10	33.3
Middle	16	44	17	56.6
Late	8	22.2	3	10

FF – farmed females, WF – wild females

fertilization rates was observed between the two broodstock groups (farmed perch: 91.9±4.4% and wild perch: 90.5±5.5%). Fertilization rates in both groups of females decreased during the season. Eggs from females spawning during the Early Period of the reproductive season achieved significantly ($P<0.05$) higher fertilization rates (farmed: 97.4±3.0%; wild: 97.9±5.5%) compared to females spawning during the Late Period (farmed: 87.1±5.6%; wild: 84.3±1.9%). The Middle Period achieved fertilization rates 91.7±4.5% in farmed perch and 88.8±3.5 in wild perch and was not significantly different with another period.

Hatching Rate

Highly significant differences were found between hatching rates of the groups. The hatching rates of eggs from the farmed females (27.9±9.3%) were significantly lower than from the wild females (62.0±9.0%). Like the fertilisation rate, the hatching rate was negatively affected by the extended spawning season in both groups. Early spawning farmed females achieved hatching rates of 43.8±9.7%, compared to 76.6±8.5% in wild females. Females spawning later had significantly lowered hatching rates (Late Period: 11.7±7.9% and 50.7±9.5%, farmed and wild perch, respectively). The Middle Period (farmed 28.2±12.5% and wild 58.6±13.8) was not significantly different from the Early and Late Periods.

Larval Total Length and Resistance to Osmotic Stress

Significantly different TLs of larvae between groups were not observed. The mean TL of larvae from farmed females was 5.88±0.55 mm compared to 5.82±0.51 mm for larvae from wild fish. The difference in TL of larvae was not substantial with respect to the date of spawning in either group (Early: TL = 5.80-5.84 mm; Middle: TL = 5.80-5.90 mm, and Late period: TL = 5.79-5.92 mm).

No significant differences were found between groups in larval resistance to osmotic stress or of larval survival rate. Larvae from farmed females reached an average survival rate of 51.1±25.2% and larvae from wild females achieved a similar average rate of survival (53.5±26.2%). Larval survival rate was not associated with the date of spawning. Larvae from early spawning farmed and wild females had average survival rates of 52.4±16.3% and 54.1±21.7%, respectively. Similar average survival rates were found in larvae from Middle and Late Periods, ranging from 48.1±18.7% to 50.6±18.4%.

Broodstock Mortality

During the reproductive season no mortality was observed in females or males of either group. However, a high mortality rate of wild perch broodstock of both sexes (females 92.5±2.5% and males 91.5±3%) was observed 14 days after the conclusion of reproduction (May 15). Farmed perch broodstock showed significantly lower mortality (females 21.5%±12.5% and males 17.5±15%).

Discussion

This study showed seasonal spawning of Eurasian perch and compared morpho-anatomical parameters, reproduction characteristics, and mortality of broodstock in farmed and wild perch.

The gonadosomatic index in wild fish (26.9±4.4%) corresponded to the results published by Migaud *et al*. (2003). These values were significantly higher than in farmed fish (18.5±2.3%), also similar to results in farmed fish recorded by Migaud *et al*., (2002). Also, HSI and VSI in this study showed significant differences between farmed and wild perch, corresponding to reports by Sulistyo *et al*. (2000) for wild perch and by Migaud *et al*. (2002) for farmed perch. Differences between farmed and wild perch have been suggested to be caused mainly by unsuitable feed for the farmed fish (Bell *et al*., 1997; Kestemont *et al*., 1999; Izquierdo *et al*., 2001; Kestemont *et al*., 2008b; Henrotte *et al*., 2010), and wild perch showed superior development of gonads under natural conditions (Kouril *et al*., 1997a). Fontaine *et al*. (2008) has drawn attention to the lower quality of reproduction in farmed perch bred under controlled conditions. These authors add that, to address lower reproduction in farmed perch, it is necessary to improve the diet by adding prey fish and a larger proportion of natural ingredients to the feed. For improving the reproduction of farmed perch, Kestemont *et al*. (2008b) recommend the use of feed with higher content of arachidonic acid (C20:4 n6).

According to Craig (2000), temperature increase in spring synchronizes spawning in Eurasian perch. However, in our study, ovarian development was not synchronous among fish, and this could explain that spawning period depended on the high variability steroid concentrations (Ciereszko *et al*., 1997a, 1997b; Migaud *et al*., 2003).

The highest spawning activity was recorded in the present study in the Middle Period, although eggs were produced in both the Early and Late Periods. More farmed perch than wild perch spawned. This may indicate that spawning in Eurasian perch, a limiting factor in fish production is affected by stress (Wang *et al*., 2003). Wang *et al*. (2003) suggested that wild broodstock possessed high cortisol levels, possibly the result of stress due to environmental conditions (handling, storage, etc.) or reproduction. The relative fecundity of wild perch observed in this study (317 054 eggs kg^{-1}) not corresponds to values reported by Policar *et al*. (2008b). Kouril and Hamackova (1999) also reported lower relative fecundity (102 100 eggs kg^{-1}) of wild fish. Fecundity

of farmed perch observed here (112 470 eggs kg^{-1}) was significantly lower than for wild perch. Similar results were also observed by Kouril and Hamackova (2000).

The fertilization rate of 91.9±4.4% and 90.5±5.5% in farmed and wild perch, respectively, was not significantly different and is in agreement with results published by Kouril *et al*. (1997b) and by Migaud *et al*. (2001). Kouril and Hamackova (1999) reported 60-95% fertilization of eggs in perch after artificial stripping. In the present study, the fertilization rate decreased over time which differs from results of Migaud *et al*. (2001).

The highest differences between farmed and wild perch in spawning quality were recorded in hatching rate. A low hatching rate (27.9% in farmed fish) could be due to poor male or female gamete quality of the farmed fish. Changes in hatching rate over time were similar to those in fertilization rate. A gradually decreasing fertilization rate and consequently a lower hatching rate were reported by Kestemont *et al*. (1999). This author also reported high fluctuations in larval resistance to starvation and osmotic stress. In the present study, fluctuations of larval resistance to starvation and osmotic stress were not observed. No differences in total length of larvae, larval resistance to starvation and osmotic stress were found between farmed and wild perch. Based on these indicators, lower reproductive success of farmed fish was not observed.

In our study, high broodstock mortality was recorded after the spawning season. The post spawning mortality of wild perch (females and males) reached 92% after two weeks and corresponded to results reported by Wang *et al*. (2003). This author reported that high mortality is probably due to elevated levels of cortisol during the spawning period. Mortality of farmed perch was 19.5%, lower than that found by Migaud (2006), who reported 36-72% mortality in farmed perch.

In conclusion, the present study showed the new data about comparison of wild and farmed perch reproduction characteristics. The highest differences between farmed and wild perch were in the morpho-anatomical parameters GSI, VSI, HSI, and in the hatching rate. Further studies are required to optimize protocols for using better artificial food in farmed perch and decreased stress level in wild perch.

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CHAPTER 3

COMPARISON ON THE EFFECT OF FOUR ANAESTHETICS ON HAEMATOLOGICAL AND BLOOD BIOCHEMICAL PROFILES IN PIKEPERCH (*SANDER LUCIOPERCA* L.)

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Comparison of the effects of four anaesthetics on haematological and blood biochemical profiles in pikeperch (*Sander lucioperca* L.)

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Abstract

OBJECTIVES: The objectives of the study were to compare the effects of Propiscin, 2-phenoxyethanol, clove oil and tricaine methane sulphonate (MS 222), anaesthetics frequently used in aquaculture.

DESIGN: The haematological and biochemical blood profiles of pikeperch (*Sander lucioperca* L.) anesthetized with Propiscin (1.5 ml L^{-1}), 2-phenoxyethanol (0.3 ml L^{-1}), clove oil (33 mg L^{-1}), MS 222 (150 mg L^{-1}) and non-anesthetized control group were tested. Each tested group was divided into two subgroups, the first subgroup was sampled in anaesthesia 10 min after application of the anaesthetic and the second one live on 24h.

RESULTS: The erythrocyte count and haematocrit was significantly decreased in 2-phenoxyethanol (24 h) compared with control group (CG). The mean corpuscular haemoglobin concentration was significantly increased in 2-phenoxyethanol (10 min), Propiscin (10 min and 24 h) compared to CG. The 2-phenoxyethanol (10 min and 24 h), MS 222 (24 h), clove oil (24 h), and Propiscin (10 min and 24 h) showed significantly lower leukocyte count compared with CG. The level of glucose was significantly ($p<0.05$) elevated with MS 222 (10 min) and clove oil (10 min) compared with CG. The 2-phenoxyethanol (10 min and 24 h), MS 222 (24 h), clove oil (24 h), and Propiscin (24 h) showed significantly lower ($p<0.01$) ammonia levels compared with CG. The triacylglycerols was significantly decreased ($p<0.01$) with Propiscin (10 min and 24 h), MS 222 (24 h), clove oil (24 h) and with 2-phenoxyethanol (24 h) compared with CG. After 24 hours MS 222 (24 h) and Propiscin (24 h) anaesthesia, fish showed significantly lower ($p<0.01$) concentration of inorganic phosphate compared with CG.

CONCLUSIONS: On the basis of this experiment, it appears that clove oil was associated with the lowest effects in pikeperch and therefore would be recommended as an alternative to MS 222, while Propiscin and 2-phenoxyethanol are not suitable for manipulation with pikeperch in aquaculture.

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Abbreviations:

CG	- control group
RBC	- erythrocyte count
Hb	- haemoglobin
PCV	- haematocrit
MCV	- mean corpuscular volume
MCH	- mean corpuscular haemoglobin
MCHC	- mean corpuscular haemoglobin concentration
GLU	- glucose
TP	- total protein
ALB	- albumin
GLOB	- total globulins
NH_3	- ammonia
AST	- aspartate aminotransferase
ALT	- alanine aminotransferase
LDH	- lactate dehydrogenase
CK	- creatine kinase
PHOS	- inorganic phosphate
ALP	- alkaline phosphatase
LACT	- lactate
ANOVA	- analysis of variance

INTRODUCTION

Anaesthetic are commonly used to reduce handling stress and are routinely administered in cases of blood sampling, measuring, tagging, sorting, photographing and the artificial reproduction procedures of fish (Cooke *et al.* 2004; King *et al.* 2005; Macova *et al.* 2008; Park *et al.* 2008; Gullian & Villanueva 2009; Kiessling *et al.* 2009; Velisek *et al.* 2009a, 2011). A variety of anti-stress agents with different properties have been used to anesthetise in aquaculture (Cho & Heath 2000; Kazun & Siwicki 2001; Velisek & Svobodova 2004; Velisek *et al.* 2005).

Currently, the commonly used anaesthetics in pikeperch (*Sander lucioperca*) are Propiscin (Zakes & Demska-Zakes 2005), 2-phenoxyethanol (Demska-Zakes *et al.* 2005), clove oil (Kristan *et al.* 2012) and tricaine methane sulphonate (Zarski *et al.* 2012).

Propiscin was developed at the Inland Fisheries Institute in Poland and is routinely used for immobilization of fish in Poland fisheries (Szkudlarek and Zakes 1996). The active substance of Propiscin is etomidate [etomidate (1)-ethyl 1-(a-methylbenzyl) imidazole-5-carboxylate] (Kazun & Siwicki 2001). The used and recommended concentration for anaesthesia of pikeperch is 1.5-2 $mL.L^{-1}$ water (Szkudlarek & Zakes 1996; Kazun & Siwicki 2001). Ethylene glycol monophenyl ether (2-phenoxyethanol) is used for short-term immobilization of fish before artificial spawning and the recommended concentration for use during artificial propagation of pikeperch is 0.1–0.4 $mL.L^{-1}$ water (Kaminnski *et al.* 2002). Clove oil is a dark brown liquid and is derived from stems, leaves and buds of the clove trees *Eugenia aromatica* and *Eugenia caryophyllata* (Sato & Burhanuddin 1995; Keene *et al.* 1998). The active ingredient of clove oil is eugenol (4-allyl-2-methoxyphenol), which makes up about 70–90% of the oil weight. It is generally used as a disinfectant and analgesic in dentistry (Curtis 1990) and as an additive in perfumes (Maura *et al.* 1989). The used and recommended concentration for percid fish is 33 $mg.L^{-1}$ (Hamackova *et al.* 2001; Kristan *et al.* 2012).

Tricaine methane sulphonate is an isomer of benzocaine with an additional sulphonate radical, making it more soluble but also more acidic in solution (Congleton 2006). It is the most commonly used anaesthetic for fish (Marking & Meyer 1985). The used concentration for anaesthesia in pikeperch is 150 $mg.L^{-1}$ water (Zarski *et al.* 2012).

Therefore, the purpose of this study was to compare the affects Propiscin, 2-phenoxyethanol, clove oil and MS 222 (relative to non-anesthetized controls) on haematological and blood plasma biochemical indices in pikeperch (*Sander lucioperca*) immediately following 10-min anaesthesia and 24 h after 10-min anaesthesia. These anaesthetics are commonly used for anaesthesia of pikeperch.

MATERIALS AND METHODS

Anaesthetics

Propiscin was supplied by the Division of Fish Pathology and Immunology at Zabieniec (Inland Fisheries Institute in Olsztyn, Poland). Clove oil (eugenol concentration 78%) was from the Kulich Company (Jan Kulich, Hradec Kralove/Ricany, Czech Republic), and 2-phenoxyethanol from MERCK – Schucherd, 85 662 Hohenbrunn, Germany. MS 222 was purchased from Sigma-Aldrich Chemicals Ltd.

Experimental procedure

For assessment of the haematological and blood biochemical profile, 72 fish (71.48±15 g body weight and 228±14.59 mm total body length) were used. To maintain growth, the fish were fed with food (Inicio Plus, BioMar) of the appropriate size at the appropriate rate during a 10-day acclimatization period. All fish were starved for 24 h before the experiments. Fish were maintained at water temperature 20.5–20.7 °C and 12:12 light: dark times during both experimental and acclimatization period. In this study, nine groups were compared, of 8 fish eachControl group – no anaesthetic, blood immediately sampled, prior to the treatment of anesthetized groups.

Four groups with blood sampled immediately after 10 min anaesthesia and designated as: Propiscin (10 min) (1.5 $ml.L^{-1}$), 2-phenoxyethanol (10 min), (0.3 $ml.L^{-1}$), Clove Oil (10 min) (33 $mg.L^{-1}$) and MS 222 (10 min) (150 $mg.L^{-1}$).

Four groups with blood sampled 24 h after 10 min anaesthesia and designated as: Propiscin (24 h), 2-phenoxyethanol (24 h), Clove Oil (24 h) and MS 222 (24 h).

The conditions were duplicated for all groups, each held in cylindrical plastic tank (volume 185 L) containing freshwater with the anaesthetic. There were no mortalities in the study.

Blood was drawn from the *caudal vessels* with heparin as an anticoagulant (Heparin inj., Leciva, Czech Republic) at a concentration of 5000 I.U. heparin sodium salt in 1 ml. Erythrocyte count (RBC), haematocrit (PCV), leucocrit (Bc), haemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) and leukocyte count (Leuko), were determined by Svobodova *et al.* (1991).

For biochemical analysis, blood was centrifuged in a cooled centrifuge (4 °C, 837 × g). The plasma was stored at –80 °C until analysis. Biochemical indices in plasma included glucose (GLU), total protein (TP), albumin (ALB), total globulins (GLOB), ammonia (NH_3), calcium (Ca^{2+}), magnesium (Mg), inorganic phosphate (PHOS), triacylglycerols (TAG), aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), creatine kinase (CK), alkaline phosphatase (ALP) and lactate (LACT) were analysed. For the biochemical analysis of blood plasma, the VETTEST 8008 analyzer (IDEXX Laboratories Inc. USA) manufactured by Medisoft was used. Sample analysis was carried out on selective testing discs (Multi-layer film slides, Kodak). QA/QC (quality assurance/quality control) measures were consistently applied within the experiment. The measurements were carried out according to validated standard operation procedures.

Statistical analysis was carried out using Statistica software 9.0 for Windows (StatSoft, Czech Republic). Data were first tested for normality (Kolmogorov–Smirnov test) and homoscedasticity of variance (Bartlett's test). If those conditions were satisfied, one-way analysis of variance (ANOVA) was employed to reveal significant differences in measured variables among control and experimental groups. When a difference was detected ($p<0.05$), Tukey's multiple comparison test was applied to identify which treatments were significantly different. If the conditions for ANOVA were not satisfied, the non-parametric Kruskal–Wallis test was used.

RESULTS AND DISCUSSION

The biochemical, haematological and histopathological profile can give important information about the internal environment of the organism and these parameters are commonly used in toxicology studies (Velisek *et al.* 2009b; Haluzova *et al.* 2010; Plhalova *et al.* 2011). For evaluation of the effect of anaesthetics are frequently used the haematological and biochemical profiles (Iwama *et al.* 1989; Velisek & Svobodova 2004; Velisek *et al.* 2005, 2006, 2007, 2009c, 2011).

In our study, we evaluated both profiles. To our knowledge, no other data on biochemical and haematological profiles in pikeperch (*Sander lucioperca*) anesthetized with Propiscin, 2-phenoxyethanol, clove oil and MS 222 are available in the literature. The biochemical profiles of control and anesthetized pikeperch are given in Table 2. The level of glucose was significantly ($p<0.05$) higher with MS 222 (10 min) and clove oil (10 min) compared to controls. On the one hand, our results corresponded to the results published by Velisek *et al.* (2009) who reported an increased glucose level with MS 222 and clove oil in perch (*Perca fluviatilis*). On the other hand, these authors also found an increase of glucose in 2-phenoxyethanol. Increase of glucose concentration after 2-phenoxyethanol was also detected by Ortuno *et al.* (2002) in gilthead sea bream (*Sparus aurata*) and Park *et al.* (2008) in kelp grouper (*Epinephelus bruneus*). However, Velisek *et al.* (2007) found no changes with 2-phenoxyethanol in European catfish (*Silurus glanis*). The increase in blood glucose concentration demonstrated the response of exposed fish to metabolic stress (Simon *et al.* 1983).

The 2-phenoxyethanol (10 min and 24 h), MS 222 (24 h), clove oil (24 h), and Propiscin (24 h) showed significantly lower ($p<0.01$) ammonia levels compared with the control group (Table 2). The concentration of ammonia do not correspond with those of Velisek *et al.* (2004, 2005, 2007, 2009a) who detected no change in level of ammonia in rainbow trout, common carp, sheat-fish and perch. Nevertheless, Gomulka *et al.*

Tab. 1. Effects of MS222, clove oil, 2-phenoxyethanol (2-PE) and Propiscin anaesthesia on haematological indices in pikeperch.

