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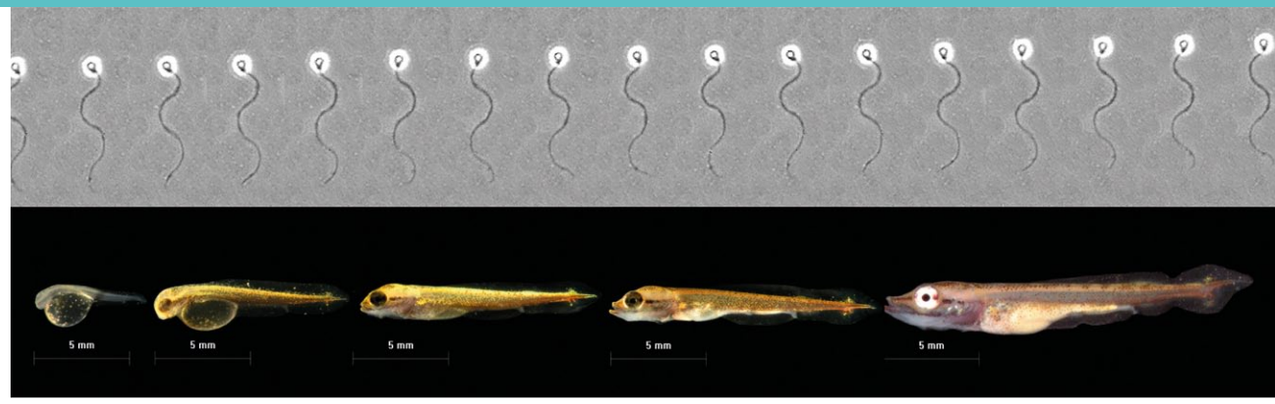
Reprodukce a intenzivní chov juvenilních ryb štiky obecné (*Esox lucius* L.)



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Czech Republic, Vodňany, 2014

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CONTENT

CHAPTER 1

GENERAL INTRODUCTION

1.1. Aquaculture – background

Aquaculture involves the rearing of aquatic organisms such as fish, crustaceans, bivalves, and aquatic plants and is currently the fastest growing sector of animal production worldwide. During the past 25 years, aquaculture production grew by up to 8.5% annually (FAO, 2013) and currently meets about half the global demand for fish and fish products for human consumption. Almost 47 million tons of fish, of which around 33 million tons are intended for direct human consumption, are captured from open water annually. This includes hundreds of species, some of which, especially predatory species, are threatened with extinction (Naylor, et al., 1998, 2000). Therefore, the constantly increasing demand for fish and fish products for human consumption must be met by fish farming.

The northern pike (*Esox lucius* L.) is a major predatory fish in Central and Eastern Europe, including the Czech Republic. Northern pike is adaptable to a wide range of natural biotopes (Crossman, 1996) and is one of the most popular fish for angling (Margenau et al., 2008). Its controlled propagation and pond rearing has a long tradition in the Czech Republic. The predatory role of northern pike has been widely used for biological control of populations of coarse fish (Adámek et al., 2010), leading to water quality improvement and improved productivity in ponds and reservoirs (Craig, 1996; Schoenebeck et al., 2008). Northern pike farming is highly valued and profitable in European countries such as Hungary, the Czech Republic, Poland, France, and Germany (Craig, 2008). The demand for northern pike is growing throughout Europe, thanks to the rapid development of sport fishing and commercial interest (FAO, 2012).

Most market-size northern pike come from lakes, rivers, and ponds, with relatively low numbers being produced by aquaculture (Figure 1). In Europe, approximately 25 100 metric tons of northern pike are harvested annually from natural waters, especially lakes and ponds (Figure 1), with only 700 metric tons coming from commercial farms (FAO, 2013).

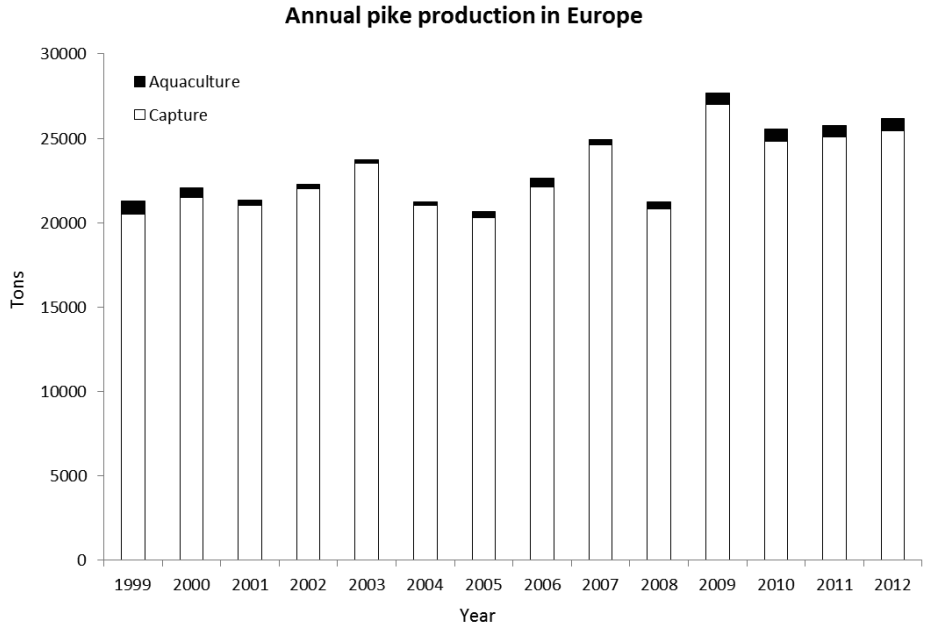

Figure 1. *Northern pike production during the past thirteen years (FAO, 2013).*

This thesis presents results of an array of investigations into aspects of intensive culture of northern pike: artificial reproduction, quality of sperm, egg incubation, and broodstock management. Aspects of culture are described in Chapter 5.

1.2. Artificial reproduction

Synthetic treatments based on superactive gonadotropin-releasing hormone analogues (GnRHa) with or without dopamine inhibitors (Mylonas and Zohar 2007; Hill et al., 2009) are used to bring about completion of final oocyte maturation (FOM) and induce ovulation in captive broodstock of many fish species (Policar et al., 2008; Podhorec et al., 2012; Křišťan et al., 2013).

Traditionally hormonal stimulation of ovulation under controlled conditions in northern pike has been mainly through "hypophysation" using dehydrated carp pituitary at a dose of 3–4 mg kg^{-1} live weight for females and 2–4 mg kg^{-1} live weight for males (Billard, 1996; Szabó, 2001). However, effects of GnRH analogues on ovulation have been assessed in northern pike (Billard and Marcel, 1980; Szabó, 2003, 2008). Billard and Marcel (1980) injected northern pike females with [D-Arp^6, Pro^{9}NEt]-mGnRH at 1 μg kg^{-1} body weight (BW) and did not observe oocyte maturation or ovulation. However, the same GnRHa formulation was effective in stimulating spermiation at the highest dose of three injections in 24 h at 10 μg kg^{-1} BW. The only known successful trial of GnRHa for activation of ovulation in northern pike was reported by Pecha et al. (1992) who observed nearly twice the ovulation rate (45%) following injection with [D-Ala^6, Pro^{9}NEt]-mGnRH at 5–10 μg kg^{-1} BW and [D-Tle^6, Pro^{9}NEt]-mGnRHa at 2.5 and 5 μg kg^{-1} BW, than found in the untreated group (25%). The effects of hormone treatments on ovulation in northern pike are reported in Chapter 2.

1.3. Sperm quality and flagella wave motility parameters

Artificial reproduction in northern pike, including thermally and hormonally induced spawning, is based on the use of either stripped or testicular sperm for egg fertilization (Billard and Marcel, 1980). The first method uses spermatozoa released from vas deferens by abdominal massage after thermal or hormonal stimulation (Billard et al., 1980). Such collection of stripped sperm has the advantage of preserving the male for further sperm production, which is not possible with testicular sperm collection. A disadvantage is that spermatozoa often becomes contaminated with urine or blood during stripping, which presents a high risk of premature activation and limited usability for fertilization of eggs (Billard, 1978; Hulák et al., 2008a,b). Testicular sperm is harvested from testes removed from the body (Billard, 1996; Lahnsteiner et al., 1998; Hulák et al., 2008a). The advantage of this method of collection lies in obtaining a larger volume of sperm with a higher concentration of spermatozoa than with stripping. Importantly, use of testicular sperm ensures clean sperm, uncontaminated by urine or blood. The major disadvantage is that the method requires killing the male, thus eliminating a broodstock fish (Hulák et al., 2008a).

A basic factor determining fertilization success in hatchery production is the availability of good quality spermatozoa (Bromage et al., 1995). Motility is a characteristic function of the male gamete that allows the spermatozoon to reach and penetrate the egg (Holwill, 1969; 1971). During movement, spermatozoon flagella present waves that are similar at a given time point after activation (Lindemann and Lesich, 2010). The characteristics of the flagellar wave change, depending on environmental conditions such as pH, salinity, and ionic

concentrations, in a homogeneous manner among the spermatozoa population (Tsvetkova et al., 1996; Cosson et al., 1999; Alavi et al., 2009).

Analysis of fish spermatozoa motility currently relies on the use of video records subjected to computer assisted sperm analysis (CASA) (Kime et al., 2001), which traces video tracks generated by head movement. Detailed information about spermatozoa movement can be obtained from analysis of flagella swimming parameters obtained by high-resolution microscopy combined with high-speed video recordings (Cosson et al., 2008).

For comparative analysis when using high-speed video records and CASA system we choose northern pike (*Esox lucius* L.) and sterlet (*Acipenser ruthenus* Brandt) because of several reason such as: the large difference in the duration of the sperm motility period and in the wave appearance: 1 – in sterlet, motility lasts approximately 3–5 minutes and 1–1.5 minutes in pike, 2 – in sterlet, flagella offer the possibility to observe and describe flagella waves alternatively as planar for some periods or as 3D during other periods (Boryspolets et al., 2013) while in contrast, pike's flagella present only planar waves (Alavi et al., 2009), 3 – the morphology of sperm: depending on species, spermatozoa have different lengths of the flagellum such as 32 μm in pike (Alavi et al., 2009) and 43 μm in sterlet (Linhartova et al., 2013). In sterlet, sperm head presents elongated shape with a functional acrosomal vesicle (Psenicka et al., 2008) while pike's sperm head has round shape and is devoid of any acrosome (Alavi et al., 2009).