Indices	Control	MS222 (10 min)	MS222 (24h)	Clove oil (10 min)	Clove oil (24h)	2-PE (10 min)	2-PE (24h)	Propiscin (10 min)	Propiscin (24h)
RBC ($T.L^{-1}$)	2.30±0.48	2.17±0.41	1.84±0.31	1.67±0.53	1.93±0.31	2.21±0.25	1.65±0.25*	2.21±0.35	1.87±0.36
Hb ($g.L^{-1}$)	40.11±6.21	32.45±5.09	45.39±12.47	37.51±9.76	44.13±4.46	50.58±3.70	39.93±6.55	47.58±6.99	46.03±8.30
PCV ($L.L^{-1}$)	0.46±0.07	0.43±0.05	0.37±0.05	0.36±0.05	0.41±0.05	0.39±0.06	0.33±0.06**	0.37±0.06	0.36±0.08
MCV (fl)	204.07±22.88	209.70±59.29	203.45±32.45	246.68±83.35	215.97±27.81	175.61±32.03	197.83±26.42	166.99±23.16	199.95±54.27
MCH (pg)	17.74±2.22	15.17±1.80	24.96±6.92	23.75±9.49	23.23±3.46	21.70±2.34	24.33±2.92	21.70±2.34	24.74±1.51
MCHC ($g.L^{-1}$)	87.55±11.43	76.42±16.79	125.98±40.21	108.00±32.49	107.47±15.79	131.79±15.46*	123.56±19.20	130.87±9.18*	131.71±32.23*

Significance levels observed are *$p<0.05$, **$p<0.01$ in comparison to the control group. All values are mean ± SD, n=8. See text for description of experimental procedure.

Tab. 2. Effects of MS222, clove oil, 2-phenoxyethanol (2-PE) and Propiscin anaesthesia on biochemical indices of blood plasma in pikeperch.

Indices	Control	MS222 (10 min)	MS222 (24h)	Clove oil (10 min)	Clove oil (24h)	2-PE (10 min)	2-PE (24h)	Propiscin (10 min)	Propiscin (24h)
GLU (mmol.L^{-1})	10.56±3.63	15.00±3.26*	7.83±1.69	15.35±2.96*	8.77±0.98	13.79±3.05	8.28±0.71	11.75±3.58	7.51±2.68
TP (g.L^{-1})	42.25±2.63	44.25±3.03	37.25±2.44	41.63±3.28	42.88±4.11	43.25±3.73	41.13±1.62	37.50±6.95	41.25±1.20
ALB (g.L^{-1})	2.88±1.62	4.13±1.27	2.50±0.86	3.25±1.85	3.50±1.66	2.88±2.03	3.88±1.05	2.00±0.87	3.38±0.99
GLOB (g.L^{-1})	39.25±2.11	40.65±2.23	34.88±1.76	38.38±2.50	39.63±3.64	40.25±2.63	37.00±1.32	36.13±6.73	37.63±1.32
NH_3 (µmol.L^{-1})	921.1±151.8	857.3±147.0	488.9±40.8**	715.3±58.8	528.1±60.7**	689.9±151.2**	506.3±69.4**	734.0±203.2	343.4±91.6**
TAG (mmol.L^{-1})	3.51±0.16	3.16±0.57	1.57±0.44**	2.81±0.60	1.74±0.60**	3.01±0.65	2.43±0.54**	1.98±0.58**	2.25±0.71**
AST (µkat.L^{-1})	2.40±0.91	2.39±0.85	1.12±0.49	1.59±0.77	1.41±0.48	2.09±0.97	2.42±1.10	2.30±0.81	1.68±0.84
ALT (µkat.L^{-1})	0.15±0.04	0.14±0.08	0.17±0.07	0.11±0.05	0.15±0.09	0.10±0.08	0.25±0.15	0.18±0.11	0.22±0.11
LDH (µkat.L^{-1})	20.14±2.54	19.23±2.80	21.12±6.23	19.80±3.03	17.75±2.78	20.04±2.22	17.94±3.43	18.90±1.56	18.98±2.60
CK (µkat.L^{-1})	14.90±2.30	14.08±1.36	14.12±2.94	14.13±1.23	15.49±3.37	14.10±1.26	14.67±2.36	14.17±1.49	15.83±1.74
Ca^{2+} (mmol.L^{-1})	2.83±0.15	3.05±0.10	2.73±0.11	2.82±0.12	2.81±0.19	2.84±0.49	2.67±0.04	2.56±0.35	2.78±0.10
Mg (mmol.L^{-1})	1.15±0.10	1.20±0.10	0.96±0.06	1.04±0.08	1.01±0.19	0.93±0.09	1.05±0.30	1.02±0.16	1.19±0.42
PHOS (mmol.L^{-1})	3.82±0.62	4.15±0.35	2.80±0.13**	3.32±0.33	3.13±0.35	3.32±0.77	3.18±0.36	3.31±0.52	2.94±0.36**
ALP (µkat.L^{-1})	1.17±0.11	0.96±0.15	1.03±0.12	0.93±0.23	0.95±0.29	0.96±0.16	0.88±0.17	0.88±0.12	1.19±0.21
LACT (mmol.L^{-1})	3.10±0.64	4.20±0.57	2.52±0.43	3.95±0.31	2.34±0.33	3.79±0.93	2.80±0.66	3.36±0.96	2.37±1.06

Significance levels observed are *p<0.05, **p<0.01 in comparison to the control group. All values are mean ± SD, n=8. See text for description of experimental procedure.

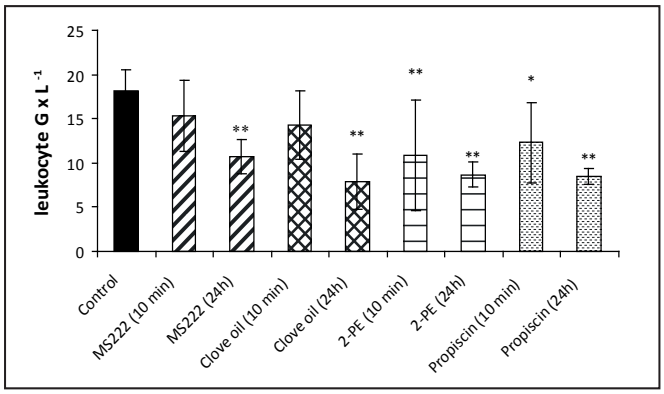

Fig. 1. Effects of MS222, clove oil, 2-phenoxyethanol and Propiscin anaesthesia on leukocytes of blood profile in pikeperch. Significance levels observed are *p< 0.05, **p<0.01 in comparison to the control group. All values are mean ± SD, n=8. See text for description of experimental procedure.

(2008) achieved also decreased of ammonia levels in Sibernia sturgeon (*Acipenser baerii*) with Eugenol and MS 222. Change levels of NH_3 in blood indicate a change in protein catabolism and/or some disturbances in NH_3 removal (Svoboda 2001).

Also the levels of triacylglycerols was significantly decreased (p<0.01) with Propiscin (10 min and 24 h), MS 222 (24 h), clove oil (24 h) and with 2-phenoxyethanol (24 h) compared with the control group (Table 2).These findings do not agree with those of Velisek *et al.* (2006) who reported increased concentration of triacylglycerols with clove oil in European catfish.

After 24 hours MS 222 (24 h) and Propiscin (24 h) anaesthesia, fish showed significantly lower (p<0.01) concentration of inorganic phosphate compared with the control group. These observations are not in agreement with Velisek *et al.* (2004, 2005, 2007, 2009a). The lower concentration of PHOS could be linked to redistribution of electrolytes between intra- and extra-cellular compartments and/or impairment of renal function (Svoboda 2001).

The values for TP, ALB, GLOB, AST, ALT, LDH, CK, ALP, Ca^{2+}, Mg and LACT were similar among all groups.

The results of haematological profiling are given in Table 1. Haemoglobin and haematocrit are often elevated during stress situations to increase oxygen carrying capacity and oxygen apply to the major organs in

response to higher metabolic demands (Rutten *et al.* 1992). The erythrocyte count ($p<0.01$) and haematocrit ($p<0.01$) was significantly decreased in 2-phenoxyethanol (24 h) compared with controls. The mean corpuscular haemoglobin concentration was a significant increase ($p<0.05$) in 2-phenoxyethanol (10 min), Propiscin (10 min and 24 h) compared to the control group. Similar results observed Velisek *et al.* (2007) who achieved also the changes of MCHC and PCV in 2-phenoxyethanol. Acute stress can cause significant changes in the white blood cell count. The response to environmental challenges often leads to leucopenia with lymphopenia and sometimes neutrophilia, which is similar to the classic leukocytic response to stress in mammals. The 2-phenoxyethanol (10 min and 24 h), MS 222 (24 h), clove oil (24 h), and Propiscin (24 h) showed significantly lower ($p<0.01$) leukocyte count compared with the control group (Figure 1), whereas, Velisek *et al.* (2005) observed no changes with clove oil in common carp, sheat-fish (2006) and with 2-phenoxyethanol in leukocyte count. The values for Hb, MCV and MCH were similar among all groups.

In summary, all tested anaesthetics were associated with changes in haematological and blood biochemical parameters in pikeperch. However, the effects of the anaesthetics were different from one another in the measured variables. On the basis of this experiment, it appears that clove oil was associated with the lowest effects in pikeperch and therefore would be recommended as an alternative to MS 222.

ACKNOWLEDGMENTS

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CHAPTER 4

ARTIFICIAL AND SEMI-ARTIFICIAL SPAWNING IN EURASIAN PERCH (*PERCA FLUVIATILIS* L.) FOR MASS EMBRYO PRODUCTION

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USE OF ARTIFICIAL AND SEMI-ARTIFICIAL SPAWNING IN EURASIAN PERCH (PERCA FLUVIATILIS L.) FOR MASS EMBRYO PRODUCTION

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1. INTRODUCTION

1.1. Current importance of Eurasian perch

Rearing of the Eurasian perch (*Perca fluviatilis* L.) has seen an expansion in European aquaculture in the past two decades (Kestemont and Mélard, 2000). High demand (up to 10 000 tonnes per year) for perch fillets is seen mainly in local markets in Alpine countries: Switzerland, Germany, France, and Austria (Watson, 2008; Policar et al., 2009). Consumers in these countries consider the Eurasian perch a delicacy due to its white, low-fat meat and lack of "Y" bones (Watson, 2008; Stejskal et al., 2010).

The Eurasian perch is also effectively used to reduce excessive populations of small and less valued cyprinids in pond culture (Policar et al., 2009; Stejskal et al., 2010), helping to maintain stable production of valuable fish species in the polyculture fish stocks. In reservoirs, Eurasian perch predation pressure ensures adequate levels of zooplankton for improved water quality (Adámek et al., 2010).

1.2. Production of Eurasian perch in Europe

Four methods of production are currently used for Eurasian perch: extensive, semi-intensive, intensive, and commercial fishing from natural waters (Policar et al., 2009).

Extensive production is based on rearing in ponds in three to four year production cycles. In this method, perch is a complementary species in polyculture with the primary production of common carp (*Cyprinus carpio*) (Bláha, 2006).

Semi-intensive production of perch uses a combination of extensive and intensive production. This method is based on broodstock production in ponds, their artificial or semi-artificial reproduction, artificial incubation of eggs and hatching of larvae, and pond rearing of larvae and juveniles to 30–50 mm total length (TL). After being harvested from the ponds, juveniles are adapted to the recirculation aquaculture system (RAS) and to a dry feed diet, and rearing follows intensive methods in RAS (Policar et al., 2009; Stejskal et al., 2010).

Intensive production of perch uses fully controlled breeding in RAS from reproduction of broodstock to the final production of market-size fish. This method uses domestic broodstock, temperature and light regime stimulation of broodstock

gonadogenesis, semi-artificial and artificial spawning, artificial incubation of eggs, rearing of larvae in controlled conditions using nauplii of *Artemia salina* and starter food mixtures (Biomar, Aller Aqua, and others), and rearing of juveniles in RAS to market size (Fig. 1) or for broodstock (Mélard et al., 1996; Kestemont et al., 2008; Policar et al., 2009). Perch are bred in optimal growing conditions (water temperature 23 °C, favourable water quality parameters and feeding), which considerably reduces the time of production compared to extensive methods. High density of intensively bred perch (up to 60 $kg.m^{-3}$) guarantees high commercial success (Mélard et al., 1996).

The final production method of market-size Eurasian perch is commercial fishing from open waters, e.g. large lakes or rivers, a system primarily used in Scandinavia and the former Soviet countries (Watson, 2008).

Figure 1. *Market-size perch (100–150 g).*

1.3. Current market size perch production in Europe

According to current FAO statistics (2011a,b), market-size perch production in 2009 was 23 524 tonnes. The largest portion (99%) (23 264 tonnes) was from wild harvest in European lakes, rivers, and reservoirs. Most were caught in Finland (10 590 tonnes), Russia (8 785 tonnes), Estonia (1 645 tonnes), Poland (838 tonnes), and Switzerland (342 tonnes) (FAO, 2011a). Total production of farmed Eurasian perch in Europe in 2009 was 260 tonnes (FAO, 2011b). Fourteen countries contributed to this production, most importantly Russia (140 tonnes), Ukraine (25 tonnes), FYROM (29 tonnes), Ireland (24 tonnes), and the Czech Republic (18 tonnes).

1.4. Production of broodstock

The successful intensive production of Eurasian perch is dependent on high quality sexually mature males and females. Currently, two methods of production of perch broodstock are used. The first is classical intensive or semi-intensive

production in ponds in polyculture fish stocks (Policar et al., 2009). In this method of production, broodstock is reared mainly on natural food in the form of small prey fish. Gonadogenesis take place under natural conditions without special intervention with respect to light or temperature regime. This allows well-developed gonads and high-quality gametes, ensuring a high rate of egg fertilization and hatching (Fontaine et al., 2008). However, this method of production has a seasonal character (Kouřil et al., 2001), as the broodstock spawn only during the natural spawning period of Eurasian perch (in Europe mostly March to June) (Ashe, 1997; Rougeot et al., 2008). In the Czech Republic, perch broodstock most often spawn from early to mid-April.

A second method of Eurasian perch broodstock production takes place under consistently controlled conditions in RAS (Policar et al., 2009). Management of controlled production must comprise: (1) optimal maintenance of broodstock, which positively affects the development and quality of gonads and gametes (Abi-Ayad et al., 1995; Fiogbé et al., 1996; Kestemont et al., 1996; Abi-Ayad et al., 1997; Fontaine et al., 1997; Kestemont et al., 2001; Xu et al., 2001; Xu and Kestemont 2002; Fiogbé and Kestemont, 2003; Kestemont et al., 2003; Mathis et al., 2003; Fontaine et al., 2008), and (2) optimal environmental conditions, especially temperature and light regime, ensuring that the broodstock enters the spawning season in optimal condition, with spermatogenesis and oogenesis complete (Fontaine et al., 2008). Detailed description of environmental stimulation of spermiogenesis and oogenesis can be found in Abdulfatah et al. (2008); Fontaine et al. (2008); Jansen and Fontaine, (2008) and Policar et al. (2009). For Eurasian perch broodstock the recommended diet includes artificial feed with adequate highly unsaturated fatty acid (HUFA), namely docosahexaenoic (DHA), eicosapentasneoic (EPA), and arachidonic (ARA) acids (Kestemont et al., 2008) at a ratio of 2DHA:1EPA:1ARA (Fontaine et al., 2008). The optimal Eurasian perch broodstock diet ensures high quality eggs, embryos, and larvae.

1.5. Successful spawning and production of high quality larvae

Successful spawning of broodstock to ensure the mass production of quality larvae is basic to successful breeding and production of market-size perch (Policar et al., 2009). Several methods of perch broodstock spawning have been tested and described, together with methods of stimulation and synchronisation of spawning (West and Leonard, 1978; Kayes and Calbert, 1979; Flajšhans and Göndör, 1989; Dabrowski et al., 1994; Kucharczyk et al., 1996, 1998; Kouřil and Linhart, 1997; Kouřil et al., 1997; Kouřil and Hamáčková, 1999, 2000; Policar et al., 2008a,b,c, 2009). This allows effective reproduction of perch broodstock by artificial, semi-artificial, or natural methods. Artificial reproduction includes hormonal induction of ovulation and spermiation, manual stripping of eggs and sperm, artificial fertilization of eggs, and artificial incubation in controlled conditions. In semi-artificial reproduction, as in artificial reproduction, the broodstock is hormonally stimulated, but spawning and fertilization take place naturally. Fertilized eggs are then collected and incubated artificially under controlled conditions. In natural reproduction, fish are not hormonally stimulated and eggs are fertilized naturally and incubated in controlled or in uncontrolled conditions (Policar et al., 2009).

To stimulate the maturation and release of gametes (eggs and spermatozoa), and to synchronize the spawning of broodstock, hormone injections of carp pituitary,

choriogonadotropins, or the synthetic analogue GnRHa in commercial products, e.g.: Supergestran, Dagin, and Chorulon (Dabrowski et al., 1994; Kucharzcyk et al., 1996, 1998; Kouřil and Linhart, 1997; Kouřil et al., 1997; Kouřil and Hamáčková, 1999, 2000; Kouřil et al., 2001; Policar et al., 2008a,b,c), or controlled temperature regime (Policar et al., 2009) can be used.

We tested methods and verified experimental findings with applied research at the Czech aquaculture facility Rybářství Nové Hrady s.r.o. Eurasian perch larvae mass production was evaluated with artificial and semi-artificial reproduction of broodstock hormonally stimulated with the commercial hormonal treatment Supergestran.

2. AIM OF STUDY

The overall goal of this study was to describe, conduct, and verify in practice technology for mass production of Eurasian perch embryos originating from hormonally stimulated artificial and semi-artificial reproduction using the commercial hormone treatment Supergestran. Through this procedure we described (1) the broodstock rearing method in ponds and (2) the preparation of broodstock for the spawning period. We evaluated (3) injection of broodstock with the hormone treatment Supergestran. During the spawning period, we observed the effectiveness (success rate), latency (the period from the hormone injection of female to spawning), the synchronization of artificial and semi-artificial spawning in females, and the fecundity of females and males. We verified (4) procedures for artificial fertilization of eggs obtained by artificial stripping, (5) artificial incubation of eggs, and (6) hatching of embryos from artificial and semi-artificial spawning, Finally (7), we evaluated the post-spawning mortality of broodstock and the use of broodstock after spawning.

3. STUDY LOCATION

The procedures were verified at the Czech fish company Rybářství Nové Hrady s.r.o. (Fig. 2) in 2009–2011. Most trials concerning broodstock pond culture, artificial and semi-artificial reproduction of broodstock, evaluation of female and male fecundity, artificial incubation of fertilized eggs, and hatching of Eurasian perch embryos were conducted at the experimental facility of the University of South Bohemia, Faculty of Fisheries and Protection of Waters (USB FFPW) in Vodňany (Fig. 3).

Figures 2 and 3. *Hatchery of Rybářství Nové Hrady s.r.o. (left) and the experimental facility of USB FFPW in Vodňany (right).*

4. TECHNOLOGY

4.1. Breeding and acquisition of broodstock for mass reproduction trials

4.1.1. Procedures

In 2009–2011, the broodstock population of Eurasian perch for these trials was bred in polyculture with other fish species, common carp (*Cyprinus carpio*), tench (*Tinca tinca*), grass carp (*Ctenopharyngodon idella*), bighead carp (*Hypophthalmichthys molitrix*), pike (*Esox lucius*), pikeperch (*Sander lucioperca*), and European catfish (*Silurus glanis*) in production ponds: Blatec (48°50′28″N, 14°44′55″E), Nakolický (48°48′21″N, 14°50′5″E), Byňovský (48°49′22″N, 14°48′15″E) and Smutný (48°50′7″N, 14°45′43″E). The ponds also contained less valuable small cyprinids such as roach (*Rutilus rutilus*), rudd (*Scardinius erythrophthalmus*), and invasive topmouth gudgeon (*Pseudorasbora parva*). These species comprised the main diet of the Eurasian perch broodstock. In 2009–2011, during the spring harvesting period, four-year-old Eurasian perch broodstock were captured, and fish in apparently good health and condition were selected and transferred to the hatchery of Rybářství Nové Hrady or to USB FFPW in Vodňany, where they were placed in storage ponds (USB FFPW) or in handling ponds (Nové Hrady) together with the forage fish topmouth gudgeon, at a ratio of 1 kg of Eurasian perch to 2 kg topmouth gudgeon.