Chapter 3 presents results of investigations of spermatozoa motility parameters in northern pike and sterlet.

1.4. Eggs incubation and quality of newly hatched larvae

Water temperature is an important factor affecting fish embryo development, duration of incubation, hatching period, and time of hatching (Penaz et al., 1983; Kucharczyk, et al., 1997; Kamler et al., 1998; Drozd et al., 2009; Bohlen, 2003; Ojanguren and Braña, 2003; Green and Fisher, 2004; Kupren et al., 2011). Therefore knowledge of temperature effects on quality of larvae is crucial to understanding the process of fish egg incubation (Ward, 1998; Klimogianni et al., 2004; Pekarik et al., 2008). Several studies have reported a range of water temperatures for egg incubation in northern pike as 4 °C to 22 °C (Lillelund, 1957, 1967; Hokanson et al., 1973).

The size of newly hatched northern pike larvae is 8.5–9 mm with a weight of 10–11 mg (Billard, 1996). Newly hatched larvae are suspended vertically from adhesive papillae that develop on the head within several hours of hatching. Attachment of northern pike larvae to a substrate occurs at approximately 130 °d. Resorption of the yolk-sac is completed within 160–180 °d post-hatching. Larvae begin to consume exogenous food before complete digestion of the yolk-sac at approximately 150–160°d post-hatching, at a size of 12–15 mm and weight of approximately 12 mg.

Chapter 4 provides information describing the effect of water temperature on egg incubation time and quality of newly hatched larvae of northern pike.

1.5. Broodstock management

Traditionally rearing of northern pike is based on pond culture, mostly co-cultured with other fish species (Milstein, 1992) such as common carp (*Cyprinus carpio* L.), common bream (*Abramis brama* L.), gibel carp (*Carassius gibelio* L.), silver bream (*Abramis bjoerkna* L.),

tench (*Tinca tinca* L.), and rudd (*Scardinius erythrophthalmus* L.). Intensive culture of northern pike has been studied in recent years in Europe, resulting in increased and stable production of high quality juveniles for ongoing culture (Kucska et al., 2005). Both traditional and intensive culture requires stable and high quality production of fertilized eggs, embryos, and larvae.

Northern pike mature at the end of the second or third (rarely fourth) year of life (Billard, 1978. Time of first breeding depends on water temperature and food availability. Growth rate also depends on local conditions and reflects the stage of maturity rather than age (Billard, 1996). Water temperature of 4–7 °C will initiate northern pike spawning (Franklin and Smith, 1963; Westers and Stickney, 1993). In Czech climatic conditions, the natural spawning season is from mid-March to mid-May. Duration of the spawning period can be from a few days to over one month, depending on the increase of photoperiod and temperature (Ivanova and Svirskaya, 2009).

Broodstock management of northern pike is presented in detail in Chapter 5.

1.6. Aims and Objectives

The aims of this study were to:

1. compare efficacy of GnRHa to that of carp pituitary and natural environmental stimulation for induction of ovulation in northern pike and develop effective methods for induction and synchronization of ovulation for artificial reproduction in northern pike broodstock under controlled conditions;
2. evaluate sperm characteristics and spermatozoa motility parameters in northern pike and develop methods of flagellar movement and sperm motility analysis by using high speed video microscopy;
3. quantify effects of water temperature on the egg incubation, embryo development, hatching, and quality of newly hatched larvae;
4. develop effective methods of high-quality juvenile production for intensive culture of northern pike.

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CHAPTER 2

EVALUATION OF TREATMENTS FOR INDUCTION OF OVULATION IN NORTHERN PIKE (*ESOX LUCIUS* L.)

Paper I:

Bondarenko, V., Podhorec, P., Svinger, V., Policar, T. Evaluation of treatments for induction of ovulation in northern pike (*Esox lucius L*). (*manuscript*)

EVALUATION OF TREATMENTS FOR INDUCTION OF OVULATION IN NORTHERN PIKE (*ESOX LUCIUS* L.)

Short title: Treatment for ovulation in northern pike

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Abstract

The effectiveness of salmon D-Arg^{6}Pro^{9}NEt-GnRHa at 50 and 100 μg kg^{-1} (groups GnRHa 1 and 2), 50 and 100 μg kg^{-1} in combination with 8 mg kg^{-1} metoclopramide (groups GnRHa 3 and 4), emulsified with Freund´s incomplete adjuvant (FIA) at 50 and 100 μg kg^{-1} (groups GnRHa 5 and 6), and emulsified with FIA at 50 and 100 μg kg^{-1} with 8 mg kg^{-1} metoclopramide (groups GnRHa 7 and 8) were tested and compared to traditional treatments: 3 mg kg^{-1} carp pituitary (Group CP), ambient outdoor conditions (Group AOC]) and indoor controlled conditions (Group ICC) for induction of ovulation in northern pike (*Esox lucius* L.).

All fish in Group CP and 70% of those in Group AOC ovulated. The latent period in Group CP was 96 ± 4 h and 264±58 h in Group AOC. One Group 3 female ovulated spontaneously 108 h post-injection, whereas no other GnRHa injected or Group ICC fish ovulated. The pseudogonadosomatic index was 19.3 ± 5.9%; 17.8 ± 7.5% and 18.5 ± 0% in the AOC, CP, and Group 3, respectively ($p < 0.05$). The fertilization rate reached 85.5 ± 12.0%, 66.0 ± 13.7%, and 88.6 ± 4.5% in AOC, CP, and Group 3, respectively. The hatching rate was 68.6 ± 9.9%; 54.4 ±8.0%, and 65.5 ± 7.5% in AOC, CP, and Group 3, respectively. The ovarian fluid pH was significantly higher (8.27 ± 0.03) in the AOC and CP groups (8.11 ± 0.02) ($p < 0.05$) compared to Group 3 (8.35 ± 0.03). There were no significant differences in egg size and weight among groups ($p > 0.05$).

Keywords: *carp pituitary, GnRHa, injection, reproduction, fertilization, hatching rate*

Introduction

Northern pike *Esox lucius* L. is an important piscivorous fish in the freshwater ecosystems of northern hemisphere temperate zones (Craig, 2008) and is popular as a food fish and for sport angling. Northern pike show synchronous development of oocytes and spawn annually in spring at a water temperature range of 5–12 °C (Farrell et al., 1996). Aquaculture production methods currently consist of capturing mature broodstock from shallow vegetated ponds in the spring. Eggs are manually stripped from naturally ovulated females and fertilized with sperm from similarly captured males (Szabo, 2001). The main drawbacks of this type of production are disparity in female maturation stages and a reliance on environmental factors. These limitations reduce the possibilities for production of same-age larvae that are needed for successful culture (Bondarenko et al., in press). A single 3 mg kg^{-1} injection of

carp pituitary is the only confirmed method of inducing mass ovulation in mature females harvested from ponds or lakes (Billard and Marcel, 1980). Pike broodstock held year-round in captivity or prematurely captured during spring do not mature in captivity (Szabó, 2008). A lack of appropriate environmental stimulation, in combination with handling stress, inhibits final oocyte maturation (FOM) and subsequent ovulation (Zohar and Mylonas, 2001).

Synthetic hormone treatments based on superactive gonadotropin-releasing hormone analogues (GnRHa) with or without dopamine inhibitors (Goren et al., 1995; Gillet et al 1996; Mylonas and Zohar, 2007; Hill et al., 2009;) are used to promote FOM and induce ovulation in captive broodstock of many fish species (Policar et al., 2008; Podhorec et al., 2012; Křišťan et al., 2013). For a variety of fish species, treatment with 10–50 μg kg^{-1} GnRHa is more effective than treatment with carp pituitary (CP), producing higher numbers of ovulated females, eggs, and hatched larvae (Mylonas and Zohar, 2007). However, replacement of CP by GnRHa with or without dopamine inhibitors is not effective for induction of ovulation in pike (Billard and Marcel, 1980; Pecha et al., 1992; Szabó, 2001; Szabó, 2003, 2008). Ineffective use of GnRHa for induction of pike ovulation is associated with low doses of GnRHa (up to 50 μg kg^{-1}) or use of emulsified GnRHa without adjuvants.

Adjuvants are generally used to initiate and augment the inflammatory reaction required for induction of an optimal innate and adaptive immune response to vaccines, as well as to ensure long-lived immunity (Safari et al., 2011). Adjuvants can also allow a lower dose and thereby increase the potency of antivenins (Pratanaphon et al., 1997) and reduce vaccine costs (Singh and O'Hagan, 1999). Freund´s incomplete adjuvant (FIA) mechanism of action is promotion of the formation of depots of antigen at a site of immunization (Guy, 2007).

Freund´s incomplete adjuvant is well known as an efficient carrier of GnRHa in rainbow trout *Oncorhynchus mykiss* (Arabaci et al., 2004; Vazirzadeh et al., 2008) and chum salmon *Oncorhynchus keta* (Park et al., 2007).

The aim of present study was to compare efficacy of induction of ovulation with CP and with housing in ambient outdoor conditions to that of administration of salmon D-$Arg^{6}Pro^{9}$NEt-GnRHa at doses of 50 and 100 μg kg^{-1} with or without FIA and dopamine inhibitor (metoclopramide) for induction and synchronization of ovulation in northern pike.

Materials and Methods

Sexually mature northern pike females (3 years, body weight [BW] = 2 852 ± 856 g and total length [TL] = 695 ± 91 mm) were collected from production ponds of Nove Hrady Ltd. fishery, Czech Republic in spring 2012 and transported to the Laboratory of Intensive Culture, Faculty of Fisheries and Protection of Waters, Vodnany. Mature females (n = 110), selected based on the migration of the germinal vesicle (Szabó, 2003), were randomly divided into eleven groups of ten. Experimental groups included nine hormone-treated groups (CP; GnRHa Groups 1–8) and an indoor controlled conditions (ICC) group injected with physiological NaCl (negative control). Groups were placed in separate flow-through 700 l indoor tanks at water temperature 9.1 ± 0.2 °C and mean dissolved oxygen saturation of 90%. After three days acclimatization, temperature was increased to 10.5 ± 0.2 °C with the same stable oxygen saturation in all groups kept under controlled conditions. Females of an eleventh group were injected with physiological saline and kept under ambient outdoor conditions (AOC) in a 500 m^2 pond with littoral vegetation covering approximately 100 m^2. Water temperature was uncontrolled and fluctuated during the day from 6 °C to 12 °C. A single group of 110 mature males (3 years, BW = 1250 ± 250 g and TL = 531 ± 52 mm) was kept under controlled conditions similar to the females in a tank with total volume 10 000 L. Fish from all grops were kept under a natural photoperiod regime for our geographic location.