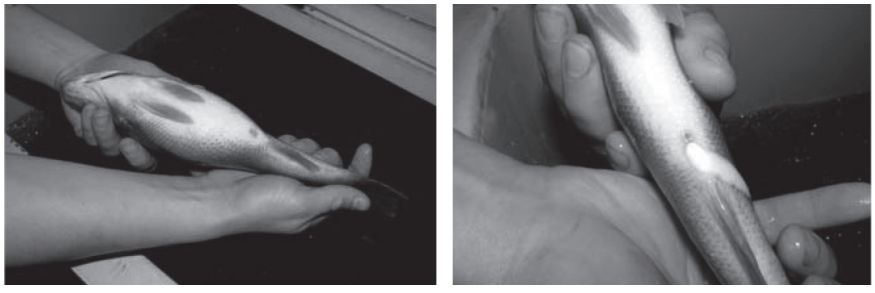
4.1.2. Results

In 2009–2011, 150–160 female broodstock (TL = 215.8 ± 24.5 mm and BW = 187.32 ± 95.0 g) and 150–160 male broodstock (TL = 196.4 ± 18.5 mm and BW = 140.3 ± 76.0 g) were obtained annually for mass artificial and semi-artificial reproduction.

4.2. Manipulation and hormone injection of broodstock

4.2.1. Procedures

After 1–3 weeks of acclimatization, high quality fish with no apparent health problems or skin damage were selected. The selection criterion for females was a full abdomen, characterizing readiness for spawning (Fig. 4). Males were selected that spontaneously released sperm without blood contamination upon abdominal massage (Fig. 5).



Figures 4 and 5. *Suitable female (left) and male (right) broodstock selected for spawning.*

Selected broodstock were divided in two groups of females (n = 60 each) and two groups of males (n = 76 and 60). Spawning took place in controlled conditions at water temperature 15.2 ± 0.2°C and oxygen saturation 8.3 mg $O_2.l^{-1}$ (c. 85% O_2).

For artificial spawning, 60 females were separated into 3 groups of 20 (Fig. 6), and each group was placed in a 0.7 m^3 tank of a semi-recirculating aquaculture system. From the group of 76 males, 60 were randomly designated to fertilize the eggs from artificial spawning and placed in a 6 m^3 tank. The remaining 16, to be used for collection of sperm to determine male fecundity, were placed in a 0.7 m^3 tank. In artificial spawning, males were held separate from female groups.

For semi-artificial spawning, the two remaining groups (n=60 males and 60 females) were separated into 6 groups of 20 broodstock each: 10 females and 10 males. The groups were placed in six 0.7 m^3 tanks.

Figure 6. *Female broodstock separated into groups for artificial spawning.*

For both spawning regimes, fish were acclimatised in tanks over the course of two days, while the water temperature was raised from 11.4 °C (natural temperature) to 15.0–15.5 °C (required temperature). Total length (TL) and body weight (BW) was recorded for all fish. For manipulation, broodstock were anaesthetized 3–4 minutes in a clove oil water bath at 0.03 $ml.l^{-1}$ (Hamáčková et al., 2001; Policar et al., 2009). Fish were tagged with VIE (Visible Implant Elastomer tag, Northwest Marine Technology, Ltd., USA), orange to indicate artificial spawning or red for semi-artificial. All females and 16 randomly selected males intended for collection of sperm to determine male fecundity were tagged for individual identification. The position of the VIE on the head represented the number of the fish within the group, so the fish could be identified during spawning (Fig. 7).

Figure 7. T*agging of broodstock with implanted elastomer tag.*

After tagging, fish were intramuscularly injected under the dorsal fin with Supergestran containing Lecirelin -mGnRHa (D- Tle^{6}, Pro^{9}, Net) containing 25 µg GnRHa.ml^{-1} (Fig. 8). Females were injected with 2 ml kg^{-1} Supergestran, which is equivalent to 50 µg kg^{-1}.GnRHa. All selected males released sperm spontaneously with no hormone stimulation.

Figure 8. *Intramuscular hormone injection.*

4.2.2. Results

Anaesthesia, obtaining biometric measurements of all 256 fish, hormone injection of females, and fish tagging was conducted by four workers over a four hour period.

For artificial and semi-artificial spawning, broodstock of equal size were selected: females TL = 236.4 ± 27.2 mm and BW = 190.2 ± 83.0 g and males TL = 184.2 ± 19.3 mm and BW = 137.6 ± 68.2 g.

No mortality of perch broodstock was observed during the procedure or on the two subsequent days. For hormone stimulation, 22.8 kg of broodstock was injected with a total of 46 ml of Supergestran. Cost of hormone injection was 1 556 CZK (Supergestran 33.82 CZK/1 ml).

4.3. ARTIFICIAL AND SEMI-ARTIFICIAL SPAWNING REGIME

4.3.1. Procedures

4.3.1.1. Control of broodstock before artificial spawning

Twenty-four hours post-injection, examination of broodstock for genital papillae and ovulation was initiated and continued every 6 h. After spawning of the first female, the broodstock were monitored at 3 h intervals. More frequent checking was necessary to prevent spontaneous spawning and release of eggs into the water. Fish were handled carefully to avoid stress and skin damage.

4.3.1.2. Artificial stripping of eggs

Females showing signs of ovulation were transferred to an anaesthesia bath, as during previous manipulation. Before stripping, the abdominal area was dried and eggs were stripped by gentle massage along the abdominal wall. Eggs were stripped into previously weighed and individually tagged dry dishes.

Eggs from each individual were weighed using a Kern PCB 800 balance with accuracy of 0.01 g. Three samples of approximately 1 g each were randomly selected and weighed using a Mettler AE 200 balance with accuracy of 0.001 g. The number of eggs in each sample was counted. To obtain the number of eggs per 1g of egg ribbon, the total number of eggs in the sample was divided by the weight of the sample in grams. To determine the absolute fecundity of a female (the total number of eggs), the number of eggs per 1 g of egg ribbon was multiplied by the total weight of the egg ribbon. The stripping data (date, time, identification number, and absolute fecundity) were recorded. After the spawning period, latency, the period from hormone injection to stripping, expressed in days, in hours, and in degree days, was calculated. The spawning success (percent of stripped females) and synchronization of spawning (number of stripped females per hour) were determined.

The dishes were covered with a damp cloth and placed in a cool area of the hatchery to preserve the eggs for up to 1 h after stripping to allow for the collection of a larger pool of eggs.

4.3.1.3. Sperm Collection

The sperm of 60 selected males was collected into 5–10 ml syringes by gentle massage of the abdomen (Fig. 9). Care was taken to avoid contamination by water, urine, or blood, and sperm was stored at 4–6 ºC. The sperm from a minimum of 3 males was used for artificial insemination of the eggs from each female.

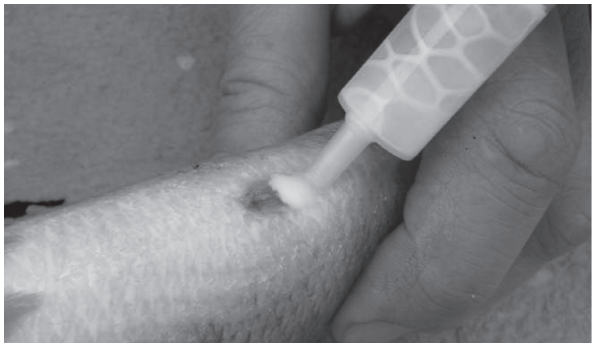
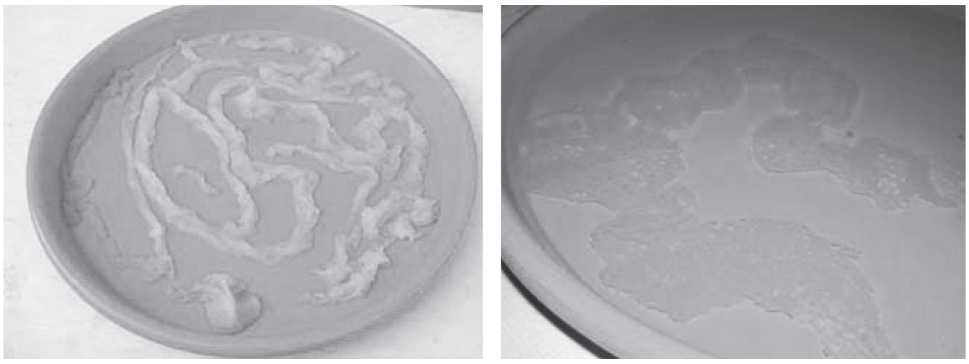


Figure 9. *Collection of sperm.*

The sperm of the 16 males selected in advance and individually tagged was collected to determine male fecundity and sperm characteristics. The volume (in ml) and density (spermatozoa per 1 ml) of collected sperm were determined according to Alavi et al. (2007). The sperm volume was measured in a syringe (accuracy 0.1 ml), and density was determined using a Bürker cell counter. Sperm was diluted 10 000 times with physiological solution (0.7% NaCl), and 10 µl of diluted sperm was put into a haemocytometer. After 10 min sedimentation, the spermatozoa per 16 squares of a haemocytometer were counted. To determine the absolute fecundity (total number of spermatozoa per male), the total volume of sperm was multiplied by the density of spermatozoa per 1 ml. To determine the relative fecundity (the number of spermatozoa per 1 kg of male) we divided the absolute fecundity by weight of each male (in g) and multiplied this number by 1 000.

4.3.1.4. Artificial fertilization of eggs

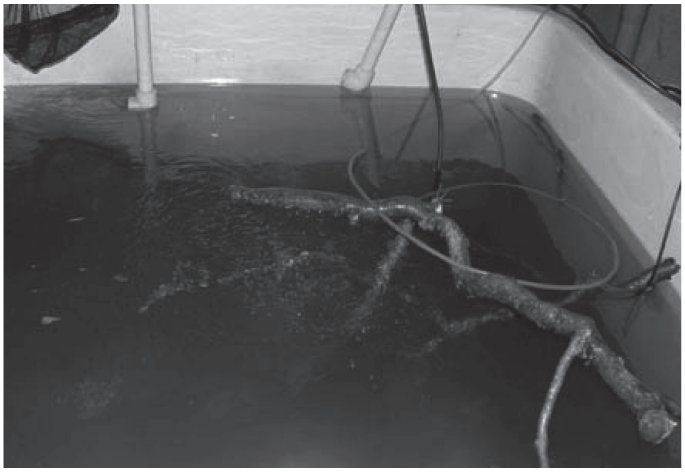
The sperm from three males, collected separately with syringe, was used for fertilization of one egg ribbon (Fig. 10). To 100 g of eggs (approx. 59 200 eggs), 2 ml of sperm (approx. 58 400 000 000 spermatozoa) was used (approx. 1 million spermatozoa/egg). Sperm was added and carefully mixed with eggs, clear hatchery water was added, and gametes were gently mixed and allowed to rest three min (Fig. 11). Eggs were rinsed with clear water and the egg ribbons were placed into plastic incubators (baskets of 300 x 200 x 80 mm) for artificial incubation (Plate 14) in RAS tanks (water temperature 15.5 ± 0.5 ºC).



Figures 10 and 11. *Egg ribbon before (left) and after (right) artificial fertilization.*

4.3.1.5. Semi-artificial spawning

During semi-artificial spawning, neither frequent checking nor the manipulation of broodstock was necessary, as females and males were stocked together. After stocking and female hormone injection, the fish spawned naturally and spontaneously. When spawning began, six dry branches approximately 80–120 cm in length of goat willow, *Salix caprea,* or European black elderberry, *Sambucus nigra*, were placed in each tank to serve as a natural spawning substrate (Fig. 12). The fish swam freely among the branches and used this substrate for fixing the egg ribbons and for the fertilization of eggs. For recording and collection of spawned and fertilized egg ribbons, the broodstock were monitored at 6 h intervals.




Figures 12 and 13. T*anks with spawning substrate for perch broodstock (left) and determination of the egg ribbon volume in semi-artificial spawning (right).*

While collecting egg ribbons, the spawned female was removed and the approximate time of spawning was recorded along with the identification number of the female. The males were kept in tanks until all females had spawned. The volume of a single egg ribbon was calculated using graduated cylinders with water according to Kouřil et al. (1998), Kouřil and Hamáčková (2000), and Kouřil et al. (2001) (Fig. 13).

The number of eggs in 1 ml of egg ribbon (see fertilization of eggs) and the total number of eggs in the egg ribbon were counted. To count the number of eggs in 1 ml of egg ribbon, a sample of approximately 1 ml eggs was taken. The volume of the sample was measured in a graduated cylinder with water. The number of eggs was determined and the number of eggs per 1 ml egg ribbon was calculated as the number of eggs in the sample divided by volume of the sample in millilitres.

The stripping data (date, time of egg collection, female identification number, and number of eggs per egg ribbon) were recorded. After the spawning period, the latency (time from hormone injection to female spawning) and synchronization of the spawning (number of spawned females per hour) was calculated according to the time of spawning. The spawning success (number and percent of spawned females) and the absolute and relative fecundity of females (total number of eggs and number of eggs per 1 kg of female BW) were determined. The egg ribbons were placed in plastic incubator baskets in RAS tanks.

4.3.2. Results

4.3.2.1. Spawning success, latency, and synchronization in artificial and semi-artificial spawning

Results indicated that 83% of females spawned with artificial spawning and 88% of females spawned in semi-artificial spawning (Table 1).

Table 1. *Efficacy, latency, and spawning synchronization of broodstock under artificial and semi-artificial spawning regimes.*

Indicator	Artificial spawning	Semi-artificial spawning
Number of spawned females	50	53
Percent of spawned females	83	88
Number of spawned males	60	Not rated
Percent of spawned males	100	
Latency (days)	3.5 ± 0.8	4.1 ± 0.7
Latency (hours)	84.0 ± 18.3	98.5 ± 17.2
Latency (degree days)	53.2 ± 11.5	62.3 ± 10.6
Synchronization of spawning (percent spawned females/hour)	83/96 h	88/72 h

All 76 males successfully released sperm during artificial spawning; for semi-artificial spawning it was not possible to determine spawning success. No problems with production or release of sperm were observed. For successful artificial spawning, it is essential to provide a sufficient number of quality males. Based on our experience, we recommend 1 : 1 males : females in both artificial and semi-artificial spawning.

Artificial spawning involves a higher demand for labour, time, and staff experience than does semi-artificial spawning, chiefly because of the necessity of more frequent examination of the females to avoid spontaneous spawning and resulting damage to eggs. Under artificial spawning, males must also be stripped of sperm, and the eggs artificially fertilized, which is time consuming and requires high level skills. These activities also significantly influence the quantity and the quality of fertilized eggs and hatched embryos. An advantage of artificial spawning is ease of monitoring production and fertilization of eggs.

The spawning of female broodstock under the semi-artificial regime began spontaneously 14.4 h later than did the artificial spawning. Latency of female perch broodstock was longer in semi-artificial spawning (4.1 ± 0.7 days/ 98.5 ± 17.2 hours/62.3 ± 10.6 °d) than in artificial spawning (3.5 ± 0.8 days/84.0 ± 18.3 hours/ 53.2 ± 11.5 °d) (Table 1).

Spawning was more highly synchronized under the semi-artificial regime, with 88% of fish spawning within a 3 day period (Table 1). Cumulative percent of spawned females under both spawning regimes is shown in Table 2. With artificial spawning, females were stripped over a 4 day period. Artificial spawning began earlier and proceeded more slowly than did semi-artificial spawning (Table 1 and 2).

Table 2. *Cumulative percent of spawned females during artificial and semi-artificial spawning.*

Spawning method	Day post-injection					
	1.	2.	3.	4.	5.	6.
Artificial spawning	0	0	22	67	75	83
Semi-artificial spawning	0	0	0	33	77	88

4.3.2.3. Female fecundity and total egg production

The absolute and relative fecundity of individual females differed considerably with both semi-artificial and artificial spawning. The mean absolute fecundity of artificially spawning females was 30 721 ± 28 796 eggs (from 5 234 to 92 003 eggs). Similar values of absolute fecundity were observed with semi-artificial spawning, with mean absolute fecundity 32 215 ± 30 128 eggs per female (from 4 567 eggs to 153 687 eggs). The average relative fecundity of females was 161 689 eggs kg^{-1} with artificial spawning and 169 552 eggs kg^{-1} with semi-artificial spawning (Table 3). Fecundity was influenced by female body size. An influence of spawning method on female fecundity was not observed.

The number of eggs in 1 g of ribbon was found to average 592.0 ± 162.4 with artificial spawning, and 1 ml of egg ribbon showed at average of 248.0 ± 79.3 eggs in semi-artificial spawning (Table 3).

In total, approximately 1 500 000 eggs were obtained through artificial spawning with 89 000 used for assessment of fertilization and hatching rates. From the 50 artificially spawning females, approximately 1 400 000 eggs were used for incubation.

With semi-artificial spawning, 1 700 000 eggs were obtained from 53 successfully spawning females; 40 000 eggs were used for assessment of fertilization and hatching rates and 1 660 000 eggs for mass incubation.

Table 3. *Absolute and relative fecundity of female broodstock with artificial and semi-artificial spawning.*

Indicator	Artificial spawning	Semi-artificial spawning
Absolute fecundity of female (number of eggs $female^{-1}$)	30 721 ± 28 796	32 215 ± 30 128
Relative fecundity of female (number of eggs kg^{-1} BW)	161 689 ± 149 500	169 552 ± 157 000
Number of eggs in 1 g of ribbon (number of eggs g^{-1} ribbon)	592 ± 162	Not rated
Number of eggs in 1 ml of ribbon (number of eggs ml^{-1} ribbon)	Not rated	248 ± 79

4.3.2.4. Male fecundity in artificial spawning

In artificial spawning, an average volume of 2.8 ± 1.5 ml sperm was collected from each male, ranging from a minimum of 0.55 ml to a maximum 6.7 ml. Average spermatozoan density was high, at $29.2 \times 10^9 \pm 15.3 \times 10^9$ spermatozoa ml^{-1} sperm. The individual minimum sperm density was 3.3×10^9 spermatozoa ml^{-1} and the maximum was 196.8×10^9 spermatozoa ml^{-1} sperm. Males released an average of 81.8×10^9 spermatozoa, or 583.0×10^9 spermatozoa kg^{-1} during artificial spawning. In semi-artificial spawning, fecundity was not evaluated.

4.4. Fertilization and hatching rates in representative samples

4.4.1. Procedures

Three samples of approximately 1 ml from each egg ribbon were taken during mass incubation to determine the fertilization rate 24 h after artificial fertilization

and 24 h after collection of egg ribbons in the semi-artificial spawning regime. Collection was as described for semi-artificial spawning. Samples were incubated in Petri dishes (diameter 120 mm, volume 75 ml) with 70 ml water. Water temperature in dishes was 16.2 ± 0.7 °C, pH = 7.5 ± 0.2, and concentration of dissolved oxygen O_2 = 7.0 ± 0.5 mg.l^{-1}. Parameters were monitored and water changed every 12 h. In each sample, the number of eggs and the number of fertilized eggs was counted. The fertilization rate was calculated as the number of fertilized eggs divided by total number of eggs, multiplied by 100. When hatching was complete, the hatching rate and the period of incubation in days, in hours, and in degree days were determined. The hatching rate was calculated as the number of hatched embryos divided by total number of eggs in the sample, multiplied by 100.

4.4.2. Results

A higher fertilization rate (85.6 ± 8.7%) was observed in samples from semi-artificial spawning than in samples from artificial spawning (67.5 ± 6.5%) (Table 4). An average incubation period of 6.5–6.8 days was observed in both spawning regimes. The period of incubation was related to water temperature. An influence of spawning method on incubation period was not observed.

The fact that the incubation period under semi-artificial spawning was 7h shorter was more likely caused by the fact that the time of semi-artificial spawning could not be accurately determined (error 1–6 h). In artificial spawning the exact time of spawning of each female was recorded (Table 4.). A significantly higher average hatching rate was observed in semi-artificial spawning (72.9 ± 12.3%) than in artificial spawning (58.4 ± 5.2%) (Table 4).

Table 4. *Fertilization rate, incubation period, and hatching rate with artificial and semi-artificial spawning.*

Indicator	Artificial spawning	Semi-artificial spawning
Fertilization rate (%)	67.5 ± 6.5	85.6 ± 8.7
Incubation period (days)	6.8 ±1.5	6.5 ± 1.3
Incubation period (hours)	163.2 ± 36	156.0 ± 30.0
Incubation period (°d)	110.2 ± 24.3	105.3 ± 20.3
Hatching rate (%)	58.4 ± 5.2	72.9 ± 12.3

4.5. Mass artificial incubation

4.5.1. Procedures

After spawning, each egg ribbon was separately incubated in a special basket (Fig. 14). After removing samples for for determination of fertilization and hatching rates, three egg ribbons were incubated together in a single basket in RAS tanks. The average water temperature during mass incubation was 15.8 ± 0.4 °C, pH = 7.4 ± 0.1, and concentration of dissolved oxygen O_2 = 8.0 ± 0.2 mg l^{-1}. The egg ribbons were placed into baskets at time of collection, which later facilitated removal of the baskets and tank maintenance. The water temperature, concentration of dissolved oxygen, and pH during incubation were monitored every 12 h. Embryo development was monitored

at the same interval, which was an important condition for successful incubation; egg ribbons with damaged or dead (white) eggs were removed from baskets to avoid water contamination. At the conclusion of mass incubation, the hatching rate and the incubation period in days, in hours, and in degree days were determined. The hatching rate was calculated as follows: number of hatched embryos divided by total number of eggs in the sample, multiplied by 100.

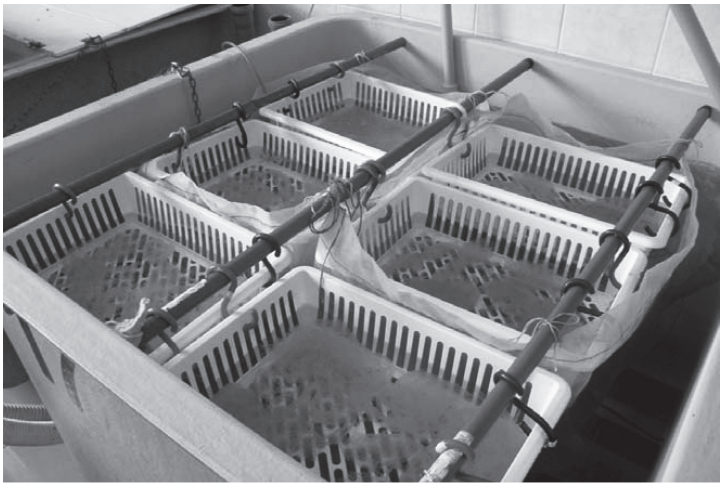


Figure 14. *Mass incubation of egg ribbons in baskets.*

4.5.2. Results

The mass incubation of eggs (Fig. 15) lasted in average 7.5 days in artificial spawning and 7 days in semi-artificial spawning (Table 5). The incubation time was slightly longer than the incubation in samples, because during the mass incubation the eggs were incubated at a water temperature 0.4 °C lower than that in samples.

Higher average hatching rate (68.0 ± 7.5%) was again observed with semi-artificial spawning than in artificial spawning (55.0 ± 9.5%) (Table 5). Hatching rates in mass incubation were lower than in the samples for both regimes, probably due to less hygienic conditions than in the controlled test.

Table 5. *Incubation period and hatching rate in artificial and semi-artificial mass incubation.*

Indicator	Artificial spawning	Semi-artificial spawning
Incubation time (days)	7.5 ± 1.5	7.0 ± 1.0
Incubation time (hours)	180.0 ± 36.0	168.0 ± 24.0
Incubation time (°d)	120.0 ± 24.0	112.0 ± 16.0
Hatching rate of embryos (%)	55.0 ± 9.5	68.0 ± 7.5

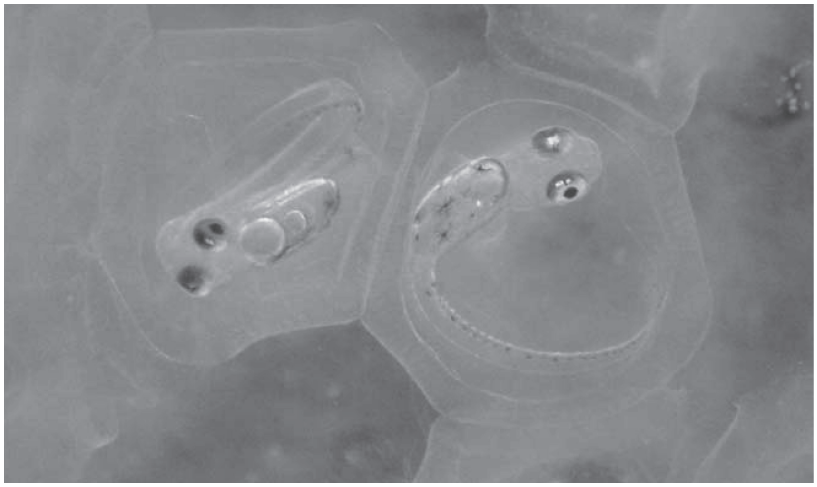


Figure 15. *Embryos of Eurasian perch.*

4.6. Mass hatching and production of embryos for breeding

4.6.1. Procedures

At the conclusion of mass incubation, the baskets were gently shaken to release the embryos. The embryos were moved to tanks with mesh of 300 µm diameter (Fig. 16) and subsequently to a bath with clean hatchery water (Fig. 17). The quantity of hatched eggs was then counted using the following method: The volume of water in the bath was decreased to 10 l, the bath was gently agitated to disperse the embryos, and five samples of 10 ml each were taken. The number of embryos in each sample was counted and average of the five samples calculated. The average number of embryos in 10 ml was multiplied by 1 000 to calculate the number of embryos in 10 l. The overall hatching rate was calculated by dividing the number of embryos by the number of eggs and multiplying by 100.

Figure 16 and 17. *Removal of embryos to tank (left) and transfer to counting bath (right).*

4.6.2. Results

At the conclusion of mass incubation, 1 930 000 embryos were obtained from all females under both spawning regimes. 1 134 000 embryos (59%) were obtained from semi-artificial spawning and 796 000 (41%) from artificial spawning. With respect to total production, semi-artificial spawning was more effective than artificial spawning, due to higher fertilization rate and hatching rate.

4.7. Mortality of broodstock during and after the spawning period

4.7.1. Procedures

The mortality of fish was investigated during the spawning period and on days 7 and 90 post-spawning. The mortality during the spawning period included fish mortality from the time of the hormone injection to spawning. Mortality was evaluated separately for the spawning regimes. After spawning, each fish was treated 5 min in an antifungal bath of potassium permanganate solution at 0.1 $g.l^{-1}$ (Fig. 18) and transferred to a plastic tank, and topmouth gudgeon (*Pseudorasbora parva*) was gradually added at a ratio of 1 kg perch to 2 kg topmouth gudgeon. The broodstock remained in this tank for 7 days after spawning of the final female, at which time mortality was recorded (Fig. 19).

The surviving fish were stocked into a 0.16 ha experimental pond with topmouth gudgeon in the same density as previously stated. Ninety days post-spawning, fish were harvested, the surviving fish were separated into groups according to sex and spawning regime, and mortality per group was determined.

Figures 18 and 19. *Potassium permanganate solution bath (left) and assessing survival of broodstock 7 days after conclusion of the spawning period (right).*

4.7.2. Results

Mortality of broodstock of both sexes during the spawning period and 7 days and 90 days after the conclusion of the spawning period is shown in Table 6. Mortality rate of females during artificial and semi-artificial spawning was 15 and 17%, respectively. No mortality was observed in males during the artificial spawning period; during semi-artificial spawning the mortality of males was 8%.

Higher mortality of broodstock was evident 7 days after the spawning period. 68% of females were lost in the 7 days following both artificial and semi-artificial spawning; 22% of males were lost within 7 days post-artificial spawning and 8% males 7 days post-semi-artificial spawning.

Ninety days after the conclusion of the spawning period, 98% mortality was observed in females, regardless of the spawning regime. High mortality was also observed in males, 92% with artificial spawning and 85% with semi-artificial spawning.

These data shows clearly that most Eurasian perch broodstock die after spawning, whether artificial or semi-artificial. It is not realistic to plan repeat use of either sex of broodstock. According to our experience, it is advantageous to kill the fish immediately or within 7 days of spawning and process it. If the fish is not used in this way, according to our experience, most of the broodstock is lost.

Table 6. *Cumulative mortality of broodstock (%) during the spawning period and 7 and 90 days after artificial and semi-artificial spawning.*

Indicator	Artificial spawning	Semi-artificial spawning
Cumulative mortality of females (%)		
During the spawning period	15	17
7 days post-spawning period	68	68
90 days post-spawning period	98	98
Cumulative mortality of males (%)		
During the spawning period	0	8
7 days post-spawning period	22	8
90 days post-spawning period	92	85

5. ECONOMIC BENEFIT OF THE TECHNOLOGY

The described technology of Eurasian perch embryo mass production will enable the fish company Rybářství Nové Hrady s.r.o. to produce several million embryos annually at minimal cost. These procedures will ensure mass production of perch embryos of similar age and positively influence the rearing of larvae and juveniles in ponds. With a sufficient food supply, fish of the same age should be of similar size, which will minimize cannibalism and increase effectiveness of rearing juveniles and, subsequently, older categories of Eurasian perch.

The aim of these procedures was to produce viable high quality Eurasian perch embryos to be reared in production ponds in a three to four year production cycle. This will increase the production of market-sized perch and, at the same time, limit the presence of less valuable fish species in the ponds to benefit production of the primary market fish, common carp.

It is difficult to calculate precisely the economic benefit of these procedures, but we estimate that, with their full employment, the aquaculture firm Rybářství Nové Hrady s.r.o. could gain several tens of thousands CZK.

6. USE OF THE TECHNOLOGY IN PERCID PRODUCTION

Procedures for mass production of high quality perch embryos described and verified in practice will be used in the aquaculture company Rybářství Nové Hrady s.r.o. In putting these procedures into effect, perch embryos, larvae, juveniles, market fish, and broodstock will be obtained for further fish production or sale.

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CHAPTER 5

HORMONAL INDUCTION OF OVULATION IN PIKEPERCH (*SANDER LUCIOPERCA* L.) USING HUMAN CHORIONIC GONADOTROPIN (hCG) AND MAMMALIAN GnRH ANALOGUE

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Hormonal induction of ovulation in pikeperch (*Sander lucioperca* L.) using human chorionic gonadotropin (hCG) and mammalian GnRH analogue

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Abstract The aim of the present study was to study spawning stimulation in artificial reproduction of females pikeperch (*Sander lucioperca* L.) using "Chorulon" containing the human chorionic gonadotropin (hCG) and compare with "Supergestran" containing a mammalian GnRH ([D-Ala^6]GnRHProNhet) analogue. The females were divided into eleven experimental groups and injected with hCG at 250, 500, 750, and 1 000 IU kg^{-1} body weight (BW) and mGnRHa at 1, 2.5, 5, 10, 25, and 50 μg kg^{-1} BW. In all treatments, a single intramuscular injection of hormone was performed. Control group was injected with 0.9 % NaCl, 0.9 cm^3 kg^{-1} BW. The average percentages of ovulating females were 88.5 ± 12.3 and 80.8 ± 10.9 % in hCG- and mGnRHa-treated groups, respectively. The average diameter of eggs was 0.95 ± 0.06 and 0.98 ± 0.06 mm in hCG- and mGnRHa-treated groups, respectively. Neither ovulation rate nor diameter of egg was statistically differed among hormonally treated groups. Statistical difference was observed only in hatching rate, where the average were 73.6 ± 14.4 and 50.6 ± 17.7 % in hCG and mGnRHa-treated groups, respectively. Among hormonally treated groups, the best results were observed in groups treated with hCG at 500 and 750 IU kg^{-1} and in groups treated with mGnRHa at 25 μg kg^{-1}. No ovulation was observed in the control group. This study indicated successful ovulation in pikeperch using a single intramuscular injection of hCG or mGnRHa analogue.

Keywords Chorulon · Egg size · Fecundity · Hatching rate · *Sander lucioperca* · Supergestran

Introduction

Pikeperch (*Sander lucioperca* L.) is a highly valuable commercial fish for inland European aquaculture (Hilge and Steffens 1996), which has an acceptable growth rate to market size

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under intensive culture (Fontaine 2009). Up to now, most market size pikeperch come from open waters (lakes, rivers, ponds, or lagoons) and relatively few are produced in fish farms under intensive and indoor conditions (FAO 2007). The stable mass production of fry for open waters or fish farms is not available yet, because the artificial reproduction of pikeperch, particularly reproductive physiology of broodfish, is not still well developed (Philipsen 2008). Therefore, the studies of optimum methods for artificial reproduction with emphasizes on broodfish reproductive physiology to achieve stable mass production of fry and grow up them to market size are still needed.

Spawning induction using hormonal injections has been frequently used to synchronize maturation of gametes (sperm or eggs) in different fish species (Bromage et al. 1992; Zohar and Mylonas 2001). There are a few studies reporting spawning induction in pikeperch using injection of carp pituitary containing gonadotropins, synthetic analogues of GnRH, human chorionic gonadotropin (hCG), or LHRH-a (Antalfi 1979; Schlumbenger and Proteau 1996; Craig 2000; Kouril and Hamackova 2005; Zakes and Demska-Zakes 2005; Rónyai 2007). Recently, a commercial Czech veterinary product approved for the use in ruminants called "Supergestran" has been successfully used in artificial propagation of perch (Kouril and Hamackova 1999, Policar et al. 2008), but the efficiency of Supergestran has not been studied in other percid fishes. The Supergestran contains mammalian GnRH ([D-Ala6]GnRHProNhet) analogue. The Chorulon has been used for artificial reproduction of African catfish (*Clarias gariepinus*) (Richter et al. 1987; Mollah and Tan 1983), rainbow sharks (*Labeo erythrurus*) (Shireman and Gildea 1989), redtail black sharks (*Labeo bicolor*) (Shireman and Gildea 1989), and of pufferfish (*Tetraodon nigroviridis*) (Watson et al. 2009).

Therefore, the present study was conducted to compare efficiency of the "Chorulon" containing hCG and the Supergestran containing mGnRHa in induction of ovulation in pikeperch under controlled conditions.

Materials and methods

Broodstock, transport, manipulation, and culture conditions

Broodfish of pikeperch were obtained from Fish farm (Rybářství Třeboň a.s., Czech Republic) during the first half of March 2010. Fish were harvested from a pond at the same time. Fish were placed in 2-m^3 plastic tank (water characteristics were: pH, 7.2 ± 0.2; oxygen saturation, 90.5 ± 4.2 %) and transferred by truck and kept in storage pond at the University of South Bohemia, Faculty of Fisheries and Protection of Waters (USB, FFPW) and fed forage fish *Pseudorasbora parva* (Total length = 40–60 mm; weight = 0.3–0.4 g) until the spawning season (April). Before the spawning season, broodfish were harvested from storage pond and sexually separated based on fish shape and genital papilla characteristics. The males (50 individuals) and females (77 Individuals) were separately kept in tank (6 m^3) of a recirculating aquaculture system (RAS) at USB, FFPW. At the time of spawning, females were divided into eleven groups and kept in 1-m^3 tanks ; each group contains seven individuals. Water characteristics were as follows: pH, 7.2 ± 0.2; oxygen saturation, 80.4 ± 3.1 %; flow rate, 12.5 L min^{-1}; temperature, 15 ± 0.5 °C; ammonia concentration, <0.02 mg L^{-1}; nitrite, <0.02 mg L^{-1}; nitrate, <0.05 mg L^{-1}; and photoperiod, 14L:10D. Water temperature was measured four times daily with an auto-recording thermometer (model RT-F5x, QiAnatytical Ltd., Czech Republic). Oxygen saturation (%) and pH were measured twice daily (06:30 and 14:00 h) with a combined pH and oxygen

meter (MultiLine P4, WTW). Other water quality parameters (nitrite, nitrate, and ammonia levels) were weekly evaluated at the chemical laboratory of USB, FFPW.

Hormonal stimulation

Before hormonal stimulation, females were tagged with PIT tags (AEG Identifikationssysteme GmbH (AEG ID), Ulm, Germany). To have no significant differences in fish size among treated groups, body weight (BW) was recorded. Maturity stage of the oocytes from each female was determined according to the method described by Kucharczyk et al. (2007). Then, females were intramuscularly under dorsal fin injected with the Chorulon containing hCG (Intervet, the Netherlands) or the Supergestran containing mGnRHa (Lecirelin (D-Tle6) GnRH*m* ProNHEt) (FERRING LÉČIVA, Czech Republic) as described by (Policar et al. 2008; Kouril et al. 2007). The biological characteristics of females in different hormonally treated groups as well as doses of injection are presented in Table 1. All individuals were anesthetized in clove oil water bath 0.03 ml L^{-1} (Dr. Kulich Ltd.) for 10 min before manipulation (Hamackova et al. 2001).

Stripping of broodfish: absolute and relative fecundity and size of eggs

Two days after hormonal treatment, females were controlled each hour by a gentle massage of the abdominal cavity. Females showing ovulation were immediately transferred to clove oil water bath, and eggs were stripped by gentle massage along abdominal cavity. Obtained eggs were weighted using a balance (PCB 1000-2, Kern, Germany with accuracy of 0.01 g) from each individual. Then, three small samples (approximately 1 g) were randomly selected and weighed using balance (ALJ 220-4, Kern, Germany with accuracy of 0.0001 g) for determination of absolute fecundity (the total number of eggs) and relative fecundity (total number of eggs per 1 kg). To measure size of eggs, pictures were taken from egg samples (100 eggs in each group) using Olympus E-510 digital camera mounted on binocular microscope Olympus BX51. Data were then collected using Quick PHOTO CAMERA 2.2 software (Olympus, Hamburg, Germany).