All males and GnRHa-treated females were intraperitoneally injected with 1 ml kg^{-1} to induce ovulation and spermiation. Groups ICC and AOC were injected with physiological saline solution (0.9% NaCl). Group CP was treated with dried carp pituitary dissolved in physiological saline solution (0.9% NaCl) at 3 mg kg^{-1}.

GnRHa groups 1 and 2 were injected with salmon D-Arg^{6}Pro^{9}NEt-GnRHa (Bachem AG, Germany) at 50 and 100 μg kg^{-1}, respectively. GnRHa groups 3 and 4 were injected with salmon D-Arg^{6}Pro^{9}NEt-GnRHa at 50 and 100 μg kg^{-1}, respectively, combined with metoclopramide (Sigma–Aldrich, USA) at 8 mg kg^{-1}. GnRHa groups 5 and 6 were injected with salmon D-Arg^{6}Pro^{9}NEt-GnRHa emulsified in FIA at 50 and 100 μg kg^{-1}, respectively. GnRHa in FIA was prepared by dissolution of GnRHa in 0.9% NaCl physiological saline and mixing with Freund´s incomplete adjuvant (FIA, Sigma Aldrich) 1 : 1v/v by using an Ika T-10 homogenizer. Groups GnRHa 7 and 8 were treated with salmon D-Arg^{6}Pro^{9}NEt-GnRHa at 50 and 100 μg kg^{-1}, respectively, in FIA combined with 8 mg kg^{-1} metoclopramide. Prior to injection, fish were anaesthetised with clove oil at a concentration of 0.033 ml l^{-1} at an exposure time of 5-8 min (Policar et al., 2011).

Females were checked for ovulation every 12 h beginning 72 h post-injection. The trial was completed 14 days (336 h) post-injection. Females without detected ovulation were identified as showing no positive response to experimental treatment. Ovulation success was defined as percentage of ovulated females within 336 h post-injection and latent period as time from injection to ovulation.

When ovulation was detected, eggs were manually stripped. The pseudogonado-somatic index (pGSI) was calculated according to the formula (weight of stripped egg/female BW before stripping) × 100. Mean egg weight to the nearest 0.0001 g was determined gravimetrically from 150 unfertilized eggs using an electronic balance (Kern and Sohn, GmbH, Balingen, Germany). Mean diameter of 150 fresh unfertilized eggs was measured for each female with using a binocular microscope (Olympus SZ 40) fitted with a phototube and digital camera (Olympus Camedia C5060WZ). The digital images were analysed with Olympus Micro Image 4.0.1. for Windows.

The pH of ovarian fluid was measured (inoLAB 720 pH meter, WTW, 823 62 Weilheim, SRN) in four areas of the freshly stripped egg mass. Average pH value was calculated from five independent measures of pH ovarian fluid.

Three samples of eggs totalling 5 g (approximately 820 eggs) were collected from each ovulated female and immediately fertilized with stripped sperm from three males. Sperm was collected according to Bondarenko et al. (in press). In total, 400 μl of sperm was mixed with 5 g of eggs, and 20.6 ml of activation solution (100 g $CO(NH_2)_2$ and 25 g l^{-1} NaCl dissolved in 5 l hatchery water) was simultaneously added and mixed with eggs and sperm. After fertilization and elimination of egg stickiness according to Bondarenko et al. (submitted), a sample of 200 eggs was collected from each fertilized egg batch. Three egg samples from each female were placed into separate transparent 2.5 l plastic incubators described by Svinger et al. (2013) for incubation and determination of fertilization and hatching rate according to Policar et al. (2010). Fertilization rate was determined under a dissecting microscope 3 days post-fertilization when the eggs were at gastrula stage. Incubators were equipped with controllable water flow at 2 l min^{-1} and water temperature 13.0 ± 0.2 °C.

All data were analysed with Statistica 9 (StatSoft, Tulsa, USA). Differences in ovulation success, latency, and pGSI were analysed using a *t*-test for independent observations. Hierarchical ANOVA was used to characteristic differences in fertilization rate, hatching rate, size and weight of eggs, and pH level of ovarian fluid. A significance level (α) of 0.05 was applied to all tests. The data are presented as the mean ± SEM (standard error of mean).

Results

Response to hormone induction of ovulation, latent period, pseudo-gonado-somatic index, fertilization rate at the gastrula stage, hatching and fertilization rate, size and weight of eggs, and pH of ovarian plasma are summarized in Table 1.

No ovulation was observed in Group ICC or in any group injected with the GnRHa formulations, with the exception of a single female in Group 3 (sGnRHa [DArg^{6}Pro^{9}Net] 50 μg kg^{-1} + Met 8 mg kg^{-1}) that ovulated spontaneously, and possibly not as a result of the treatment. All females in Group CP ovulated, and 70% ovulated in Group AOC. The latent period in Group AOC was significantly longer at 264 ± 58 h (mean ± SD) than in Group CP (96 ± 4 h) and Group 3 (108 h). The value of pGSI did not significantly differ among groups. The mean fertilization rate (FR) and hatching rate (HR) was significantly higher in Group GnRHa 3 (FR = 88.6 ± 4.5% and HR = 65.5 ± 7.5%) and AOC (FR = 85.5 ± 12.0%; HR = 68.6 ± 9.9%) than in the CP group (FR = 66.0 ± 13.7%; HR = 54.4 ± 8.0%). There was no significant difference in egg size and weight among groups AOC, CP, and Group 3. The pH of ovarian plasma was significantly higher in GnRHa Group 3 (8.35 ± 0.03) than in groups CP and AOC (8.11 ± 0.02 and 8.27 ± 0.03, respectively).

Discussion

Single injections (10–50 μg kg^{-1}) with mammalian GnRH analogues have been able to overcome reproductive issues related to captivity and induce ovulation in various fish species (Mikolajczyk et al., 2008). In contrast, a single injection of mGnRHa or sGnRHa in northern pike has not been shown effective (Billard and Marcel, 1980; Szabó, 2003). Northern pike is a coldwater spawning species and may require a prolonged time with elevated luteinising hormone (LH) levels to complete the final stages of gametogenesis, as is the case with another cold water species, the winter flounder *Pseudopleuronectes americanus* (Harmin and Crim, 1992). After a single injection of GnRHa, the duration of GnRHa circulation in blood may be insufficient to stimulate the surge of LH (Crim et al., 1988; Mikolajczyk et al., 2003, 2007; Podhorec and Kouril, 2009) that is a prerequisite for completion of final oocyte maturation and ovulation in northern pike. Other factors that can considerably influence the final stages of gametogenesis are handling stress (De Montalembert et al., 1978; Breton et al., 1983) and stage of gonad development (Billard and Marcel, 1980). The solution to this inadequate release profile of GnRHa could be the utilisation of GnRHa delivery systems that are able to stimulate sustained elevation of plasma LH and therefore induce the natural progression of plasma steroid increase that is associated with FOM and ovulation (Mylonas and Zohar, 2001; Fornies et al., 2003).

Amplification of GnRHa potency with a dopamine inhibitor is commonly used in culture of fish such as Cyprinidae, but the benefits of dopamine inhibitor use in northern pike remains questionable (Szabó, 2003).

In the CP group, all females ovulated on day 4 post-injection. This is consistent with results of other studies that have used carp pituitary or other hormone gonadotropin-containing preparations (Billard and Marcel, 1980; Brzuska and Malczewski, 1989; Szabó 2001, 2003, 2008). In the AOC group, 70% of females ovulated during the experiment. However, De Montalembert et al. (1978) showed that non-stimulated females in captivity do not ovulate for various reasons: stress, photo-thermal regimes, lack of spawning substrates. It was shown by Ivanova and Svirskaya (2009) that duration of spawning period can range from a few days up to one month or more, depending on photoperiod and temperature. Our results indicate

that housing pike females in ambient environmental conditions with natural littoral vegetation is suitable for stimulation of final ovary maturation; however synchronization of ovulation is low. The synchronization of ovulation in carnivorous fish species is an important aspect of their culture, since a long spawning period may increase cannibalism during larval or juvenile culture because of different ages and sizes of larvae (Ivanova, 2009). Therefore applications of hormone treatments are widely used (Křišťan et al., 2013). In our experiment, in Group AOC ovulation was not synchronized, and the spawning period was significantly longer than in the CP group (264 ± 58 h).

The pGSI value in our study was similar to that reported by Szabó (2003). We did not find significant differences in pGSI among groups. Higher fertilization and hatching rates were found in Group AOC and Group GnRHa 3 than in Group CP (Table 1). Our hatching and fertilization results were higher than those reported by other authors (De Montalembert et al., 1978; Billard and Marcel, 1980; Horváth, 1983; Szabó, 2001; 2003; 2008). Billard and Marcel (1980) describe low efficacy of carp pituitary at 3 mg kg^{-1} in induction of ovulation in northern pike, reporting oocyte maturation but no ovulation. Low doses of carp pituitary consistently stimulate final maturation of oocytes, but do not always induce ovulation (Horváth, 1983).

In Group GnRHa 3, mean egg weight was 20–25% greater and size 12% larger than was reported by Murry et al. (2008) for weight and by Benzer et al. (2010) for diameter. However, egg weight and size in groups AOC and CP were similar to those reported by the cited authors.

The pH is considered one of the main indicators of ovarian plasma quality, but no information on this is available for northern pike. In rainbow trout, pH below 7.4 is considered to indicate low quality ovarian plasma (Wojtczak et al., 2007), while an ovarian plasma pH range of 8.44–8.57 is considered high quality (Lahnsteiner et al., 1999). Low ovarian plasma pH has a negative effect on sperm motility and velocity during artificial insemination of eggs (Wojtczak et al., 2007). Decreasing of the pH can appear when the sour contain of the eggs escape (6.47 according to Dietrich et al., (2007)) to the ovary plasma. It can happen when the eggs are overripened. (Lahnsteiner, 2000) or mechanically destroyed (Dietrich et al., 2007). In the present study, the values of the ovary pH ranged between 8.11 and 8.35 and significantly higher were in Group GnRHa 3. Were found positive dependence of ovary plasma pH of fertilization rate in gastrula stage (Figure 1) but this result shut be understand only as an indicative due to low number of observations. It is necessary to conduct more experiments with a larger number of females to determine optimal pH value, which could help to establish good conditions for artificial fertilization.