Table 1 Details of hormonally treated groups of pikeperch (*Sander lucioperca* L.) used in the present study

Group	Number of females	Mean ± SD body weight (g)	Treatment	Dose
Group 1	7	1,202 ± 259	hCG (IU kg^{-1} BW)	250
Group 2	7	1,188 ± 199	hCG (IU kg^{-1} BW)	500
Group 3	7	1,204 ± 295	hCG (IU kg^{-1} BW)	750
Group 4	7	1,309 ± 359	hCG (IU kg^{-1} BW)	1 000
Group 5	7	1,265 ± 565	mGnRHa (μg kg^{-1})	1
Group 6	7	1,104 ± 384	mGnRHa (μg kg^{-1})	2.5
Group 7	7	1,212 ± 392	mGnRHa (μg kg^{-1})	5
Group 8	7	1,268 ± 396	mGnRHa (μg kg^{-1})	10
Group 9	7	1,238 ± 412	mGnRHa (μg kg^{-1})	25
Group 10	7	1,283 ± 197	mGnRHa (μg kg^{-1})	50
Group 11	7	1,247 ± 282	0.7 % NaCl	

There was no significant difference in body weight

In vitro fertilization

To study whether different hormone affect fertilization rate of obtained eggs, in vitro fertilization test was performed on collected eggs. Sperm of 4 males was collected with a syringe after bathing in clove oil (Hamackova et al. 2001). Genital papilla of males was firstly dried to avoid sperm contamination by water, urine, and blood. Sperm quality was then tested after activation in hatchery water under microscope (Alavi et al. 2009). Samples with 90 % of motile spermatozoa were chosen for fertilization test. The sperm from three males (without hormonal injection) was directly added into the batches of eggs (50 μL of sperm to 100 eggs), and then hatchery water was added and mixed for 2 min. After fertilization, the eggs were washed to remove adhesiveness of the fertilized eggs with milk and talc (Gela et al. 2003). Then, three samples from each female, each contains 100 eggs, were separately incubated in experimental small cages (water temperature: 14 ± 0.7 °C and oxygen saturation: 88.5 ± 2.9 %). The incubation cages were connected to the re-circulating system. The hatching rate was determined as follows:

$$\text{Hatching rate} = (\text{Hl}/\text{Te}) \times 100$$

where "Hl" is the number of hatched larvae and "Te" is number of eggs at the beginning of fertilization.

Statistical analysis

The reproductive performances of the fish were evaluated with program Statistica 9.0 (StatSoft, Inc., Czech Republic). Reproductive parameters (latency time, relative and absolute fecundity, size of eggs, and hatching rate) were statistically analyzed by one-way analyses of variance, ANOVA ($P < 0.05$) followed by the post hoc Tukey's multiple-comparison tests. Percentage data were transformed prior to analyses with arcsin function. Differences between hormonal preparations were statistically analyzed by one-way analyses of variance ANOVA ($P < 0.05$) by the post hoc mean comparisons (Unequal N HSD comparison test).

Results

Ovulation rate, latency time, and hatching rate

The all females had oocytes exclusively in maturity stage II or in stages III and IV. The highest ovulation rate (100 %) was observed in females treated with mGnRHa (25 μg kg^{-1}) or with hCG (500 and 750 IU) (Table 2). The average percentages of ovulated females were 88.5 ± 12.3 % in the hCG-treated groups and 80.8 ± 10.9 % in mGnRHa-treated groups. Ovulation rate did not differ among hormonally treated groups, statistically. The latency time ranged from 78.05 to 89.29 h after injection (Table 2), but no significant difference was observed among hormonally treated groups. Hatching rate showed significant differences among treated groups (Table 2). The highest hatching rate was observed in groups stimulated with hCG at 500 and 750 IU. The average hatching rate was 73.6 ± 14.4 and 50.6 ± 17.7 % in hCG- and mGnRHa-treated groups, respectively.

Table 2 Ovulation rate, latency time, and hatching rate in females of pikeperch (*Sander lucioperca* L.) treated with single intramuscular injections of Chorulon containing human chorionic gonadotropin (hCG) and Supergestran containing mammalian GnRH without dopamine inhibitor

Group ($n = 7$)	Ovulation (%)	Latency time (h)	Hatching rate (%)
hCG 250 IU	71	84.98 ± 9.89	70.9^{bc} ± 4.1
hCG 500 IU	100	78.05 ± 6.93	84.2^{c} ± 6.2
hCG 750 IU	100	78.59 ± 7.48	86.8^{c} ± 3.8
hCG 1 000 IU	83	88.0 ± 12.05	52.5^{b} ± 4.5
mGnRHa 1 μg kg^{-1}	86	89.29 ± 11.73	52.3^{b} ± 5.5
mGnRHa 2.5 μg kg^{-1}	71	84.42 ± 11.46	65.5^{b} ± 2.5
mGnRHa 5 μg kg^{-1}	71	83.90 ± 21.64	51.1^{b} ± 3.6
mGnRHa 10 μg kg^{-1}	71	79.4 ± 9.24	52.2^{b} ± 4.5
mGnRHa 25 μg kg^{-1}	100	83.0 ± 9.13	60.5^{b} ± 6.4
mGnRHa 50 μg kg^{-1}	86	86.46 ± 27.67	22.10^{a} ± 7.2
Control	n.d.	n.d.	n.d.

Data are shown as mean ± SD

n.d. values are not determined due to no ovulation of females

There was no significant difference in latency period among treated groups

Values within the column with different superscripts are significantly different ($P < 0.05$)

Weight of eggs, relative fecundity, absolute fecundity, and size of eggs

The percentage of weight of eggs was similar among treated groups and ranged from 5.22 % observed in group treated with mGnRHa at 50 μg kg^{-1} to 10.89 % observed in group treated with mGnRHa 2.5 μg kg^{-1} (Table 3). No significant differences were found between groups in terms of relative fecundity (ranged from 88.0 to 144.6 × 10^3 eggs kg^{-1}) and absolute fecundity (ranged from 164.6 to 208.3 × 10^3 eggs) (Table 3). Size of non-fertilized eggs did not also differ among treated groups and ranged from 0.917 to 1.012 mm. Average diameter of eggs was 0.949 ± 0.06 mm in the hCG-treated groups and 0.976 ± 0.057 mm in the mGnRHa-treated groups.

Discussion

The present study showed successful ovulation induction in pikeperch hormonally treated with a single intramuscular injection of Supergestran and Chorulon. All females were determined in stages II, III, IV before injection, and during the ovulation were also observed stages V and VI (Zarski et al. 2012). The ovulation rate observed in the present study (88.5 and 80.8 % in the hCG- and mGnRHa-treated groups) is similar to those of Zakes and Demska-Zakes (2005) and Rónyai (2007), where two injections were performed for stimulation of ovulation. Therefore, a single injection is suggested to reduce stress in females due to hormonal treatment, which may affect the quality of ovulated eggs (Schreck et al. 2001).

It is shown that Supergestran, containing mGnRHa, could be introduced for artificial reproduction of fish in hatcheries as has been used for induction of ovulation in perch (Policar et al. 2008). In the present study, fully ovulated females of pikeperch were observed when hCG at 500–750 IU kg^{-1} was injected. Very similar ovulation rate (93 %)

Table 3 Weight of eggs, relative fecundity, absolute fecundity, size of eggs in females of pikeperch (*Sander lucioperca* L.) treated with single intramuscular injections of Chorulon containing human chorionic gonadotropin (hCG) and Supergestran containing mammalian GnRH without dopamine inhibitor

Group	Percentage eggs (% of female BW)	Relative fecundity (number of eggs $\times 10^3$ per 1 kg)	Absolute fecundity (number of eggs $\times 10^3$)	Size of egg (mm)
hCG 250 IU	$8.08^a \pm 1.3$	$88.7^a \pm 18$	$180.7^a \pm 99$	$1.011^a \pm 0.6$
hCG 500 IU	$7.00^a \pm 3.0$	$101.3^a \pm 23$	$189.2^a \pm 64$	$0.971^a \pm 0.5$
hCG 750 IU	$9.21^a \pm 5.3$	$116.7^a \pm 58$	$181.6^a \pm 89$	$0.982^a \pm 0.5$
hCG 1 000 IU	$6.51^a \pm 2.6$	$106.1^a \pm 45$	$168.8^a \pm 50$	$0.946^a \pm 0.5$
mGnRHa 1 μg kg^{-1}	$9.26^a \pm 2.8$	$120.1^a \pm 64$	$208.3^a \pm 86$	$0.928^a \pm 0.5$
mGnRHa 2.5 μg kg^{-1}	$10.89^a \pm 1.8$	$88.0^a \pm 82$	$124.5^a \pm 73$	$0.949^a \pm 0.4$
mGnRHa 5 μg kg^{-1}	$9.52^a \pm 6.2$	$133.9^a \pm 60$	$175.0^a \pm 78$	$1.012^a \pm 0.6$
mGnRHa 10 μg kg^{-1}	$7.93^a \pm 2.5$	$142.3^a \pm 79$	$168.7^a \pm 86$	$0.978^a \pm 0.2$
mGnRHa 25 μg kg^{-1}	$7.58^a \pm 3.9$	$144.6^a \pm 82$	$196.0^a \pm 80$	$0.920^a \pm 0.5$
mGnRHa 50 μg kg^{-1}	$5.22^a \pm 3.5$	$127.6^a \pm 67$	$164.6^a \pm 82.6$	$0.917^a \pm 0.1$
Control	n.d.	n.d.	n.d.	n.d.

Data are shown as mean ± SD

n.d. values are not determined due to no ovulation of females

There was no significant difference in any measured parameters among treated groups

has been reported at 700 IU kg^{-1} (Kucharczyk et al. 2008). It is well known that hCG directly acts at the level of gonads and does not require the existence of LH stores or activation of the pituitary gonadotropins (Zohar and Mylonas 2001). The higher cost of Chorulon preparation is a disadvantage compared with that of Supergestran. Better ovulation rate, spawning synchronization, and hatching rate were observed in treated group with Chorulon (hCG) than with Supergestran, but it is possible that the treated females do not response to hormonal treatment in the next spawning season (Van der Kraak et al. 1989; Watanabe et al. 1998). This is probably due to the large size of the GtH molecule and its heterogenous nature, which may consequently result in inducing the immune response of some fish species. (Mylonas and Zohar 1997). It is also shown that that low dose of mGnRHa (25 μg kg^{-1}) could induce ovulation in a substantial number of pikeperch. This is lower than the dose that has been used for ovulation in perch (50–125 μg kg^{-1}) (Policar et al. 2008; Kouril and Hamackova 1999). Schlumbenger and Proteau (1996) also showed ovulation induction in pikeperch at 100 μg kg^{-1} mGnRHa. In the control group, no ovulated female was observed, suggesting no possible effects pheromones in ovulation of pikeperch (Rónyai 2007).

Several studies show shorter latency period in females treated with hormones acting on gonads (such as hCG, carp pituitary) compared with hormones acting on brain (GnRHa) (Zohar and Mylonas 2001). On the other hand, no differences were found between hCG- and mGnRH-treated groups in this study. Latency time in the hCG-treated groups was similar to the results reported by Rónyai (2007), who performed two injections. Nevertheless, latency time in the mGnRH was also similar to the results described by Policar et al. (2008), who used only one injection.

The relative fecundity (from 88.0 to 144.6 $\times 10^3$ eggs kg^{-1}) of pikeperch obtained in this study is lower compared with Lappalainen et al. (2003) (250 $\times 10^3$ eggs kg^{-1}) and to Schlumbenger and Proteau (1996) (200 $\times 10^3$ eggs kg^{-1}). The absolute fecundity (from

164.6 to 208.3 $\times$ 10^3 eggs) of pikeperch recorded in this study is similar to the results reported by Demska-Zakes and Zakes (2002) and Lappalainen et al. (2003). Also, size of eggs showed no difference with the previous studies (Horvath et al. 1984; Schlumbenger and Proteau 1996; Lappalainen et al. 2003). The size of unfertilized eggs is very constant and not responded results corresponded by Demirkalp (1992) and Barus and Oliva (1995), which reported ranged size of unfertilized eggs (0.5–1.4 mm).

In conclusion, our study on pikeperch demonstrated that a single hormonal injection of Chorulon containing hCG or Supergestran containing mGnRHa could be used for induction of ovulation. Although we observed better efficiency of hCG compared with that of mGnRHa, further studies are needed to look at reproductive status of broodfish in the next spawning season. This study showed the highest quality of eggs at 500 and 750 IU kg^{-1} hCG and in result at 25 μg kg^{-1} mGnRHa. The Supergestran is suggested to be used in fish farm due to lower price and its availability in the Czech Republic. Further studies are required to optimize protocols for using Supergestran in artificial reproduction of pikeperch via looking at physiological functions of hypothalamus–pituitary–gonad axis.

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CHAPTER 6

SPERM MORPHOLOGY, FINE STRUCTURE AND MOTILITY IN PIKEPERCH, *SANDER LUCIOPERCA* (PERCIDAE, TELEOSTEI) USING DIFFERENT ACTIVATION MEDIA

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SPERM MORPHOLOGY, ULTRASTRUCTURE, AND MOTILITY IN PIKEPERCH, *SANDER LUCIOPERCA* (PERCIDAE, TELEOSTEI) ASSOCIATED WITH VARIOUS ACTIVATION MEDIA

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ABSTRACT

Spermatozoa morphology, ultrastructure, and spermatozoa motility traits were studied in pikeperch (*Sander lucioperca*) after activation in various media (AM 1, 45 mM NaCl, 5 mM KCl, 20 mM Tris, pH 8.5; AM 2, 100 mM Sucrose, 20 mM Tris, pH 8.5; AM 3, 100 mM sucrose, 1 mM $CaCl_2$, 20 mM Tris, pH 8.5) during a 48 h storage period. The spermatozoon was acrosomeless and differentiated into a spherical nucleus (head), midpiece, and flagellum. The nucleus length and width measured 1.83 ± 0.03 and 1.63 ± 0.02 mm, respectively. The midpiece was located lateral to the nucleus and possessed proximal and distal centrioles and 2–4 mitochondria. Flagellar length was 33.2 ± 0.90 µm, and a pair of lateral fin-like structures projections was observed. The axoneme consisted of nine peripheral doublet microtubules and a single central pair. After 24 h storage in all activation media at all sampling times post-activation (15, 45, 90, and 120 s), spermatozoa motility was significantly decreased. Spermatozoa were motile after 48 h storage at all sampling times post-activation only in AM 3. After 48 h storage, no motile spermatozoa were observed in AM 2 and AM 1 at 90 and 120s post-activation, respectively. Differences in spermatozoa velocity varied with activation medium during storage. After 48 h storage in AM 1 and AM 2, decrease of spermatozoa velocity at 15 s post-activation was observed, but, in AM 3, velocity was decreased only after 48 h storage. Pikeperch spermatozoa morphology and ultrastructure was found similar to that of most freshwater teleosts, with differences in arrangement of midpiece, number of mitochondria, and position of centrioles. Viable pikeperch sperm was observed after 48 h storage. Motility of spermatozoa was improved by addition of Ca^{2+} to the activation medium, where higher spermatozoa velocity was observed.

Key words: *Spermatozoa motility, Spermatozoa velocity, Calcium, Storage time*

INTRODUCTION

Pikeperch is a highly valuable fish in commercial aquaculture. Presently, most market size pikeperch are caught from open waters (lakes, rivers, ponds, and lagoons), and relatively few are produced under intensive and/or indoor conditions (Kucharczyk et al., 2007). The total catch of pikeperch was reported about 50 000 t in 1950 and 18 000 t in 2007 (FAO, 2008). Global aquaculture production for this species increased from 200 t in 1990 to about 500 t in 2007 (FAO, 2008). In pikeperch breeding and culture, most studies have focused on improving methods for stimulating ovulation and increasing the quality of the eggs and larvae (Zakes Demska-Zakes, 2005; Ronyai, 2007; Zakes, 2007). Less is known about the biology of sperm and its quality.

In fish farming, good broodstock management is a prerequisite for successful artificial breeding and is important for ensuring consistent production to meet market demand (Billard et al., 1995; Alavi et al., 2008; Mylonas et al., 2010). To date, a large number of studies have focused upon the management of female broodstock in fish farms. However, it is vital to also manage male broodfish, especially in species with low potential for producing semen and a high risk of sperm contamination by urine during stripping and failure of sperm release in captivity due to stress (Alavi et al., 2008).

Spermatozoa motility in fish, particularly freshwater species such as pikeperch, lasts from several seconds to several minutes (Alavi et al., 2008; Cosson, 2010), and is a key factor in fertilization rate (Billard et al., 1995; Linhart et al., 2008). Due to this short duration of motility and the physiology of the micropyle, which becomes blocked after release of the ova into the aquatic environment (Billard et al., 1995; Alavi et al., 2008; Mylonas et al., 2010), spermatozoa should penetrate the egg within a few seconds of activation (Kudo, 1991). Therefore, use of an activation medium with high potential for triggering activation and identification of factors influencing the viability of sperm after stripping are invaluable for artificial reproduction (Billard et al., 1995; Alavi et al., 2008).

In pikeperch, the quality of spermatozoa differs widely among individuals, and semen production is low (Cejko et al., 2008; Korbuly et al., 2009). The urinary bladder is close to the urogenital pore, which may decrease the initial quality of sperm (Perchec Poupard et al., 1998). Some characteristics of seminal plasma and spermatozoa m motility in pikeperch have been reported by Cejko et al. (2008). Significant decrease of spermatozoa m viability and motility has been reported after 3 h storage of non-diluted sperm or of sperm diluted in various immobilizing media (Korbuly et al., 2009).

Knowledge of spermatozoa morphology and ultrastructure provides information for fish phylogeny and taxonomics and aids in identification of relationships among spermatozoa morphology, reproductive biology, and spermatozoa motility and velocity (Jamieson, 1991, 2009; Lahnsteiner and Patzner, 2008). Spermatozoa morphology and ultrastructure can also be an indicator of male fertility when fish are exposed to endocrine disrupting chemicals or when spermatozoa are manipulated for genome banking such as cryopreservation (Billard et al., 2000; Hatef et al., 2010a; Butts et al., 2011).

The present study was conducted to describe the morphology and ultrastructure of spermatozoa using scanning electron microscopy and to investigate spermatozoa

motility and velocity during short-term storage. Ultrastructure of pikeperch spermatozoa has been studied by Lahnsteiner and Mansour (2004). Our results add complementary information to previous studies, particularly by including morphological parameters of spermatozoa and determining motility and velocity within 48 h of sperm collection.

MATERIALS AND METHODS

Broodfish and sperm collection

Sperm was collected from 12 mature pikeperch (body mass: 871.58 g ± 31.00 (mean ± standard deviation) and total length: 46.69 ± 0.41) 48 h after spermiation induction using a single intramuscular injection of hCG at 500 IU kg^{-1}(Křišťan et al., 2013). Spermatozoa concentration was assessed by light microscopy, using a haemocytometer, following the method of Alavi et al. (2009a).

Sperm morphology

Sperm from four males was fixed in with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.5) and stored at 4 °C until transfer to the Electron Microscopy Laboratory, University of South Bohemia in České Budějovice. Samples were post-fixed and washed repeatedly for 2 h in 4% osmium tetroxide at 4 °C and dehydrated through an acetone series. Samples for scanning electron microscopy (SEM) were dehydrated in a critical point dryer, Pelco CPD 2 (Ted Pella, Inc., Redding, California, USA). Sperm samples were coated with gold under vacuum with an SEM Coating Unit E5100 (Polaron Equipment Ltd., England) and examined using a JSM 7401-F (JEOL Ltd., Akishima, Tokyo, Japan) equipped with a Sony CCD camera. Samples for transmission electron microscopy (TEM) were embedded in resin (Polybed 812). A series of ultrathin sections were cut using a Leica UCT ultramicrotome (Leica Mikrosysteme Gmbh, Austria) and double-stained with uranyl acetate and lead citrate. Samples were viewed in a TEM JEOL 1010 (JEOL Ltd., Tokyo, Japan) operated at 80 kV. Micrographs were evaluated using the Olympus MicroImage software (version 4.0.1. for Windows) to measure spermatozoa morphological parameters.

Sperm motility assessment

Sperm motility and velocity of non-diluted sperm were analysed at room temperature during short-term storage (6 °C), at 0, 6, 24, and 48 h after collection. At each storage time, sperm motility was directly activated, without dilution in immobilizing medium, in the following activation media: Activation medium 1 (AM 1) was composed of ionic compounds (45 mM NaCl, 5 mM KCl), while activation medium 2 (AM 2) was a non-ionic compound (100 mM Sucrose). Activation medium 3 (AM 3) was composed of a non-ionic compound (100 mM sucrose) plus 1 mM $CaCl_2$. All activation media were buffered by adding 20 mM Tris, and pH was adjusted to 8.5. A small drop of sperm was directly diluted in 50 µl of the activation medium at a sperm: AM of about

1 : 2 000. Bovine serum albumin (BSA) was added to the activation medium at a final concentration 0.1% w/v to avoid sticking of the sperm cells onto the slide. Immediately after addition of sperm to the drop, sperm motility was recorded 2 min per observation using a three CCD video camera (SONY DXC-970MD, Japan) mounted on a dark-field microscope (Olympus BX50, Japan) and a DVD-recorder (SONY DVO-1000 MD, Japan). The microscope was equipped with a stroboscopic lamp with frequency adjusted to 50 Hz. A computer-assisted image analysis (Olympus Micro Image 4.0.1 for Windows) was used to measure percent sperm motility and sperm velocity ($\mu m.s^{-1}$) from five successive video images, which showed positions of sperm heads following Hatef et al. (2010b). Five video frames were captured from a DVD-recorder (SONY DVO-1000 MD, Japan) and accumulated in real time post-activation. The output frame showed positions of heads of motile spermatozoa in five spots (red-green-green-green-blue), while the immotile spermatozoa appeared as white. The percent motile spermatozoa was calculated by counting red or blue spots versus the number of white spots. To measure velocity, the distance between red and blue spots (five head positions) were measured and divided into the duration of travel. Velocity was calculated only for motile spermatozoa (50–60 spermatozoa per treatment).

DATA ANALYSIS

Homogeneity of variance was tested for all data using Levene's test. Repeat measures of analysis of variance (ANOVA) were used to understand the effects of storage time and activation medium and their interactions on sperm motility and velocity measured at different times post-activation; alpha was set at 0.05. When significance of storage time was observed, the model was revised to individual ANOVA models for each activation medium to analyse the effects of storage time on spermatozoa motility and velocity using a single-factor ANOVA followed by Tukey's post-hoc test. All data are presented as mean ± standard error of mean (S.E.M.).

RESULTS

Sperm morphology and ultrastructure

The pikeperch spermatozoon is a primitive acrosomeless aquasperm differentiated into a nucleus (head), a small midpiece, and a flagellum (Figs. 1a–c, 2a–d). The nucleus dimensions were measured at 1.83 ± 0.03 mm length and 1.63 ± 0.02 mm width. The midpiece was located lateral to the nucleus (Fig. 1a–c). Longitudinal (Fig. 2a,b) and cross (Fig. 2c) sections of midpiece showed the presence of proximal and distal centrioles consisting of nine triplets of microtubules, at right angle to each other. In some cases, the proximal centriole was inclined to the distal centriole at an angle 110° (Fig. 2b). The distal centriole served as the basal body of the flagellum. Two to four mitochondria were observed in the midpiece between the plasma membrane and centriole complex (Fig, 2a–c). A cytoplasmic channel was clearly visible close the plasma membrane around midpiece and flagellum. A lateral pair of fin-like projections was observed along the flagellum, originating from extension of the plasma membrane around the flagellum. The flagellum, 33.2 ± 0.90 µm in length, was composed of a typical eukaryote microtubule-based axoneme. The axoneme

was composed of nine peripheral doublet microtubules and a single central pair (Fig. 2d). Outer and inner dynein arms, radial spokes, and other fine structure of the axoneme were observed in pikeperch spermatozoa (Fig. 2d).

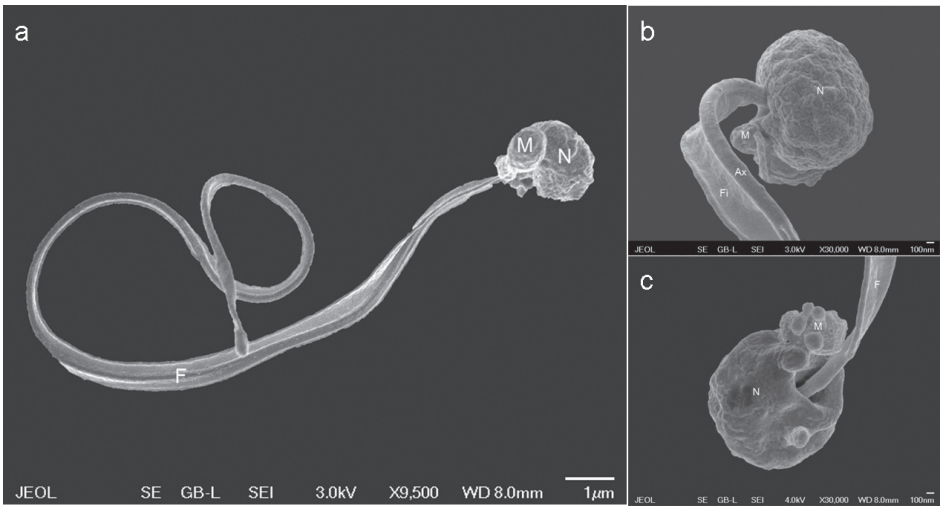

Figure 1. *Scanning electron micrograph of pikeperch (Sander lucioperca) spermatozoon. A, b and c show position of midpiece. N, nucleus; M, midpiece; F, flagellum; Ax, axoneme; Fi, fin-like projection.*

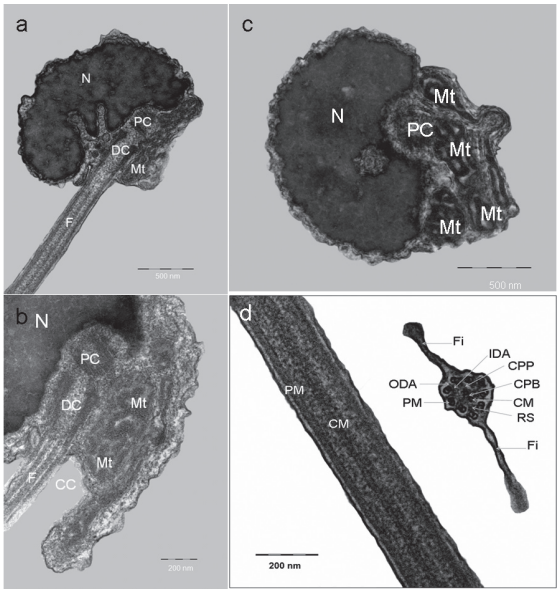

Figure 2. *Transmission electron micrograph of longitudinal section (a, b), cross section in the midpiece region (c), and axonemal ultrastructure of pikeperch (Sander lucioperca) spermatozoon. N, nucleus; Mt, mitochondria; CC, cytoplasmic channel; DC, distal centriole; PC, proximal centriole; F, flagellum; Fi, fin-like projection; IDA, inner dynein arm; ODA, outer dynein arm; CPB, central pair bridge; CM, central microtubule; PM, peripheral microtubule; RS, radial spoke; CPP, central pair projections.*

Sperm concentration, motility and velocity

Mean ± S.E. sperm concentration was found to be 14.87 ± 1.36 per ml. Only storage time showed significant effects on spermatozoa motility at different times post-activation (Table 1). Therefore, the model was re-run with the storage time × activation medium interaction effects removed and the effects of storage time and activation medium were interpreted. Results showed significant effects of storage time on sperm motility ($F_{df,\ 15\ s\ post\text{-}activation} = 29.68_3$; $F_{df,\ 45\ s\ post\text{-}activation} = 27.06_3$; $F_{df,\ 90\ s\ post\text{-}activation} = 29.64_3$; $F_{df,\ 120\ s\ post\text{-}activation} = 13.33_3$ ($p < 0.001$). Activation media did not influence sperm motility ($F_{df,\ 15\ s\ post\text{-}activation} = 0.07_2$; $F_{df,\ 45\ s\ post\text{-}activation} = 0.06_2$; $F_{df,\ 90\ s\ post\text{-}activation} = 0.43_2$; $F_{df,\ 120\ s\ post\text{-}activation} = 0.11_2$ ($p > 0.05$). Therefore, the models were revised to calculate the effects of storage time on motility after activation in each activation medium separately for each time post-activation. At all times post-activation (15, 45, 90, and 120 s), motility was significantly affected by storage time in all media (Table 2) and decreased at 24 h storage (Fig. 3). Spermatozoa were motile after 48 h storage in AM 3 at all times post-activation (Fig. 3). After 48 h storage, no motile spermatozoa were observed in AM 2 at 90 s post-activation and in AM 1 at 120 s post-activation, (Fig. 3c–d).

Table 1. *Summary of statistics (Fdf) obtained from repeated ANOVA models used to study the effects of storage time, activation medium, and their interaction (storage time × activation medium) on spermatozoa motility and velocity in pikeperch (Sander lucioperca) at various times post-activation.*

Parameter	Time post-activation (s)	Storage time	Activation medium	Storage time × Activation medium
Spermatozoa motility	15	26.513***	0.752	0.506
	45	22.123***	0.192	0.576
	90	23.053***	0.302	0.506
	120	10.443***	0.052	0.426
Spermatozoa velocity	15	18.573***	8.422***	1.376
	45	6.723***	10.482**	0.626
	90	7.103***	4.472	0.386
	120	3.913*	0.972	1.196

*, P < 0.05; **, P < 0.01; ***, P < 0.001

Table 2. *Summary of statistics (Fdf) obtained from ANOVA models used to study the effects of storage time on spermatozoa motility and velocity in pikeperch (Sander lucioperca) at various times post-activation in different activation media.*

Parameter	Time post-activation (s)	Activation medium		
		AM 1	AM 2	AM 3
spermatozoa motility	15	4.623*	4.493*	4.403*
	45	7.583**	5.633*	4.563*
	90	5.873*	5.493*	7.263**
	120	5.333*	4.813*	8.143**
spermatozoa velocity	15	4.013*	2.493*	52.423***
	45	3.563*	2.243*	2.533*
	90	12.203**	0.073	4.253**
	120	1.003	0.023	5.533**

*, P < 0.05; **, P < 0.01; ***, P < 0.001

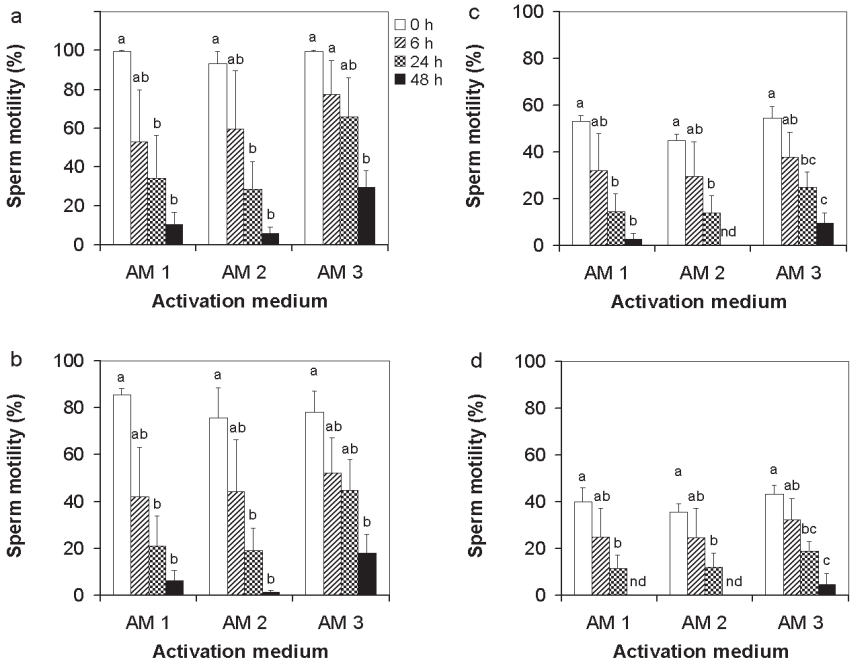

Figure 3. *Spermatozoa motility in pikeperch (Sander lucioperca) under 4 storage periods measured at 15 (a), 45 (b), 90 (c) and 120 (d) s post-activation in different activation media (AM 1, 45 mM NaCl, 5 mM KCl, 20 mM Tris, pH 8.5; AM 2, 100 mM Sucrose, 20 mM Tris, pH 8.5; AM 3, 100 mM sucrose, 1 mM $CaCl_2$, 20 mM Tris, pH 8.5). For each activation medium, values with similar superscripts are not significantly affected by incubation time (P > 0.05).*

Significant effects of both storage time and activation medium were observed on spermatozoa velocity (Table 1). As no significant effect of storage time × activation medium interactions was observed (Table 1), this effect was removed from the model, and the effects of storage time and activation medium were interpreted. Results showed significant effects of storage time on spermatozoa velocity at all evaluated times post-activation ($F_{df,\ 15\ s\ post\text{-}activation} = 11.39_3$, $p < 0.001$; $F_{df,\ 45\ s\ post\text{-}activation} = 5.13_3$, $p < 0.01$; $F_{df,\ 90\ s\ post\text{-}activation} = 7.49_3$, $p < 0.001$; $F_{df,\ 120\ s\ post\text{-}activation} = 3.96_3$, $p < 0.05$). Activation media influenced spermatozoa velocity at 45 s ($F_{df} = 7.13_2$, $p < 0.01$) and 90 s ($F_{df} = 3.39_2$, $p < 0.05$) post-activation, but not at 15 s ($F_{df} = 2.21_2$, $p > 0.05$) and 120 s ($F_{df} = 0.56_2$, $p > 0.05$) post-activation. Therefore, for each time post-activation, the models were revised to separately analyse the effects of storage time on velocity after activation in each medium. At 15 and 45 s post-activation, spermatozoa velocity was significantly affected by storage time in all activation media (Table 2). After 48 h storage, activation in AM 1 resulted in decreased spermatozoa velocity at 15, 45, and 90 s post-activation, but no difference was observed among storage times at 120 s post-activation (Fig. 4). After 48 and 24 h storage in AM 2, spermatozoa velocity at 15 s and 45 s post-activation, respectively, was significantly decreased, while no velocity were observed at 90 and 120 s post activation (Fig. 4). Spermatozoa velocity showed significant decrease at 15 s post-activation after 24 h storage (Fig. 4).

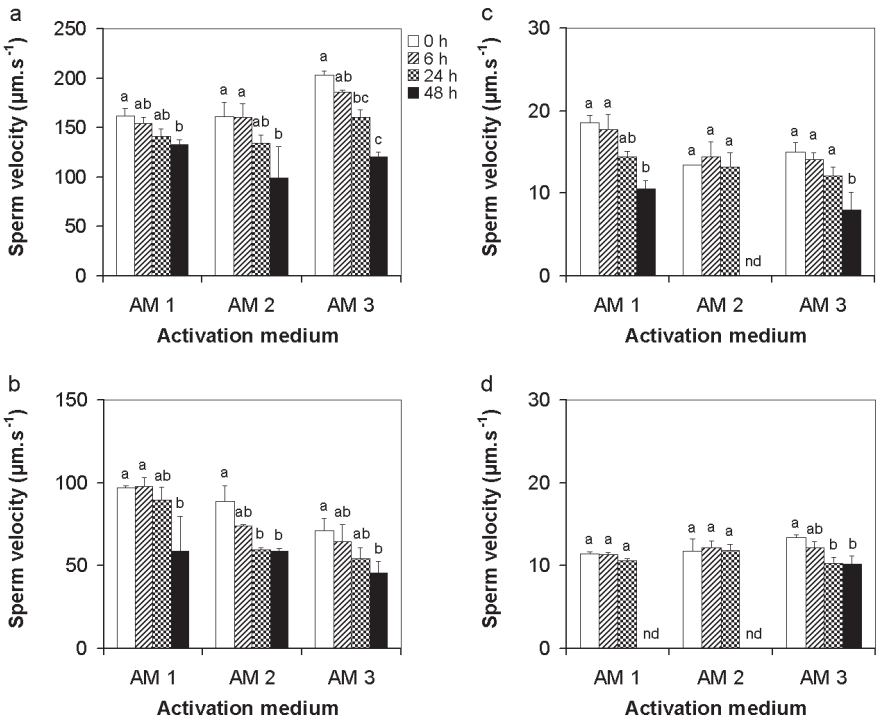

Figure 4. *Spermatozoa velocity in pikeperch (Sander lucioperca) under 4 storage periods measured at 15 (a), 45 (b), 90 (c) and 120 (d) s post-activation in different activation media (AM 1, 45 mM NaCl, 5 mM KCl, 20 mM Tris, pH 8.5; AM 2, 100 mM Sucrose, 20 mM Tris, pH 8.5; AM 3, 100 mM sucrose, 1 mM $CaCl_2$, 20 mM Tris, pH 8.5). For each activation medium, values with similar superscripts are not significantly affected by incubation time (P > 0.05).*

DISCUSSION

In the present study, spermatozoa morphology, ultrastructure, and motility traits were studied in pikeperch after activation in different media during storage period. The motility of spermatozoa was evaluated within 2 min of activation during the storage period. Storage time showed significant effects on motility, and both storage time and activation medium showed significant effects on sperm velocity.

To date, among 159 species in 9 genera of Percidae (Lahnsteiner et al., 2008), spermatozoa morphology and ultrastructure have been studied only in Eurasian perch *Perca fluviatilis* (Lahnsteiner et al., 1995; Hatef et al., 2010b) and pikeperch (Lahnsteiner and Mansour, 2004). Nucleus shape seems to be similar in Eurasian perch and pikeperch, both exhibiting a spherical nucleus (Lahnsteiner et al., 1995; Lahnsteiner and Mansour, 2004; Hatef et al., 2011b). Previous studies have shown a larger spermatozoa nucleus in Eurasian perch (1.9 x 1.8 µm) (Lahnsteiner et al., 1995) compared to that of pikeperch (1.6 x 1.3 µm) (Lahnsteiner and Mansour, 2004), and the present study show similar dimensions (1.8 x 1.6 µm). The minimal difference observed between the present study and that of Lahnsteiner and Mansour (2004) might be related to inter-individual differences as has been previously reported (Alavi et al., 2008; Psenicka et al., 2008; Hatef et al., 2011). Midpiece in pikeperch was found to be located lateral to the nucleus, in agreement with Lahnsteiner and Mansour (2004), while in Eurasian perch it is located at the base of the nucleus where the flagellum originates (Lahnsteiner et al., 1995; Hatef et al., 2011). The present study and that of Lahnsteiner and Mansour (2004) showed similar ultrastructure of centrioles consisting of nine triplets of microtubules, but the organization of centrioles seems to differ between Eurasian perch and pikeperch. The proximal centriole was inclined to the distal centriole under angle 90 or 110° in pikeperch (Lahnsteiner and Mansour, 2004; the present study), but the angle was 90° in Eurasian perch (Lahnsteiner et al., 1995; Hatef et al., 2010b. One or 2 mitochondria has been previously reported in Eurasian perch (Lahnsteiner et al., 1995; Hatef et al., 2011), but 2–4 mitochondria were observed in the present study. In both species, cytoplasmic channel has been observed between the flagellum and midpiece. The present study presents the first record of flagellar length at 33.2 µm, similar to that of Eurasian perch at 30–35 µm (Lahnsteiner et al., 1995). In both Eurasian perch and pikeperch, the axoneme consists of 9 peripheral doublet microtubules and a central pair, similar to other eukaryotes (Inaba, 2008). A lateral fin-like projection was observed in pikeperch, but not reported in Eurasian perch (Lahnsteiner et al., 1995; Hatef et al., 2010b). This structure has been previously reported in sturgeon (Psenicka et al., 2008; Hatef et al., 2011) and pike, *Esox lucius* (Alavi et al., 2009).