Conclusion

GnRH analogues were ineffective under the protocol used in the present study. However, these products or other GnRHa formulations may be more effective with different protocols. Assessment of LH levels during oocyte maturation and ovulation may aid in developing more effective induction protocols. Future research with northern pike should investigate protocols using sustained release methods of GnRHa variants.

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Table 1. *Effectiveness of treatments [ambient environment, carp pituitary, and sGnRHa (DArg^{6}Pro^{9}Net) at 50 or 100 µg kg^{-1} with or without metoclopramide or Freund's incomplete adjuvant] on induction of ovulation in northern pike (Esox lucius L.). Data are presented as mean ± standard error of mean (SEM).*

Group	Treatment / dose	Ovulation success (%)	Latent period (h)	pGSI (%)	Fertilization rate in gastrula stage (%)	Hatching rate (%)	Size of eggs (mm)	Weight of eggs (mg)	pH of ovarian plasma
ICC	Saline solution 3 mg kg^{-1}	0							
AOC	Saline solution 3 mg kg^{-1}	70	264^{c}± 58	19.3^{a} ± 5.9	85.5^{b} ± 12.0	68.6^{b} ± 9.9	2.7^{a} ± 0.21	6.15^{a} ± 0.26	8.27^{a} ± 0.03
CP	Carp pituitary 3 mg kg^{-1}	100	96^{a} ± 4	17.8^{a} ± 7.5	66.0^{a} ± 13.7	54.4^{a} ± 8.0	2.7^{a} ± 0.16	6.11^{a} ± 0.35	8.11^{a} ± 0.02
GnRHa group 1	sGnRHa (DArg^{6}Pro^{9}Net) 50 µg kg^{-1}	0							
GnRHa group 2	sGnRHa (DArg^{6}Pro^{9}Net) 100 µg kg^{-1}	0							
GnRHa group 3	sGnRHa (DArg^{6}Pro^{9}Net) 50 µg kg^{-1} + Met 8mg kg^{-1}	10	108^{b}	18.5^{a} ± 0	88.6^{b} ± 4.5	65.5^{b} ± 7.5	2.8^{a} ± 0.25	6.4^{a} ± 0.38	8.35^{b} ± 0.03
GnRHa group 4	sGnRHa (DArg^{6}Pro^{9}Net) 100 µg kg^{-1} + Met 8mg kg^{-1}	0							
GnRHa group 5	sGnRHa (DArg^{6}Pro^{9}Net) 50 µg kg^{-1} + FIA	0							
GnRHa group 6	sGnRHa (DArg^{6}Pro^{9}Net) 100 µg kg^{-1} + FIA	0							
GnRHa group 7	sGnRHa (DArg^{6}Pro^{9}Net) 50 µg kg^{-1} + Met 8mg kg^{-1} + FIA	0							
GnRHa group 8	sGnRHa (DArg^{6}Pro^{9}Net) 100 µg kg^{-1} + Met 8mg kg^{-1} + FIA	0							

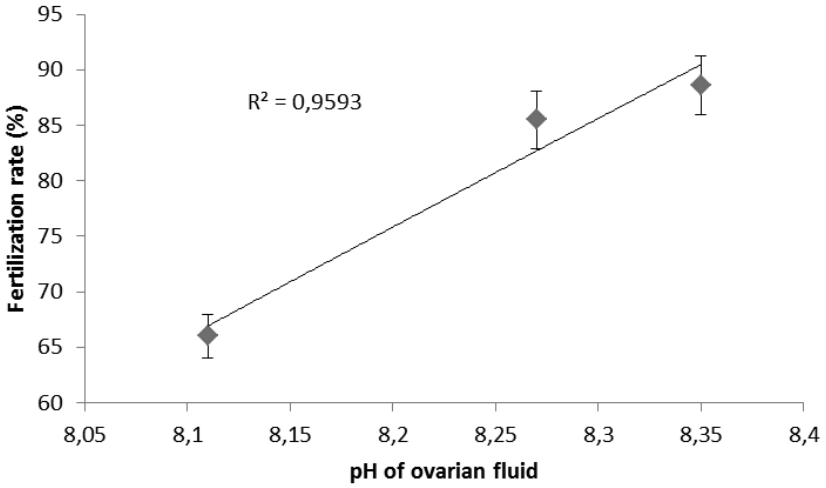

Figure 1. *Relation between ovarian fluid pH and fertilization rate.*

CHAPTER 3

COMPARISON OF SWIMMING FLAGELLA CHARACTERISTICS IN NORTHERN PIKE (*ESOX LUCIUS* L.) AND STERLET (*ACIPENSER RUTHENUS* BRANDT) SPERMATOZOA USING CASA ANALYSIS AND HIGH SPEED VIDEO MICROSCOPY

Paper II:

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COMPARISON OF SWIMMING FLAGELLA CHARACTERISTICS IN NORTHERN PIKE (*ESOX LUCIUS* L.) AND STERLET (*ACIPENSER RUTHENUS* BRANDT) SPERMATOZOA USING CASA ANALYSIS AND HIGH SPEED VIDEO MICROSCOPY

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Flagella movement in pike and sterlet sperm

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Keywords: *sperm motility, quality assurance, wave parameters*

List of abbreviations: *CASA – Computer assisted sperm analysis; DHT – diameter of head tracks; FBF – flagella beat frequency; HO – head orientation; HSVR – high speed video records; HV – head velocity; LIN – linearity of the curvilinear path; NOC – number of curvatures; SE – swimming efficiency; VAP – average path velocity of sperm. WA – wave amplitude; WD – waves damping; WL – wave length; WV – wave velocity*

Abstract

In fish reproduction, evaluation of sperm quality varies broadly depending on technologies used for the goal and needs of fish farmers. Two methods of sperm motility analysis were used in the present study. Northern pike (*Esox Lucius* L.) and sterlet (*Acipenser ruthenus* Brandt) sperm characteristics were analyzed and compared by traditional CASA (Computer Assisted Sperm Analysis) analysis with tracks of head and new and modern high-speed video microscopy records with high definition image of fish sperm flagella. Average pike and sterlet sperm velocity were found such as 160.28 ± 11.4 μm/sec and 193.3 ± 17.8 μm/sec at 10 seconds after sperm motility activation. At 45 seconds after sperm activation, 10% of motile spermatozoa were found in pike and in sterlet after 210 seconds. Linearity was 0.66 in pike and 0.76 in sterlet at 10 seconds after sperm activation. High speed video recordings analysis measured little different values of average sperm velocity at 10 seconds after sperm activation such as: 183.48 ± 15.11 μm/sec in pike and 234.57 ± 26.13 μm/sec in sterlet. Average value of wavelength for pike sperm was 6.10 ± 0.07 μm and for sterlet was 4.91 ± 0.09 μm. Average value of wave amplitude was 3.07 ± 0.04 μm for pike and 4.27 ± 0.08 μm for sterlet respectively. Average wave velocity was 879.54 ± 69.37 μm/sec in pike spermatozoa and 1 144.92 ± 89.63 μm/sec in sterlet spermatozoa respectively. Value of swimming efficiency was 4.052 for pike and 4.401 for sterlet spermatozoa. Number of curvatures per flagella was 4.5 ± 0.25 for pike and 7.7 ± 0.51 for sterlet spermatozoa. Head orientation angle was 15.5° for pike and 8.3° for sterlet.

Introduction

Fish sperm motility is lasting for short period of time from 40 seconds up to a few minutes and rapid with 50 to 90 Hz initial frequency of flagellar beating (Morisawa, 1994; Cosson, 2010). During this brief period of sperm activity, all swimming parameters are decreasing very rapidly and leading to a lower and lower progressive efficiency (Cosson et al., 1999). During spermatozoa movement flagella presents waves, which are very similar from sperm to sperm cell at a given time point after activation (Lindemann and Lesich, 2010). The flagella wave parameters are changing depending on different environmental condition (pH, salinity, ionic concentration etc.) in a homogeneous way among the sperm cell population (Cosson et al., 1999).

Northern pike (*Esox lucius* L.) and sterlet (*Acipenser ruthenus* Brandt) presented several advantages for description of flagella parameters of sperm in movement: both type of spermatozoa have quite long flagellum such as 32 μm in pike (Alavi et al., 2009) and 43 μm in sterlet sterlet (Linhartova et al., 2013). In sterlet, sperm head presents elongated shape with an acrosomal vesicle (Psenicka et al., 2008) while head is round shape in pike but without acrosome (Alavi et al., 2009). In sterlet, flagella present the possibility to describe planar waves during some brief periods and 3D waves during other periods (Boryspolets et al., 2013a) while in contrast, pike's flagella present only planar waves. The motility period duration is approximately 3–5 minutes in sterlet and 1–1.5 minutes in pike.

Current method used for sperm motility analysis is CASA (Computer Assisted Sperm Analysis), which mainly allows obtaining information about the head movement (number of motile sperm heads, their velocity, linearity etc. (Boryshpolets et al., 2013a; Amann et al., 2014). Much more information can be acquired from the analysis of flagella swimming parameters obtained by high-resolution microscopy combined with high speed video microscopy (Cosson et al., 2008). This information is dealing with flagellar parameters such as: wave amplitude, wave length, wave velocity, general curvature of flagellum, number of bends along the flagellum, amplitude of successive bends, etc.

The main goal of the present study is to describe new and modern ways to appreciate and quantify with more details the sperm quality of cultured fish and to compare the sperm swimming parameters in Northern pike and sterlet, used as model species, by application of high speed video microscopy in complement to the CASA analysis.

Materials and methods

Broodstock fish preparation

Northern pike and sterlet were used such as experimental species in this study. In total, 10 mature males of pike (BW = 1600 ± 150 g and TL = 235 ± 30 mm) were collected from production pond near Nove Hrady, Czech Republic (GPS: 48°48′6.395″N, 14°46′55.138″E) in spring 2012 and transported to the Laboratory of Intensive Aquaculture, Faculty of Fisheries and Protection of Waters in Vodnany, Czech Republic (IA, FFPW). In total, 10 mature males of sterlet (BW = 1300 ± 150 g and TL = 470 ± 45 mm) were used for this experiments from the Genetic Fisheries Center, FFPW in Vodnany, Czech Republic. Sperm samples from both species were collected during natural spawning season after hormonal injection by carp pituitary in dose 3 mg kg^{-1} for pike (Bondarenko et al., in press) and 4 mg kg^{-1} for sterlet (Dzyuba et al., 2010).

Sperm collection

Sperm samples from pike were obtained by abdominal massage 96 hours after hormonal injection (sperm was collected in the syringe). Sperm from sterlet was obtained from urogenital papilla by plastic catheter (4 mm diameter) and collected directly into 50 mL plastic tubes. All attempts were made to avoid contamination of sperm by urine, blood or water during stripping. After collection of sperm samples, sperm from both species was stored on ice no longer than 2 hours under aerobic conditions till sperm dilution and analysis.

Activation of motility

Sperm of both species was diluted 1 : 1000 in swimming solution containing: 20 mM $CO(NH_2)_2$ and 5 mM NaCl, osmolality – 200 mOsmol kg^{-1} for pike (Hulak et al., 2008; Alavi et al., 2009) and 10 mM NaCl, 1 mM $CaCl_2$, 10 mM Tris-Cl pH 8.5 for sterlet (Dzyuba et al., 2010). Sperm motility in both species was assessed at room temperature under the microscope (18–20 °C).

Analysis of the video records

Video-microscopy records of swimming spermatozoa were recorded after 10 seconds after the sperm activation by application of the following methods:

Computer assisted sperm analysis

CASA (Computer Assisted Sperm Analysis) analysis was used for regular sperm observations by an Olympus BX50 microscope with dark-field optics (objective 20×), which was illuminated by stroboscopic LED illumination unit ExposureScope 0.1 (FROV, JU, Czech Republic). This technique was combined with video recording by a CCD video camera (SONY SSC-DC 50 AP, Japan) and DVD video-recorder (SONY SVO-9500 MDP, Japan) and computed from five successive frames by a micro image analyzer (Olympus Micro Image 4.0.1. for Windows). Such video records provide 50 half frames per second with 720 x 576 pixels (PAL 4 : 3) spatial resolution per frame (Figure 1) (Boryshpolets et al., 2013b). At least 700 spermatozoa were analyzed per one used male of each species. CASA analysis leads to information regarding to percentage of motile cells, sperm velocity and linearity of sperm movement. All these parameters were measured according to Wilson-Leedy and Ingermann (2007).

Data obtained from CASA video records

The next parameters were measured by CASA: average path velocity of sperm (VAP), percentage of motile spermatozoa and linearity of the curvilinear path (LIN) (Amann and Waberski, 2014).

High speed video records analysis

High speed video records (HSVR) with detailed images of spermatozoa moving were obtained by using of Olympus BX50 microscope with 100× phase contrast optics (Zeiss Ph 3 NeoFluar 100×, Oil). Spermatozoa movement was recorded with a high-speed video camera (Olympus *i*-speed TR) providing 848 x 688 pixels spatial resolution, 1000 frames per second. Records were analyzed by image analysis software (Olympus Micro Image 4.0.1. for Windows)

on successive images covering one beat cycle (usually at least 20 successive images). At least 10 spermatozoa were analyzed per each male in both species. Analyses were could not be done automatically which explains why a restricted number of sperm flagella was analyzed in this preliminary study.

Data obtained from HSVR

The video sequences of interest, especially those where the individual sperm flagella were recorded in correct focus, were retained and the following parameters were measured on each successive image: wave length (WL, μm), wave amplitude (WA, μm), wave velocity (WV, μm/sec), waves damping (WD), head velocity (HV, μm/sec), swimming efficiency (SE), flagella beat frequency (FBF, Hz), head orientation (HO), number of curvatures (NOC) and diameter of head tracks (DHT).

WL was measured at 10 seconds after sperm activation in each successive image and corresponds to the distance in the intersection between the reference line and the flagellum crest of wave 1 (Figures 2a, 2b, 2c).

WA was measured at the same moment such as WL in each successive images such as distance from reference line (mid line of the flagellum) to the crest of the corresponding wave (Figures 2a, 2b, 2c).

WV was observed such as the displacement covered by the wave crest during one beat cycle (Figures 2a, 2b, 2c).

WD was illustrated by the fact that a decrease of wave amplitude appears along the distance from the head on the flagellum.

HV was measured at 10 seconds after the sperm activation in each successive image during one beat cycle such as distance covered by spermatozoa during one beat cycle.

Value of SE was obtained by calculating the ratio between HV and WV by following formula:

$$SE = HV/WV.$$

FBF was obtained by the evaluation of the number of images necessary to cover a full beat cycle of the flagellum. This parameter showed how many images are needed to observe the same flagellar image with bends in the same position, the time elapsed between two successive images representing a known period of the time.

The HO value was obtained such as the angle of the head oscillation according to the drawing in Figures 3A and 3B for sterlet and 3C and 3 D for pike. The amplitude of the head angle is related to the local curvature of the flagellum at the head/tail junction.

The NOC per flagellar length was measured on several video images (at 10 seconds after the sperm activation) of individual sperm flagella. This parameter is schematically shown in Figure 2C.

DHT was measured on several video images (at 10 seconds after the sperm activation) of individual sperm flagella, which is schematically shown in Figure 4.

Statistical Analyses

Data were analyzed by Statistica 10 software (StatSoft Inc., Tulsa, USA). Factorial ANOVA ($P < 0.05$) was used to characterize differences in the sperm velocity, percentage of motile sperm, linearity, wave length, wave amplitude, wave efficiency and head oscillation. Data in text, figures and tables are presented as mean ± S.D.

Results

Summary of results from CASA

A summary of the values of various parameters obtained in this study by CASA for pike and sterlet sperm is shown in Figure 5. Duration of sperm motility in pike was circa 45 seconds. Average pike sperm velocity was found 160.28 ± 11.4 μm/sec at 10 seconds after the sperm activation and less than 15.3 ± 3 μm/sec after 45 seconds after sperm activation. Duration of sperm motility in sterlet was approximated 210 seconds. Average sterlet sperm velocity was significantly higher 193.3 ± 17.8 μm/sec at 10 seconds after activation of motility, 137.55 ± 11.7 μm/sec after 45 seconds and 14.33 ± 3.7 μm/sec at the end of motility sperm period (Figure 5a). Percentage of motile spermatozoa (Figure 5b) was similar in pike and sterlet at 10 seconds after the sperm activation such as: 90% and 85% for pike and sterlet, respectively. Less than 10% of motile sperm was found at 45 and 210 seconds after sperm activation in pike and sterlet spermatozoa, respectively. The linearity index was 0.66 and 0.76 in pike and sterlet at 10 seconds after sperm activation, respectively. A similar linearity index 0.75 was observed in pike spermatozoa from 25 seconds till 45 seconds after the sperm activation. A stable linearity index 0.85 was observed in sterlet spermatozoa from 90 seconds till 210 seconds after the sperm activation (Figure 5c).

Summary of results from HSVR

The variation of wave length as a function of time during one beat cycle for pike and sterlet spermatozoa is presented in Figure 6 such as a function of time during one beat cycle for pike and sterlet spermatozoa. Average value of WL for pike was 6.10 ± 0.07 μm, maximum value was 9.43 ± 0.3 μm. Value of WL in sterlet was significantly lower. Average value of WL for sterlet was 4.91 ± 0.09 μm, maximum value was 7 ± 0.16 μm.

Typical values of wave amplitude for sterlet and pike spermatozoa during one beat cycle are presented in Figure 7. Average value was 3.07 ± 0.04 μm, maximum value of WA for pike spermatozoa was 4.94 ± 0.07 μm. Value of WA in sterlet is significantly higher. Average was 4.27 ± 0.08 μm and maximum value was 7.59 ± 0.14 μm.

The average wave velocity values (Figure 8) was 879.54 ± 69.37 μm/sec in pike spermatozoa and 1144.92 ± 89.63 μm/sec in sterlet spermatozoa (Table 1).

The representative results of WD are shown in Figure 9.

Value of HV obtained by CASA (Figure 5a) were 160.28 ± 11.4 μm/sec and 193.3 ± 17.8 μm/sec at 10 seconds after sperm activation, in contrast value of the same parameters obtained by method of HSVM were 183.48 ± 15.11 μm/sec and 234.57 ± 26.13 μm/sec for pike and sterlet respectively (Table 1).

The values of SE were 4.052 for pike and 4.401 for sterlet sperm (Table 1).

As mentioned in Table 1, the initial FBF has value of 52 Hz in sterlet and 30 Hz in pike sperm.

The representative values of NOC per flagellar was 7.7 ± 0.51 for sterlet spermatozoa and 4.5 ± 0.25 for pike spermatozoa.

The HO angle was 8.3º for sterlet and 15.5º for pike. The results of HO measurement are shown in Figure 10.

The presence of blebs, loops etc., appearing during motility period due to low osmolality exposure can also be detected by high-speed video images as shown for example in Figure 11.

Discussion

Some of the original features of fish spermatozoa, especially their flagellar characteristics have been qualitatively described in few previous publications (Cosson, 2008a, 2010; Ishijima, 2012). While some description can be found for sterlet sperm in Gillies et al., (2013), only very restricted information was published in case of pike spermatozoa (Alavi et al., 2009). In the present paper we propose a new approach for quantification of flagella parameters using dynamic morphometry from images obtained from high-speed video records at high resolution. Among other advantages, as shown by our results, the use of the new method describing flagella parameter is complementary to the usual CASA analysis. Nevertheless our present set of observations constitutes only a prospective study due to the restricted number of samples analyzed.

So far, the most previous studies on swimming fish spermatozoa mainly made use of CASA, a method which takes into account the characteristics of sperm head tracks (Kime et al., 2001; Wilson-Leedy and Ingermann, 2007). The validity and the limits of the evaluation in the different CASA parameters related to flagellar parameters (velocity, linearity, etc) was discussed in Wilson-Leedy and Ingermann (2007) and Boryshpolets et al. (2013).

The main motor component in a flagellum is the axoneme, a macromolecular assembly of microtubule assorted of ATP consuming molecular motors (Gibbons, 1981; Cosson, 2008a). This organelle develops waves, which, in fish spermatozoa, propagate from sperm head/tail junction to tip of flagellum (Cosson, 2008a). The viscous friction of the flagellum waves on the surrounding fluid constitutes the basis of the mechanical power, which propels sperm cells forwardly (Gibbons, 1981). Therefore a better description of essential flagella parameter leads to an improved characterization of movement of the whole sperm cell (Gray and Hanckock, 1955). Some head track parameters are only grossly reflecting flagella behavior: more information is potentially gained directly at the production source of mechanical power, i.e. the flagellum.