Initial spermatozoa motility in pikeperch observed in the present study is similar to that of Eurasian perch, but velocity seems to be higher (160 vs. 115–130 µm.s^{-1}) (Lahnsteiner et al., 1995; Alavi et al., 2007). The difference in velocity between Eurasian perch and pikeperch spermatozoa might be related to the presence of a lateral fin-like projection, which enhances flagellar movement during sperm activation, or to initial ATP content (Cosson, 2010).

Motility in pikeperch spermatozoa observed in the present study was higher than that reported by Cejko et al. (2008). They reported sperm motility of 15–24% after activation in water, but, in the present study, initial motility ranged from 92–100%. This difference may be related to initial quality of sperm rather than the hypo-

osmolality required for sperm activation in freshwater teleosts (Alavi and Cosson, 2006; Morisawa, 2008). Perchec-Poupard et al. (1995, 1998) showed significant effects of urine on both spermatozoa motility and velocity via modulation of ATP content of spermatozoa, which is required for sperm activation after triggering by a hypo-osmotic signal.

Storage period significantly affected both motile cells and velocity in pikeperch spermatozoa. Initial sperm motile cells and velocity, analysed at 15 s post-activation, was 92–99 % and 161–204 $\mu m.s^{-1}$ at 0 h storage and decreased to 6–30% and 9–132 $\mu m.s^{-1}$ at 48 h storage. At 120 s post-activation, spermatozoa motility was decreased from 36–43% (0 h storage) to 0–5% (48 h storage of sperm), but sperm velocity remained unchanged (11–13 $\mu m.s^{-1}$ at 0 h and 10 $\mu m.s^{-1}$ after 48 h). Decrease in spermatozoa motility and velocity have been frequently documented in fish species including common carp, *Cyprinus carpio* (Saad et al., 1988), halibut (Hippoglossus stenolepis) (Billard et al., 1993); turbot, *Psetta maxima* (Chereguini et al., 1997), tench, *Tinca tinca* (Rodina et al., 2004), and Eurasian perch (Hatef et al., 2011). These decreases have been attributed to damage of spermatozoa ultrastructure and decrease of ATP content (Billard et al., 1995; Hatef et al., 2011). Rodina et al. (2004) observed lower fertilizing ability of stored sperm of tench and suggested use of an immobilizing medium with osmolality similar or slightly higher than that of seminal plasma for sperm storage. In pikeperch, Korbuly et al. (2009) studied incubation of sperm using various immobilizing media and observed better spermatozoa motility after 3 h incubation compared to a control when the sperm was previously diluted in Ringer's solution or PBS. In the present study, the sperm was not pre-diluted in immobilizing medium, as the aim was to quantify viability of sperm during storage. The results showed that pikeperch spermatozoa usually live for 24 h after sperm collection, and very low sperm motility could be achieved at 48 h storage after activation in medium containing 1 mM Ca^{2+}. This suggests participation of Ca^{2+} in spermatozoa activation, as has been reported in freshwater teleosts (Alavi and Cosson, 2006; Morisawa, 2008).

The present study showed spermatozoa activation in pikeperch both after dilution in an ionic medium (AM 1 and AM 3) and in non-ionic medium (AM 2). This suggests a similar mechanism of sperm activation in Eurasian perch and pikeperch (Alavi et al., 2007). However, activation media in the present study influenced velocity, but not motility. Spermatozoa motility evaluated at 15 s post-activation did not differ with respect to activation media at 0 h storage, but, after 48 h storage, the highest motility was observed with activation in AM 3 (30%) compared to that in AM 1 (10%) and AM 2 (6%). At 120 s post-activation, spermatozoa motility did not differ with activation media at 0 h storage, but, after 48 h storage, motile spermatozoa were observed only with activation in AM 3. At 15 s post-activation, the highest spermatozoa velocity was observed after activation in AM 3 (204 $\mu m.s^{-1}$), compared to AM 1 (162 $\mu m.s^{-1}$) and AM 2 (161 $\mu m.s^{-1}$) at 0 h storage, and did not differ at 48 h storage (132, 99, and 120 $\mu m.s^{-1}$ in AM 1, AM 2, and AM 3, respectively). The observed higher velocity with AM 3 might be related to the presence of Ca^{2+}. It is known that Ca^{2+} enhances axonemal beating. Similar effects of Ca^{2+} have been reported in velocity of Eurasian perch spermatozoa (Alavi et al., 2007).

The present study showed similar morphology and ultrastructure of Eurasian perch and pikeperch, with differences observed in arrangement of midpiece and centrioles and the presence of fins along the flagellum. The present study suggests use of an activation medium containing Ca^{2+} for activation of short-term stored sperm.

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CHAPTER 7

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

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

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ABSTRACT

Elimination of egg stickiness is one of the most important factors in artificial reproduction of pikeperch (*Sander lucioperca* L.). The efficacy of Alcalase for removing adhesive from pikeperch eggs was investigated. The eggs of 10 female and 10 male pikeperch were treated with Alcalase at 0.5, 1.0, 1.5, 2.0, or 5.0 mL L^{-1} or a milk/talc solution 2 min post-insemination. Duration of exposure was 2 min and 60 min in Alcalase and milk/talc, respectively. The highest 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 rate were significantly lower compared other groups following the 5.0 mL L^{-1} (56.4%) and in milk/talc (61.3%) treatments. Incubation time was significantly longer compared to other Alcalase groups and those with milk/talc treatment (126° D). The shortest incubation time (110° D) was with the 5.0 mL L^{-1} Alcalase treatment. All Alcalase treatments led to significantly lower incubation times compared to 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*

1. INTRODUCTION

Pikeperch (*Sander lucioperca* L.) is a promising species for inland European aquaculture (Fontaine, 2009; Kristan et al., 2012a; Policar et al., 2012). Increased production of fish that are valuable economically and ecologically 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 in fish culture, and optimizing the method employed increases hatching rate (Linhart et al., 2000; Gela et al., 2003).

The first attempts to remove adhesive from pikeperch eggs were made with a suspensions of mud, starch, or charcoal (Sakowicz 1928; Demska-Zakes et al., 2005). The traditional methods for elimination of fish egg stickiness involve stirring the fertilized eggs into Woynarovich solution (Woynarovich and Woynarovich, 1980), milk powder (Khan et al., 1986), milk (Billard et al., 1995), talc or clay suspensions (Gela et al., 2003; Mizuno et al., 2004), or tannic acid (Dumas and Brand, 1972; Colesante and Youmans, 1983; Demska-Zakes et al., 2005).

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 proteolytic enzyme was first used for elimination egg stickiness in European catfish *Silurus glanis* (Horvath, 1980; Proteau et al., 1994) and walleye, Sander vitreus (Krise et al., 1986, 1988). Currently, Alcalase is commonly used for elimination of egg stickiness in tench *Tinca tinca* (Linhart et al., 2000, 2003a,b; Gela et al., 2003), European catfish (Linhart et al., 1997, 2002, 2003b, 2004), and common carp *Cyprinus carpio* (Linhart et al., 2003b,c). It is routinely used in hatcheries in the Czech Republic and in France (Linhart et al., 2002, 2003c).

The primary aim of the current study was to assess the efficacy of Alcalase enzyme® (Merck EC 3.4.21.14) in removing adhesive from eggs of pikeperch, and to identify the optimum treatment concentration, and compare exposure time with the traditional milk-talc treatment. This offers the potential for employing recently developed methods of intensive pikeperch production, such as triploidization and production of an all-female population.

2. MATERIALS & 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.

2.1. Fish handling and gamete collection

Broodstock of pikeperch were obtained from the fish farm Rybářství Třeboň Inc. and kept in a storage pond at USB FFPW during March, 2011. 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 et al., 2012a) and given a single intramuscular injection of hCG (500 $IU.kg^{-1}$) and stripped as described by (Kristan et al., 2012b).

2.2. Experimental procedure

Eggs from 5 females were mixed and divided into six batches of 5 g (~ 6 000 eggs) and inseminated with the mixed sperm from the 10 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 of eggs. Treatments to eliminate the adhesiveness of eggs were administered 2 min post-fertilization.

Two methods with several variants (6 treatments × 3 replicates = 18 groups) 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, Inc., Nový Bydžov, Czech Republic)] and a talc solution [100 g to 10 L of hatchery water (M. Rada Inc., Litomyšl, Czech Republic)]. The eggs were stirred 60–80 min, rinsed in hatchery water, and transferred to incubators and Zug jars.
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 to 999.5, 999, 998.5, 998 and 995 mL 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 Zug jars in incubators.

Batches of 80–250 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, 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.

2.3. Statistical analysis

Statistical analysis was carried out using Statistica software 9.0 for Windows (StatSoft, 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.

3. RESULTS

3.1. Fertilization and hatching rate

Adhesive elimination was successful for all groups of eggs. Neither destruction of egg envelopes nor larval malformations were observed. The fertilization rate (88.2–91.2%) was not significantly different among groups (Table 1). Hatching rate showed

significant differences 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.

3.2. Incubation time in degree days

The incubation time ranged from 110 to 126°D (Fig. 1.) in 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.

Table 1. *Fertilization rate, hatching rate, and egg mortality in pikeperch eggs under treatment to remove adhesive.*

Treatment	Enzyme L^{-1} of dilution solution(mL L^{-1})	Number of stocked eggs	Number of dead eggs	Fertilization rate (%)	Hatching rate (%)
Alcalase	0.5	159.5 ± 32.3	34.5 ± 14.3	91.2 ± 6.3	79.9 ± 6.0^{c}
	1.0	159.5 ± 1.2	38 ± 6.5	89.6 ± 2.9	76.1 ± 5.2^{bc}
	1.5	182 ± 23.7	25.5 ± 2.0	91.1 ± 4.2	85.4 ± 3.7^{c}
	2.0	150 ± 51.4	24 ± 4.1	90.3 ± 1.8	82.3 ± 4.1^{c}
	5.0	167 ± 66.1	68.5 ± 21.6	88.2 ± 0.9	56.4 ± 5.3^{a}
Milk/talc	0	169 ± 13.1	66 ± 9.8	90.0 ± 2.3	61.3 ± 3.4^{ab}

Data are shown as mean ± S.D.
Values within the column with different superscripts are significantly different ($P < 0.05$).

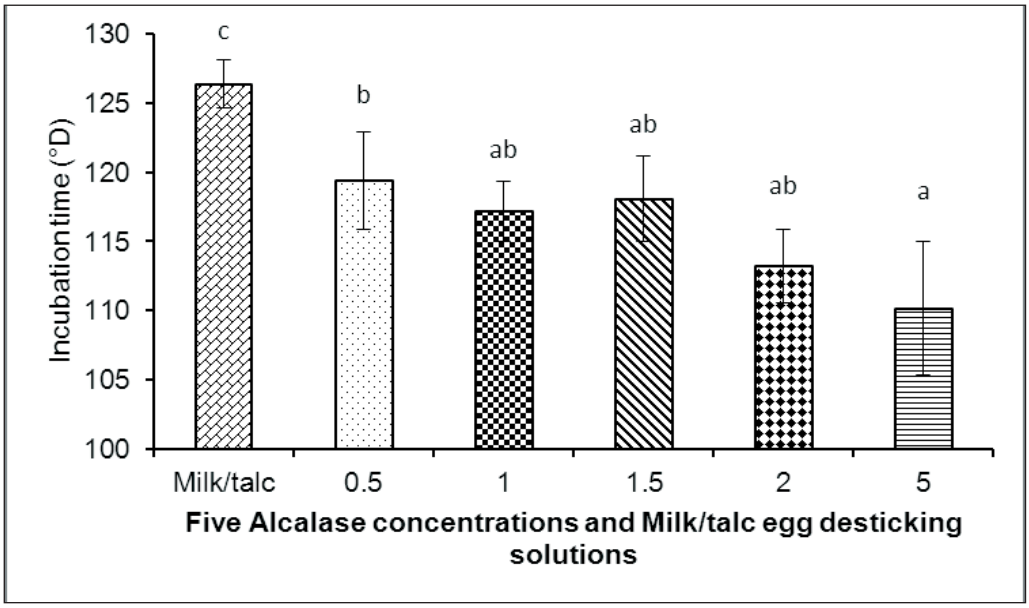

Figure 1. *Incubation time in degrees days (°D) for pikeperch eggs treated for elimination of egg stickiness. All values are means ± SD. Values within the column with different superscripts are significantly different (P < 0.05).*

4. DISCUSSION

Because fish species may differ widely in their response to elimination of egg stickiness, the screening of treatment concentrations and time of exposure is necessary for improvement of hatching rate and larva production.

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 60–80 min (Kristan and Blecha, pers. comm., 2013). Another method involves a talc/sodium chloride solution (100 g NaCL + 25 g talc + 10 L water) for 45–60 min (Schlumpberger and Schmidt, 1980), which gave hatching rates of approximately 60%. The disadvantage of these methods is that the duration of stirring leads to premature hatching of embryos due to damage to the external membrane by bacteria.

The third possibility is the use of tannic acid as is routinely employed in Poland (Demska-Zakes et al., 2005, Zakes et al., 2007; Kucharczyk et al., 2007; Kujawa et al., 2010; Zarski et al., 2012). Colesante and Youmans (1983) observed 65% hatching of walleye eggs when treated with a solution of 250 mg tannic acid L^{-1} H_2O for 4 min. In pikeperch, 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 1 000 mg L^{-1} for 5 min. These results are in accordance with the study by Krise et al. (1986) and Waltemyer (1975), who indicated that tannic acid has a detrimental effect during

incubation. Mizuno et al. (2004) also showed that the traditional method of kaolin treatment resulted in higher hatching rates and lower mortality during incubation compared to tannic acid-treated eggs in shishamo smelt

Spirinchus lanceolatus.

In the current study, it was indicated that 0.5–2.0 mL L^{-1} of Alcalase for 2 min may increase pikeperch egg hatching success (to 80–85%) compared to milk-talc solution. This method also allows significantly reduced time for eggs rinsing and incubation. Similar results were achieved in tench (Linhart et al., 2003a) and in European catfish (Linhart et al., 2004). These studies produced similar results (80–87%) but with higher concentrations (5–20 mL L^{-1}) of Alcalase. 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.

The use of chemical treatment to remove egg stickiness has been shown to reduce the growth of viral pathogens, bacteria, and fungi more effectively than mechanical methods (Krise et al., 1986; Kujawa et al., 2010), as well as requiring less time than the traditional milk/clay/talc methods. Use of Alcalase for elimination of egg stickiness in pikeperch can facilitate the application of physical shock for chromosome manipulation as in tench (Flajšhans et al., 1993; Gela et al., 2010).

5. CONCLUSIONS

The application of Alcalase enzyme can be successfully used for elimination of pikeperch egg stickiness, and requires considerably less time than the traditional milk/clay/talc methods. This provides the potential for employing recently developed methods of intensive pikeperch production such as triploidization and production of an all-female population. Further studies are required to optimize the protocol for using Alcalase in artificial reproduction of pikeperch in hatchery conditions.

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CHAPTER 8

GENERAL DISCUSSION
ENGLISH SUMMARY
CZECH SUMMARY
ACKNOWLEDGEMENTS
LIST OF PUBLICATIONS
TRAINING AND SUPERVISION PLAN DURING STUDY
CURRICULUM VITAE

GENERAL DISCUSSION

Reproduction plays an important role in pond production, especially in intensive production of percids (Kucharczyk et al., 2007; Policar et al., 2008a; Zarski et al., 2012). Successful control of broodstock reproduction and mass reproduction are basic prerequisites for profitable intensive percid production (Fontaine et al., 2008).

Seasonal spawning, reproduction characteristics, and broodstock mortality of pond cultured and intensively farmed perch were investigated (Chapter 2, Křišťan et al., 2012a). It was surprising that the success of ovulation without hormone treatment was greater in farmed perch broodstock. This may indicate that spawning in Eurasian perch, a limiting factor in fish reproduction, is affected by stress (Wang et al., 2003). However, the morpho-anatomical parameters (gonadosomatic, hepatosomatic, and viscerosomatic indices), fecundity, and hatching rate were significantly lower in farmed perch. These differences have been suggested to be caused mainly by unsuitable feeding of farmed perch (Kestemont et al., 2008; Henrotte et al., 2010). Henrotte et al. (2010) recommend the use of feed with specific ratios of highly unsaturated fatty acids (HUFA) (docosahexaenoic DHA/eicosapentaneoic EPA/arachidonic ARA acids). Their results indicated that a ratio of 3DHA/2EPA/2ARA was effective in obtaining eggs and larvae of good quality. On the other hand, Fontaine et al. (2008) reported that the enrichment of dry diets with additives such as vitamin E, vitamin C, or mixtures of HUFAs does not significantly improve reproductive performance or the hatching rate of larvae. These authors add that, to address lower reproduction in intensively farmed perch, it is necessary to improve the diet by adding prey fish and a larger proportion of natural ingredients to the feed. However, Křišťan et al. (2012a) found that adding prey fish to farmed perch diets did not increase the hatching rate in intensively farmed perch. Comparison of post-spawning mortalities confirmed our hypothesis that post-spawning mortalities are significantly higher in pond farmed perch broodstock. Wang et al. (2003) suggests that this phenomenon may also be due to higher cortisol levels in pond farmed perch.

Anaesthetics are routinely used to reduce handling stress and are commonly administered in artificial and semi-artificial reproduction procedures of percids (Kouril et al., 1997, Kucharczyck et al., 2007). Tricaine methane sulphonate (MS222), clove oil, propiscin, and 2-phenoxyethanol are used in percid aquaculture. The effects of these anaesthetics were compared in pikeperch (Chapter 3, Kristan et al., 2012b), since it has been shown that the physiological effects of anaesthetics are species-specific (Celik, 2004; Gomulka et al., 2008; Velisek et al., 2011). Haematological and biochemical profiles are frequently used for evaluation of the effect of anaesthetics (Iwama et al., 1989; Velisek and Svobodova, 2004). All anaesthetics tested in the present study were associated with changes in haematological and blood biochemical parameters in pikeperch. The greatest differences of 2-phenoxyethanol, Propiscin, MS222, and clove oil, compared to the control group were seen in leukocyte count, which was significantly lower in 2-phenoxyethanol and Propiscin but not in MS222 and clove oil. According to these results, we can recommend clove oil as an alternative to MS222. On the other hand, Velisek et al. (2009) recommended Propiscin as the optimal anaesthetic for Eurasian perch. Only MS222 is licensed for use in food-fish in the US and the United Kingdom (Velisek et al., 2009). However, clove oil, Propiscin, and 2-phenoxyethanol, which sees some use in non-food applications and in research,

have been evaluated experimentally (Coyle et al., 2004). The use of these products is not permitted in foods under EEC Regulation 2377/90, as no maximum residue levels have been established.