Most of the results described in present study mainly concentrate on the events during earliest part of the sperm motility period during which all swimming parameters and consequently the velocity are at their highest level (Cosson, 2004; Cosson, 2010). The flagellar beat frequency (FBF) corresponds to the number of beat cycles that the flagellum describes per second (Cosson et al., 1985; Cosson, 2008b): in our study, the time duration needed to cover one beat cycle is directly measured on series of successive high-speed video images. In the case of sterlet, our results lead to values FBF ranging 52 Hz during the earliest part of the sperm. These results are similar to those previously published in studies on *Acipenser baerii* spermatozoa: 48–42 Hz in Tsvetkova et al. (1996) or 55–60 Hz in Billard et al. (1999). In pike spermatozoa, we observe a beat frequency ranging 30 Hz but no previous data on this point were found in literature.

The wave amplitude in combination with the number of waves per flagellar length mainly contributes to the total power pushing forwardly the sperm cell in movement (Gray and Hancock, 1955). The values of WA obtained for pike and sterlet in their earliest period of motility are ranging 4.94 ± 0.07 μm and 7.56 ± 0.14 μm respectively. These values can be compared with WA measured for sperm flagella of other species: 4–4.6 μm in turbot (Cosson et al., 2008), 5.5 μm in sea bass (Cosson, 2010) and 8 μm in *Merluccius merlicius* (Cosson, 2010) at the earliest part of the sperm motility period.

For geometrical reasons (total length of flagellum), the wave-length (WL) is also closely related to the WA and the number of waves. In our study, the values of 9.43 ± 0.3 μm and 7 ± 0.16 μm are observed for pike and sterlet respectively. These values are to be compared

with those published for spermatozoa of other species: 9–12 μm in turbot (Cosson et al., 2008), 10.5 μm in sea bass (Cosson, 2010) and 21 μm in *Merluccius merluccius* (Cosson, 2010).

Another characteristic of fish sperm flagella is the change of the flagellar shape which occurs during the sperm motility period: wave attenuation, so called damping, mostly appears in form of a decrease of WA as a function of the distance from the head (Tombes et al., 1987). This feature was already described in spermatozoa of various fish species such as turbot (Chauvaud et al., 1995), sterlet (Tsvetkova et al., 1996), trout (Cosson et al., 2008a) and it is observed in the present paper for pike spermatozoa as well. During the sperm motility period, this partial decrease in WA towards the distal part of the flagellum contributes to lower the global flagella propulsive efficiency (Tombes et al., 1987) and could be related to a lack of ATP in the portion of the flagellum distal to the mid-piece where are located mitochondria, which constitute the ATP source (Tombes et al., 1987; Cosson, 2010; Cosson, 2012a).

In the most fish species, sperm flagella are ribbon-shaped as they present membrane folds flanking both sides of axoneme, so called fins (Cosson et al., 1999). By computational simulation, the presence of flagella fins was shown to improve the hydrodynamic properties of spermatozoa (Gillies et al., 2013). These modifications of hydrodynamic behavior are reflected in the wave parameters described in the present paper. It will be interesting to experimentally compare finned flagella with those of cylindrical flagella present in a few other fish species by using flagella parameters such those shown to be accessible in the present paper.

The description of flagellar parameters also leads to additional potent applications: fish spermatozoa have to cope with various physiological situations, which greatly result in a change of the flagellar shape. This is the case when the are exposed to changes in external and internal ions concentration (Cosson, 2004), intracellular ATP level (Perchec, 1995; Cosson et al., 2012a; Cosson, 2012b) or facing hypo or hyper-osmotic conditions. Detailed analysis of such changes in flagellar morphology constitutes a reservoir of new pieces of information as detailed below and so far lowly exploited.

The role of calcium ions in the linearity of the flagellar shape is known to lead to a circularization of tracks described by sperm cells. It well established that Ca^{2+} ions are mainly involved in the control of the degree of curvature of the tracks, both at the external level (Brokaw et al., 1974; Brokaw, 1991) and consequently at the Ca^{2+} internal level, especially in trout sperm (Cosson and Billard, 1989), as well as ultimately at the axonemal level (Cosson and Cosson, 1991).

Ca^{2+} ions are also known to be involved in the acrosomal reaction: the two species used in our study were chosen because one, pike, is lacking this sperm organelle, the acrosome. In case of sterlet the presence of an acrosome in its reacted state (filament protruding at the tip of the head) greatly influence the swimming ability of the sperm cell (Psenicka et al., 2008, 2011) and almost completely prevents its fertilizing ability when the acrosome filament is triggered prior to gametes meeting.

In fish sperm, many effects of other ions on flagellar parameters were described by Alavi and Cosson (2006). These other ions (such as K^{+}) greatly influence the flagellar shape and consequently modify the swimming ability.

Another characteristic of fish spermatozoa motility is their sensitivity to osmolality: as example, freshwater spermatozoa become motile when exposed to low osmolality (fresh water being almost devoid of ions) and this feature greatly influences the wave shape (Dreanno et al., 1999a). But at the same time, such exposure to low osmolality constitutes an osmotic shock of large amplitude, when sperm switch at spawning from quite high osmotic situation (seminal fluid) to surrounding fluid (fresh water): this leads to appearance of blebs or of loops at flagellar tip (Dreanno et al., 1999b). These defects can be visualized on high-

resolution video images (Figure 11) (Tsvetkova et al., 1996) and such defects obviously also affect swimming parameters (Table 1).

In a similar way, other physical conditions of the surrounding swimming fluid such as viscosity are also affecting flagella morphology and consequently swimming performances (Cosson, 2008a). Such viscous conditions are encountered by sperm cells when swimming in the ovarian (coelomic) fluid. Also, pollutants affect fish sperm swimming performance (Alavi et al., 2009) and their effects on flagellar wave's characteristics would gain better quantification by using methods of dynamic morphometry as we briefly describe here from high speed video images.

Ultimately, our results also lead to better basic understanding of flagellar mechanics (Gibbons et al., 1983; Inaba, 2007). As example, one can address the question on the way by which 3D movement (rotation of the whole spermatozoon) is generated? This situation, quite specific to spermatozoa of some fish species (Boryshpolet et al., 2013b), is comparable to the helical movement of eel spermatozoon (Gibbons et al., 1981; Wooley, 1998).

Use of high-speed video allows recording successive individual positions of flagellum during one or several full beat cycles. Such data will lead to more accurate modeling of flagellum behavior during fish sperm motility period and provide a deeper understanding of basis of sperm motility. In addition, these data also allow the development of new approaches for simulating fish sperm flagellum movement by computerization methods.

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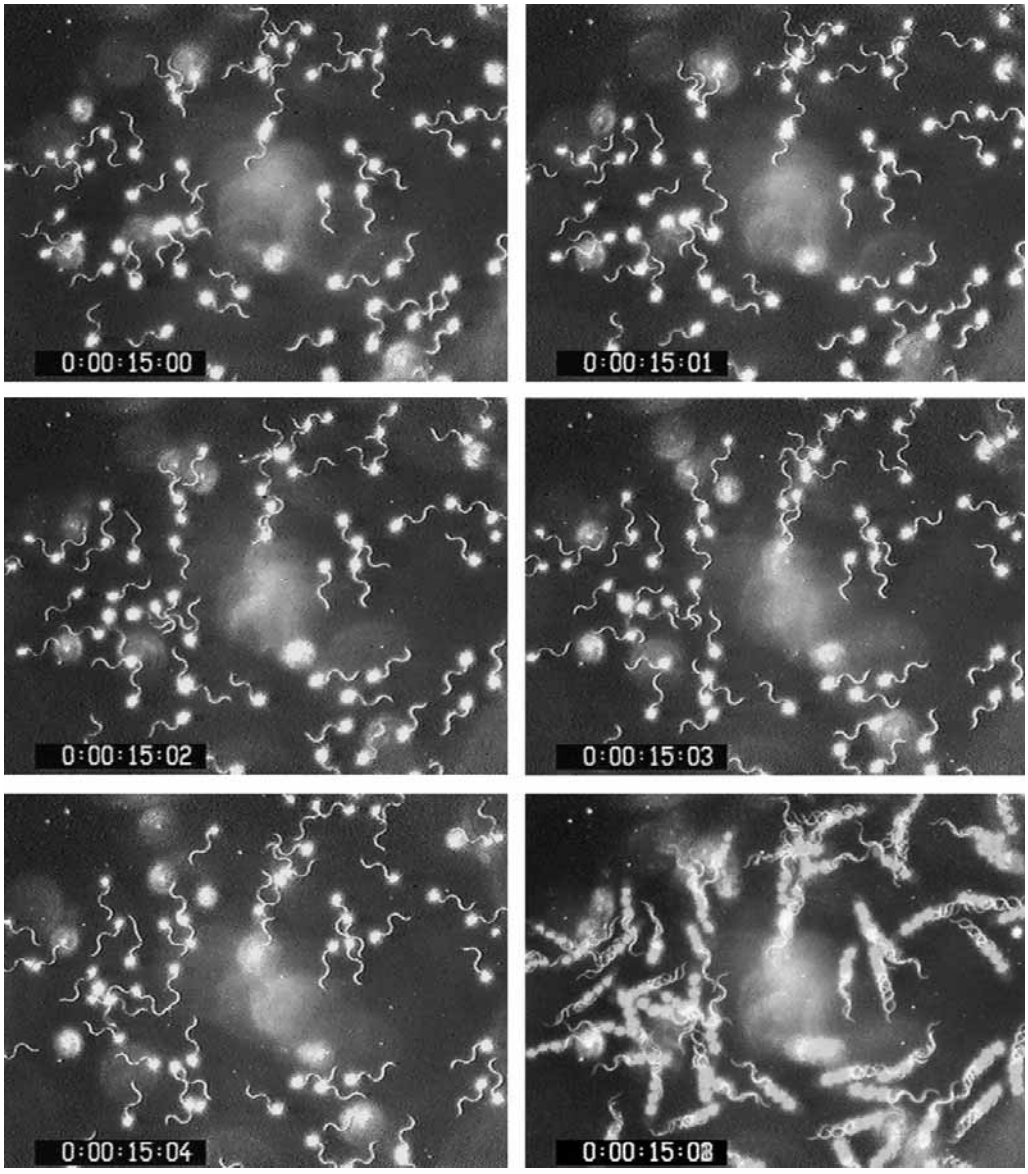

Figure 1. *Example of video image obtained by a regular video camera within CASA analysis. CASA uses group of five successive images (see timer on bottom right) to obtain velocity of sperm. The 5 images are overlapped and trace of each sperm head is automatically identified as blue (origin) and red (last), while intermediate positions are green. Original magnification 20×.*

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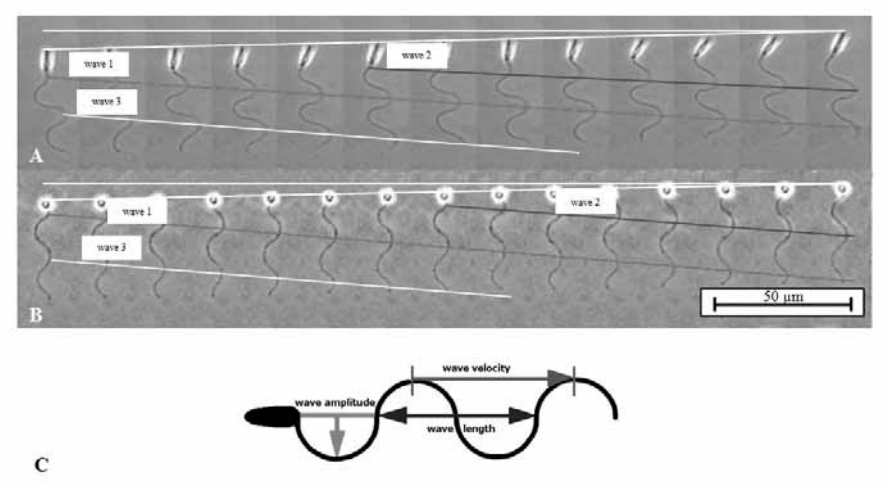

Figure 2. *Sterlet (Acipenser ruthenus Brandt) (A) and Northern pike (Esox lucius L.) (B) spermatozoon. Video was recorded at 1 000 frames per second. The time interval between two images = 2 µsec., time duration of one full beat cycle = 26 µsec. corresponding to 13 above images.*
C – The determination of the wave parameter from each single image. The wave amplitude corresponds to the distance from reference line (mid line of the flagellum) to the crest of the corresponding wave. The wavelength corresponds to the distance the intersection between the reference line and the flagellum. The wave velocity is the displacement covered by the wave crest during one beat cycle (expressed per the unit of time, generally one second). Original magnification 100×.

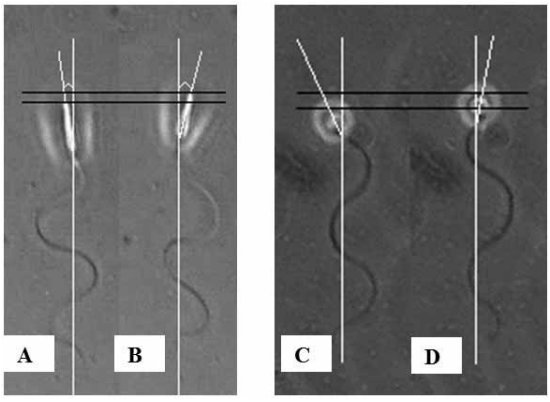

Figure 3. *Head orientation during one beat cycle in sturgeon (Acipenser ruthenus Brandt) (A, B) and in Northern pike (Esox lucius L.) spermatozoon (C, D). The time interval between two images is 12 msec. The distance covered by the sperm head is 1.6 µm. The angle between reference line and sperm head axis in (A, B) panels is 8.3º and is 15.5º in (C, D). Original magnification 100×.*

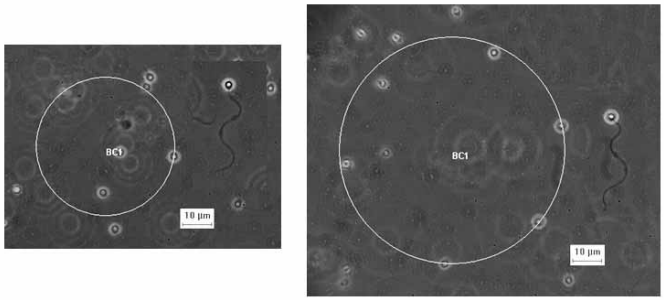

Figure 4. *Diameter of sperm head tracks. Two examples showing successive head positions of the same Northern pike (Esox lucius L.) spermatozoon identified by CASA software (blue red and green spots). A circle is best fitted to measure the head track diameter. Global curvature of the flagellum is in relation with the circularity of the corresponding track. Original magnification 100×.*

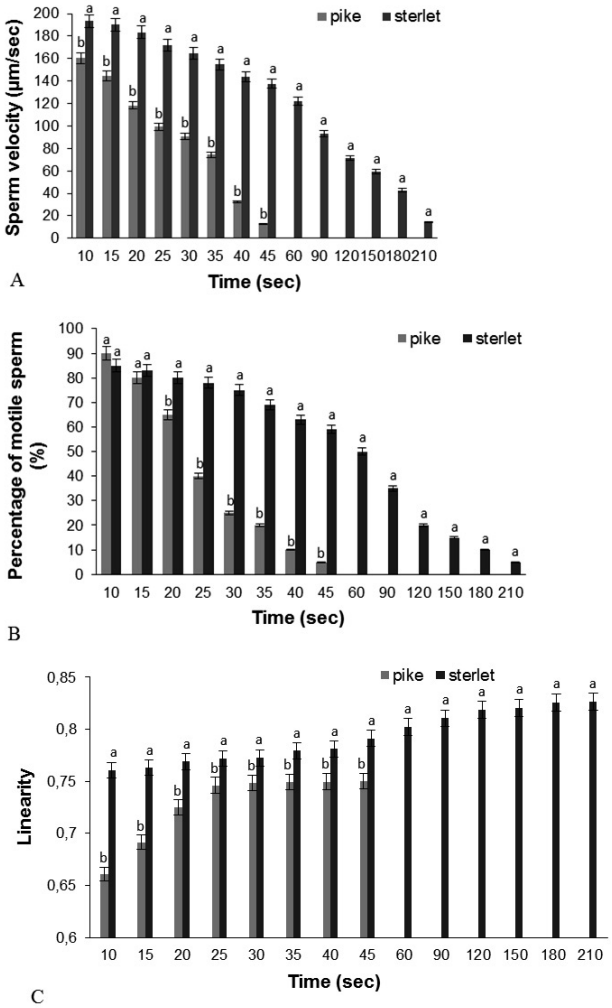

Figure 5. *Average value of velocity (A), percentage of motile spermatozoa (B) and linearity of the curvilinear path (C) in Northern pike (Esox lucius L.) and sterlet (Acipenser ruthenus Brandt).*

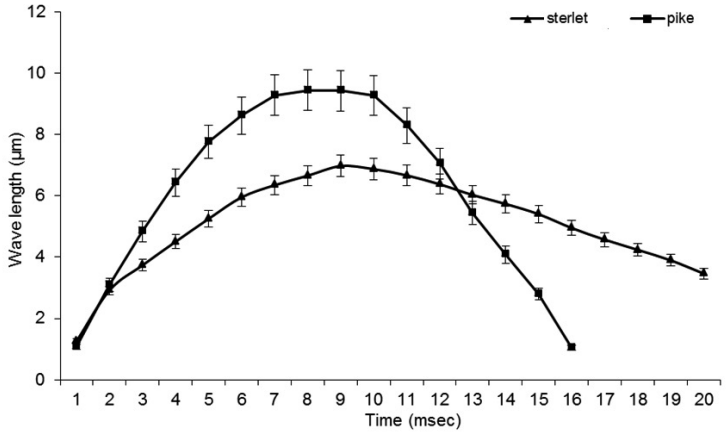

Figure 6. *Variation of wave length of Northern pike (Esox lucius L.) and sterlet (Acipenser ruthenus Brandt) sperm flagella during one beat cycle.*

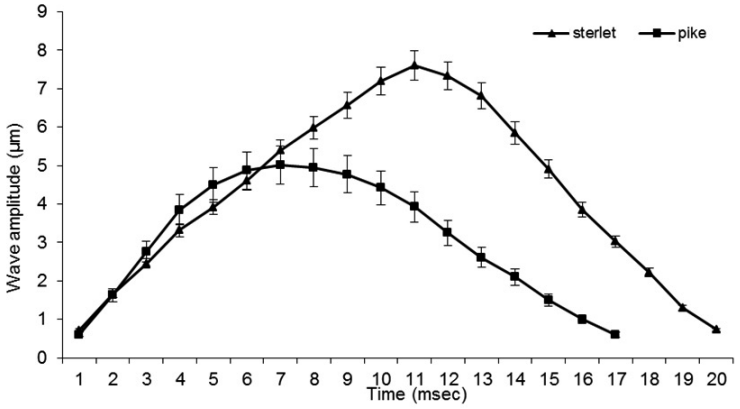

Figure 7. *Wave amplitude of flagella measured on successive images during one beat cycle.*

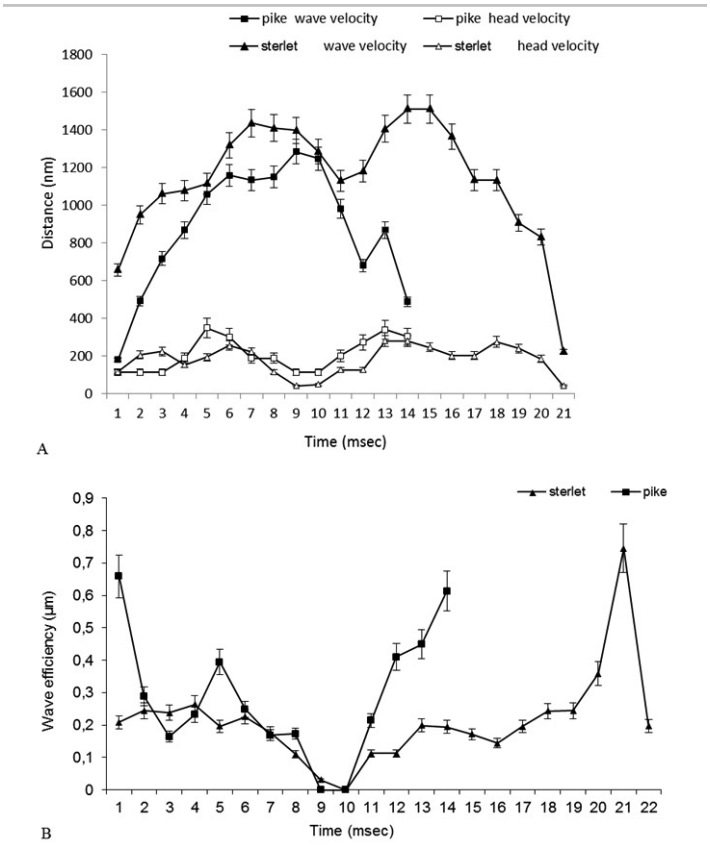

Figure 8. *The local velocity of the crest of wave number 1 is represented as a function of time during one beat cycle (top graphs); the bottom graph represents the wave efficiency during the same beat cycle.*