The comparison of artificial and semi-artificial reproduction of perch broodstock using the commercial hormone Supergestran for the mass production of embryos was evaluated (Chapter 4, Policar et al., 2011). The spawning of female broodstock began spontaneously 14.4 h later in semi-artificial spawning than in artificial spawning. Latency of female perch broodstock was greater with semi-artificial spawning (4.1 ± 0.7 days/98.5 ± 17.2 h/62.3 ± 10.6 °d) than with artificial spawning (3.5 ± 0.8 days/84.0 ± 18.3 hours/53.2 ± 11.5 °d). Similar results were achieved by Kouřil et al. (2011), who reported 90–113 hour latency at 14°C. The spawning of broodstock was more highly synchronized with semi-artificial spawning, with 88% of fish spawning within 3 days compared with 4 days in artificial spawning. The absolute and relative fecundity was influenced by fish body size, and no influence of the spawning method on female fecundity was observed. Higher fertilization rates (85.6 ± 8.7%) and hatching rates (72.9 ± 12.3%) were observed in controlled testing of egg samples from semi-artificial spawning than in those from artificial spawning (fertilization rate = 67.5 ± 6.5%, hatching rate = 58.4 ± 5.2%). The average water temperature during mass incubation was 15.8 ± 0.4 °C, and mass incubation lasted, on average, 7.5 days in artificial spawning and 7 days in semi-artificial spawning. Artificial spawning increases demands of labour, time, and staff experience relative to semi-artificial spawning, chiefly due to the necessity for frequent monitoring of the ovulation status of females (Policar et al., 2008b). The procedures evaluated in this study will ensure the mass production of perch embryos a single age cohort, which will positively influence the rearing of larvae and juveniles in ponds. With sufficient feed supply, fish of the same age will be similar in size, which will minimize the cannibalism and increase the effectiveness of rearing of juveniles and older categories of Eurasian perch.

To investigate the optimal hormone treatment for pikeperch, we evaluated and compared Chorulon and Supergestran (Chapter 5, Kristan et al., 2013a). In Czech hatcheries, Supergestran containing a mammalian GnRH ([D-Ala6]GnRHProNhet) analogue is commonly used in Eurasian perch (Policar et al., 2008c), but the efficacy of Supergestran has not been studied in other percids. Chorulon, containing the human chorionic gonadotropin (hCG), is used for many fish species including pikeperch (Zakes and Demska-Zakes, 2005; Kucharczyck et al., 2007). In this study, the potential of different single doses of these preparations to stimulate spawning of pikeperch females was tested. The highest ovulation rate (100%) was observed in females treated with mGnRHa (25 μg kg^{-1}) and with hCG (500 and 750 IU). Similar results were obtained by Kucharczyck et al. (2008), who reported results of 700 IU hCG. This study (Kristan et al., 2013a) also shown that that the low dose of mGnRHa (25 $\mu g.kg^{-1}$) could induce ovulation in a substantial number of pikeperch. This is lower than the dose that has been used for ovulation in perch (50 $\mu g.kg^{-1}$) (Kouril and Hamackova, 1999; Policar et al., 2008c). Latency in the hCG-treated groups was found similar to that with mGnRHa, in contrast to results of Zohar and Mylonas (2001), who reported a shorter latent period in females treated with hormones acting on gonads, such as hCG and carp pituitary, compared with hormones acting on the brain as does GnRHa. Latency with the single injection was similar to results of Zakes and Demska-Zakes (2005) and Rónyai (2007), who used a double injection.

The application of a single dose can reduce stress in females and increase quality of eggs (Schreck et al., 2001). The hatching rate was significantly higher with Chorulon than with Supergestran. However, Supergestran is a Czech product and is easily available in the Czech Republic. Based on the results obtained in this study, we can recommend administration of either hormone treatment as a reliable and efficient ovulation-inducing therapy in pikeperch under optimal temperature and light conditions.

Fertilization success depends on the availability of good quality sperm (Bromage et al., 1995). The quality of semen is mainly determined by density and the morphology, velocity, and motility of spermatozoa. These parameters, relative to short-term storage and activation media, were investigated in sperm of pikeperch (Chapter 6, Kristan et al., 2013b). The quality of male broodstock is highly family-specific and depends upon gonad morphology, neuro-endocrine regulation of spermatogenesis, and physiology and biochemistry of sperm (Alavi et al., 2008). Pikeperch possess a primitive acrosomeless aquasperm, similar to most freshwater teleosts, consisting of a spherical nucleus, midpiece, and flagellum. One or two mitochondria have been reported in Eurasian perch (Lahnsteiner et al., 1995; Hatef et al., 2011), but 2–4 mitochondria were observed in pikeperch in this study. The observed morphology was similar to that reported by Lahnsteiner and Mansour (2004). The present study represents the first report of pikeperch sperm flagella length at 33.2 μm, similar to that of Eurasian perch 30–35 μm (Lahnsteiner et al., 1995). A lateral fin-like structure on the flagellum was observed in pikeperch, which is not present in Eurasian perch (Hatef et al., 2011). This structure has been previously reported in a few species such as sturgeon (Psenicka et al., 2008) and pike, *Esox lucius* (Alavi et al., 2009), and probably increases sperm velocity. Initial sperm motility in pikeperch observed in this study was similar to that of Eurasian perch, but sperm velocity was higher (160 vs. 115–130 $\mu m.s^{-1}$) (Alavi et al., 2007). Short-term storage significantly affected both percent sperm motility and velocity in pikeperch. In the present study, sperm motility and velocity evaluated at 15 s post-activation was decreased from 92–99% and 161–204 $\mu m.s^{-1}$ at 0 h incubation to 6–30% and 99–132 $\mu m.s^{-1}$ after 48 h storage. Pikeperch spermatozoa were generally still living 24 h after sperm collection, and very low sperm motility could be achieved after 48 h with activation in AM3 containing 1 mM Ca^{2+}. This suggests participation of Ca^{2+} in sperm activation, as has been reported in freshwater teleosts (Alavi and Cosson, 2006; Morisawa, 2008).

In artificial reproduction, the final step before incubation is the removal of egg adhesive. The efficacy of artificial reproduction is measured by the number of larvae obtained. In some fish species such as common carp, *Cyprinus carpio*; European catfish, *Silurus glanis*; tench, *Tinca tinca;* and pikeperch, *Sander lucioperca*, this is partially dependent on the method by which the adhesiveness of the eggs is removed. The adhesive chorion that occurs in these fish species allows the eggs to attach to various substrate types (Riehl and Patzner, 1998). Adhesiveness removal efficacy and hatching rate of larvae exposed to five concentrations of Alcalase enzymes under controlled conditions, with a control group using talc and dry milk solution were evaluated (Chapter 7, Kristan et al., 2013c). The application of Alcalase enzyme to pikeperch eggs was previously studied. A further method for removal of pikeperch egg adhesive is the use of tannic acid (Demska-Zakes et al., 2005).

In the current study, 0.5–2.0 ml l^{-1} of Alcalase for 2 min may increase pikeperch egg hatching success to 80–85%, compared to use of a milk-talc solution (60–65%). This

method also allows significantly reduced time for egg rinsing and incubation. Similar results were achieved in tench (Linhart et al., 2003) and in European catfish (Linhart et al., 2004). Elimination of egg stickiness by the enzyme Alcalase compared to the traditional methods reduces the time of elimination eggs stickiness. The application of Alcalase enzyme can be successfully used for elimination of pikeperch egg stickiness. This gives the potential for utilization of modern methods of intensive pikeperch production such as triploidization (Malison et al., 2001, Garcia-Abiado et al., 2001) and production of an all-female population (Stejskal et al., 2009).

This thesis has identified possible problems in percid reproduction, and presented and discussed factors having the potential to improve embryo production for pond or intensive percid aquaculture.

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ENGLISH SUMMARY

Optimization of reproduction and gamete quality in percid fish

Jiří Křišťan

Pond and intensive aquaculture of percids relies mainly on juveniles supplied by semi-artificial and artificial reproduction. Fish hatcheries do not always provide optimal conditions for percid reproduction. This project was undertaken to optimize reproduction methods of Eurasian perch (*Perca fluviatilis* L.) and pikeperch (*Sander lucioperca* L.).

Reproduction parameters were compared in farmed and pond-reared perch broodstock without hormone treatment. Spawning was successful without hormone induction at 92.3% and 76.9% in farmed and pond-reared perch, respectively. Significant differences in morphological characteristics (gonadosomatic, hepatosomatic and viscerosomatic indices), fecundity, hatching, and post-spawning mortality were detected. All results, with the exception of spawning success and post-spawning mortality, were superior in pond reared broodstock. Further studies are required to optimize protocols for using better artificial food in farmed perch and decreased stress and post-spawning mortalities in pond reared perch broodstock.

The possibilities of using clove oil, Propiscin, or 2-phenoxyethanol as an alternative to tricaine methane sulphonate (MS222) as anaesthetic, particularly with respect to reducing fish stress, were investigated. The haematological and biochemical profiles of pikeperch anaesthetized with clove oil (33 $mg.l^{-1}$), Propiscin (1.5 $ml.l^{-1}$), 2-phenoxyethanol (0.3 $ml.l^{-1}$), and MS222 (150 $mg.l^{-1}$) were determined. Each tested group was divided into two subgroups; the first subgroup was sampled 10 min after application and the second 24 h post-application. Clove oil was shown to be associated with the lowest effects in pikeperch, and therefore could be recommended as an alternative to MS222, while Propiscin and 2-phenoxyethanol were shown to be unsuitable for use with pikeperch.

In Eurasian perch the use of semi-artificial reproduction for mass production of embryos was shown to be simpler and more effective. With mass incubation, 1 134 000 embryos (59% of total production) were obtained with a semi-artificial spawning regime and 796 000 embryos (41% of total production) with artificial spawning. Based on these results, we can recommend the semi-artificial spawning for production of perch embryos.

Differing doses of two commonly used hormone preparations: human chorionic gonadotropin (hCG) and mammalian GnRHa (Supergestran) were tested for induction of ovulation in pikeperch. A broad range of effective doses is used in aquaculture of pikeperch. The minimal effective dose based on ovulation success is still missing. Hormone therapy applied to excess can result a detrimental impacts on broodstock fecundity and quality of eggs. Results indicated that a single injection of hCG or Supergestran could be used for successful ovulation, while no spawning was observed in a control group treated by saline solution. Among hormone treated groups, the best results were observed in groups treated with hCG at 500 and 750 IU kg^{-1} BW and in groups treated with mGnRHa at 25 $\mu g.kg^{-1}$ BW.

The sperm of pikeperch and Eurasian perch show similar morphology and fine structure, with the exception of differences in arrangement of midpiece and

centrioles and the presence of a fin-like structure on the flagellum in pikeperch. Our results suggest use of an activation medium containing Ca^{2+} for activation of short-term stored sperm.

Efficacy of five concentrations of Alcalase enzyme for elimination of egg stickiness compared to a milk/talc suspension was determined.

Results indicated that 0.5–2.0 $ml.l^{-1}$ of Alcalase for 2 min may increase pikeperch egg hatching success (80–85%) compared to the milk-talc suspension. The highest hatching rate (85.4%) was observed with 1.5 $ml.l^{-1}$ of Alcalase. The application of Alcalase successfully eliminated pikeperch egg stickiness in 2 min compared to 60 min with traditional milk/clay/talc methods.

CZECH SUMMARY

Optimalizace reprodukce a kvality gamet u okounovitých ryb

Jiří Křišťan

Rybniční a intenzivní akvakultura okounovitých ryb je založena zejména na produkci juvenilních ryb pocházejících z poloumělé a umělé reprodukce. Rybí líhně ne vždy poskytují optimální podmínky pro reprodukci okounovitých ryb. Cílem této studie je vylepšit, optimalizovat a doplnit poznatky o reprodukci okouna říčního (*Perca fluviatilis* L.) a candáta obecného (*Sander lucioperca* L.).

První studie porovnává reprodukční charakteristiky u generačních ryb z farmového a rybničního odchovu bez použití hormonálních přípravků. Výtěr bez hormonální indukce byl úspěšný v 92,3 % u intenzivně chovaného okouna a v 76,9 % u rybničně chovaného okouna. Byly zjištěny významné rozdíly v morfologických charakteristikách (v gonadosomatických, hepatosomatických a viscerosomatických indexech), v plodnosti, v líhnivosti embryí a v povýtěrové mortalitě. S výjimkou úspěšnosti výtěrů a povýtěrové mortality jsme ve všech případech dospěli k lepším výsledkům u generačních ryb pocházejících z rybničního odchovu. Z výsledků je patrné, že další studie by se měly věnovat výživě farmových ryb a snažit se zredukovat stres a povýtěrovou mortalitu u rybničně odchovaných jedinců.

Dále jsme zkoumali možnost nahrazení anestetika MS 222 hřebíčkovým olejem, Propiscinem nebo 2-phenoxyethanolem, a to zejména s ohledem na snížení stresu ryb. U candáta obecného ošetřeného hřebíčkovým olejem (33 mg L^{-1}), Propiscinem (1.5 ml L^{-1}), 2-phenoxyethanolem (0.3 ml L^{-1}) a MS 222 (150 mg L^{-1}) byl stanoven hematologický a biochemický profil. Každá z testovaných skupin byla rozdělena na dvě podskupiny, první podskupina byla vzorkována ihned po desetiminutové anestézii a druhá podskupina byla vzorkována 24 hodin po desetiminutové anestézii. Hodnoty získané v průběhu tohoto experimentu ukazují, že hřebíčkový olej ovlivňuje candáta obecného nejméně a může být doporučen jako alternativa k MS 222, zatímco Propiscin a 2-phenoxyethanol nejsou pro manipulaci s candátem vhodné.

Výsledky masové produkce embryí okouna říčního ukázaly, že poloumělý výtěr je jednodušší a efektivnější. Na konci masové inkubace bylo celkem získáno 1 930 000 embryí od všech jikernaček v rámci obou způsobů výtěru, 1 134 000 embryí (59 % z celkové produkce embryí) bylo získáno z poloumělých výtěrů a 796 000 embryí (41 % z celkové produkce embryí) bylo získáno z umělých výtěrů. Na základě této studie můžeme doporučit poloumělý výtěr pro masovou produkci embryí okouna říčního.

Pro úspěšnou indukci ovulace candáta obecného byly testovány různé dávky dvou běžně užívaných hormonálních přípravků, lidského choriogonadotropinu (hCG) a Supergestranu obsahujícího savčí GnRHa. Výše účinné dávky je jedním z nejvýznamnějších faktorů ovlivňujících úspěšnost hormonální indukce ovulace. Nadměrně vysoká dávka zbytečně zatěžuje rybí organizmus a může mít negativní dopad na pohlavní produkty, zatímco nízká dávka nevyvolá odezvu v podobě ovulace. Výsledky ukazují, že jednorázová hormonální injikace hCG nebo Supergestranu může být užita pro úspěšnou ovulaci generačních ryb. Mezi hormonálně ošetřenými skupinami byly nejlepší výsledky pozorovány ve skupinách ošetřených hCG v dávkách 500 a 750 IU kg^{-1} a ve skupině ošetřené savčím GnRH analogem v dávce 25 $\mu g.kg^{-1}$.

Spermie candáta obecného a okouna říčního vykazuje podobnou morfologii i přes rozdíly pozorované v „ploutvičkách" podél bičíku a v uspořádání střední části a centriol. Tato studie také navrhuje použití aktivačního média obsahujícího Ca^{2+} pro aktivaci spermatu v případě jeho krátkodobého uchování.

Dále bylo porovnáváno pět koncentrací enzymu alkaláza se suspenzí mléka a talku pro zbavení lepivosti jiker u candáta obecného. Bylo zjištěno, že užití 0,5–2,0 $mL.L^{-1}$ alkalázy po dobu 2 minut může zvýšit líhnivost oproti suspenzi mléka a talku až na 80–85%. Nejvyšší líhnivosti (85,4 %) bylo dosaženo při koncentraci 1,5 $mL.L^{-1}$ alkalázy. Alkaláza úspěšně zbavila jikry candáta lepivosti v kratším časovém intervalu (2 min) než při tradičním užití suspense mléka a talku (60 min).

Veškeré výsledky, stejně jako nalezení optimálního způsobu výtěru okouna říčního, představují významný přínos pro rybářskou a líhňařskou praxi.

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LIST OF PUBLICATIONS

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Kristan, J., Alavi, S.M.H., Stejskal, V., Policar, T., 2013. Hormonal induction of ovulation in pikeperch (*Sander lucioperca* L.) using human chorionic gonadotropin (hCG) and mammalian GnRH without dopamine inhibitor. Aquaculture International 21, 811–818.

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Policar, T., **Kristan, J.,** Blaha, M., Stejskal, V., 2012. Juvenile production in pikeperch for ongrowing culture. In: New, M. (Chairman AQUA 2012): AQUA 2012 – Global Aquaculture, Securing Our Future, USB of Abstracts, Prague, Czech Republic, p. 887.

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Křišťan, J., Alavi, S.M.H., Hatef A., Policar, T. 2011. Short-term storage of pikeperch sperm. 3rd International Workshop on the Biology of Fish Gametes. Budapest, Hungary, 7–9 September 2011, p. 156 (poster presentation)	2011
Křišťan, J., Stará, A., Turek, J., Policar, T., Velíšek, J., 2012. Comparison of the effects of four anaesthetics on blood biochemical profiles in pikeperch (*Sander lucioperca* L.). In: Book of abstracts. 17th Interdisciplinary Toxicology Conference TOXCON 2012. 27–31 August 2012, Stará Lesná, Slovakia, Interdisciplinary Toxicology 5 (Supll. 1), 46–47. (poster presentation)	2012
Křišťan, J., Stejskal, V., Policar, T., 2012. The alcalase enzyme treatment for elimination of egg stickness in pikeperch Sander lucioperca L. In: WAS and EAS (eds.): AQUA 2012. Prague (Czech Republic), 1–5 September 2012, p. 591. (poster presentation)	2012

Křišťan, J., Zarski, D., Palińská, K., Krejszeff, S., Nowosad, J., Alavi, S.M.H., Policar, T., Kucharczyck, D., 2012. The effect of sperm to egg ratio on fertilization success in artificial insemination of pikeperch Sander lucioperca. Domestication in Finfish Aquaculture. Olsztyn, Poland, 23–25 October 2012, p. 70. (poster presentation)	2012

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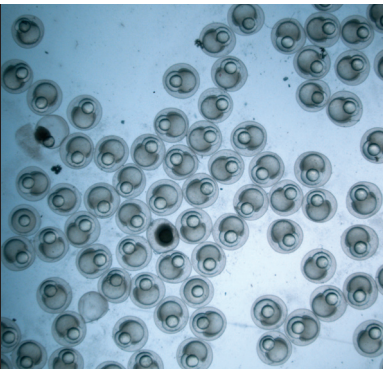

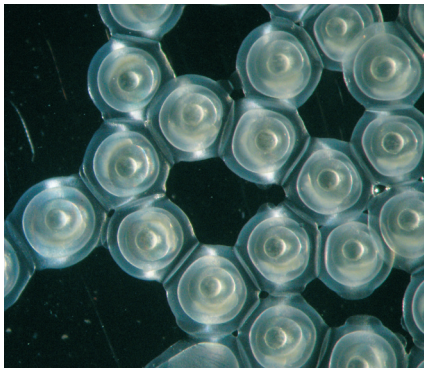

Fakulta rybářství
a ochrany vod
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