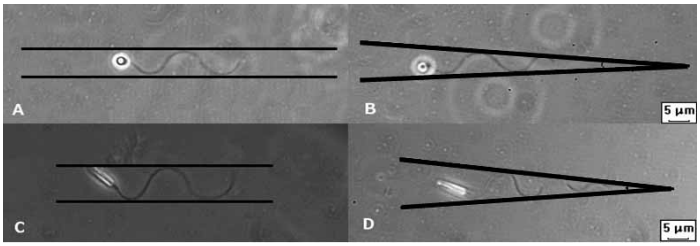

Figure 9. *Wave damping in Northern pike (Esox lucius L.) (A and B) and sterlet (Acipenser ruthenus Brandt) spermatozoa (C and D). The amplitude of first wave (closest to head) is larger than second, and so on to the tip of flagellum (B). This damping effect is absent in the earliest period after motility activation (A and C) and appears during the progress of the motility period (B and D). Original magnification 100×.*

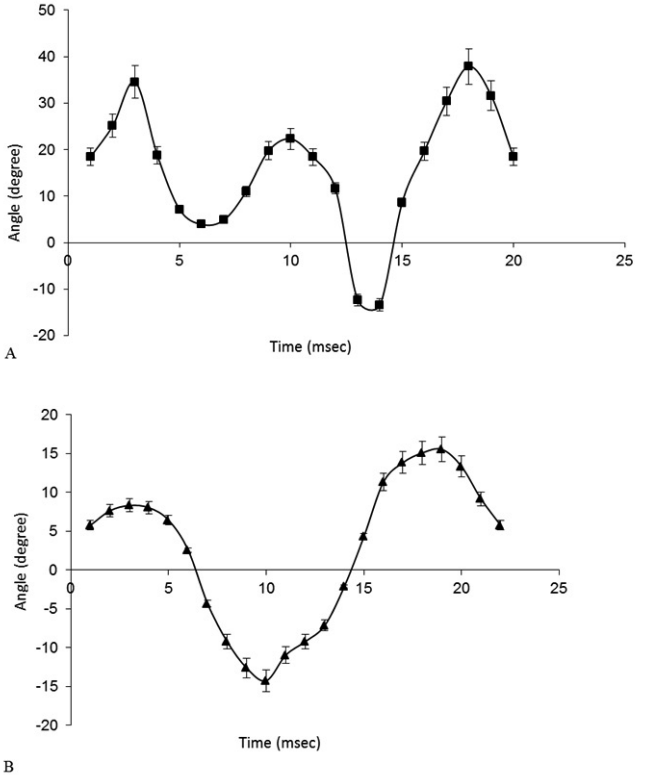

Figure 10. *The variation of head angle during one beat cycle for Northern pike (Esox lucius L.) (A) and sterlet (Acipenser ruthenus Brandt) (B) spermatozoa.*

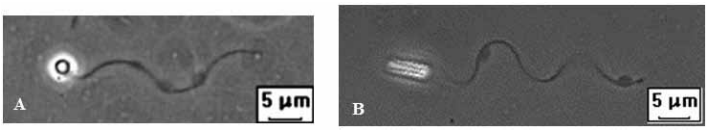

Figure 11. *Presence of abnormalities appearing along flagella in Northern pike (Esox lucius L.) (A) and sterlet (Acipenser ruthenus Brandt) (B) during the motility period in low osmotic swimming media. Original magnification 100×.*

Table 1. *Summary of data obtained from high speed video images at high resolution (10 seconds after activation).*

Parameters	**Northern pike (*Esox lucius* L.)**	**Sterlet (*Acipenser ruthenus* Brandt)**
Number of curvatures per length	4.5 ± 0.25	5.7 ± 0.5
Beat frequency, Hz	30	52
Rotation frequency, Hz	0	25
Average sperm velocity, μm/sec	183.48 ± 15.11	234.57 ± 26.13
Average wave velocity, μm/sec	879.51 ± 69.37	1144.92 ± 89.63
Average head velocity, μm/sec	207.32 ± 26.8	272.79 ± 34.2
Swimming efficiency	4.052	4.401

CHAPTER 4

EFFECT OF WATER TEMPERATURE ON EGG INCUBATION TIME AND QUALITY OF NEWLY HATCHED LARVAE OF NORTHERN PIKE (*ESOX LUCIUS* L.)

Paper III:

Bondarenko, V., Drozd, B., Policar, T. Effect of water temperature on egg incubation time and quality of newly hatched larvae of northern pike (*Esox lucius* L.) Journal of Applied Ichthyology. (*accepted for publishing 16.04.2014*)

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EFFECT OF WATER TEMPERATURE ON EGG INCUBATION TIME AND QUALITY OF NEWLY HATCHED LARVAE OF NORTHERN PIKE (*ESOX LUCIUS* L.)

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Summary

This study examined the effect of temperature on the egg incubation period, survival of eggs during embryo development and quality of newly hatched larvae of northern pike (*Esox lucius* L.) under laboratory conditions. Eggs of similar size (diameter 2.7 ± 0.16 mm and weight 6.11 ± 0.35 mg) from five females were incubated at 3 °C, 6 °C, 10 °C, 14 °C, and 18 °C (groups A, B, C, D, and E, respectively). The lowest fertilization (FR) and hatching rates (HR) were observed in larvae incubated in group A, 44.6 ± 3.2% and 18.26 ± 2.25%, respectively. The highest FR and HR were found in group B (FR, 71.3 ± 4.3%; HR, 56.2 ± 3.21%) and C (FR, 65.6 ± 3.1%; HR, 65.5 ± 5.41%). Time of incubation period varied from 38 ± 0.33 days (120 ± 1.03°d) with 5% hatched larvae to 46 ± 0.42 days (144 ± 1.31°d) with 95% hatched larvae in group A and 2.5 ± 0.08 days (44.67 ± 1.42°d) with 5% hatched larvae to 3.42 ± 0.06 days (61.11 ± 1.07°d) with 95% hatched larvae in group E. Larvae from groups A, D, and E were characterized by the lowest values of variables such as resistance to osmotic stress after 90 min of exposure to saline solution (OS = 54 ± 3 – 76 ± 3%), frequency of normally developed larvae (FNL = 23.8 ± 4.14 – 87. 1 ± 2.42%), and yolk sac volume (YsV = 3.41 ± 0.44 – 3.89 ± 0.45 μl^3). Larvae showing the best quality were recorded in groups B and C: OS = 92 ± 3% and 80 ± 4 %, FNL = 89.7 ± 3.62% and 93.8 ± 3.17, YsV= 3.3 ± 0.66 μl^3 and 3.04 ± 0.42 μl^3). Fertilization and hatching rates and quality of larvae showed that optimal temperature for successful egg incubation and production of high quality larvae ranges 6 to 10 °C. Relationship between ontogenic rate and temperature predicts development of the pike embryo to be hypothetically stopped at 3.3ºC or below.

Introduction

Current European inland aquaculture requires new and rapid development with respect to diversification of fish species, aquaculture products, and markets (Policar and Adamek, 2013). Freshwater fishes such as European perch (*Perca fluviatilis* L.) (Stejskal et al., 2009), pikeperch (*Sander lucioperca* L.) (Policar et al., 2013), burbot (*Lota lota* L.) (Wocher et al., 2012), and northern pike (*Esox lucius* L.) (Kucska et al., 2005) are promising species for diversification of intensive European aquaculture using recirculating aquaculture systems (RAS).

Northern pike is an important piscivorous species in freshwater ecosystems (Craig, 2008) and a popular sport fish providing valuable meat (Salam and Davies, 1994; Mann, 1996; Margenau et al., 2008).

Traditional polyculture of pike for restocking and the problem of market limited by cannibalism (Wright and Giles, 1987; Muscalu-Nagy et al., 2011), can be eliminated by adequate grading and feeding under intensive culture using recirculating aquaculture systems (RAS) and artificial feeding (Kucska et al., 2005). Intensive culture of pike, including hormone-

induced spawning (Szabo, 2008), weaning of larvae onto commercial food (Szczepkowski, 2009), optimized intensive juvenile culture up to body weight (BW) of 27 g (Kucska et al., 2005), and on-growing culture up to 250-300 g BW (Muscalu-Nagy et al., 2011) has been developed in Central Europe in the past decade.

To be economically viable, intensive culture needs a stable, high quality, year-round supply of larvae (Müller-Belecke and Zienert, 2008). Generally, it is possible to stagger time of fish reproduction by techniques such as out-of-season spawning (Migaud et al., 2004; Ronyai, 2007; Policar et al., 2010), shifting the spawning period (Ronyai, 2007; Muscalu-Nagy et al., 2011) and modifying egg incubation times (Kucharczyk, et al., 1997; Policar et al., 2004; 2009; Drozd et al., 2009; Kupren et al., 2011). Water temperature is one of the most important factors affecting fish embryo development as well as incubation and hatching period (Kucharczyk, et al., 1997; Drozd et al., 2009; Kupren et al., 2011).

The aim of the present study was to determine the effect of water temperature on duration of embryo ontogenesis and hatching time, fertilization at gastrula stage, hatching rates, and quality of larvae including larval size, development, and resistance of larvae to osmotic stress under controlled conditions for northern pike.

Materials and methods

Five females (BW = 2450 ± 350 g and TL = 70 ± 4.5 cm) and 15 males (BW = 960 ± 180 g and TL = 54 ± 3.5 cm) of northern pike were collected on March 12, 2012 from ponds near Nove Hrady, Czech Republic (GPS: 48.802016,14.781997) and transported to the Laboratory of Intensive Aquaculture, Faculty of Fisheries and Protection of Waters in Vodnany (IA, FFPW). Females were held in a 750 L plastic tank indoors with a semi-recirculating system of IA FFPW. Males were held in three similar tanks. Total water volume of the recirculation system was 30 m^3; flow rate was 8 L^{-1}; about 10% of the water was exchanged daily with tap water. Water temperature and dissolved oxygen saturation were measured daily and maintained at 8–10°C and 90 ± 3.0%, respectively. After 24 h acclimatization, all fish were intra-peritoneally injected with carp pituitary at dose 3.0 mg kg^{-1} BW (Billard and Marcel, 1980) after sedating with clove oil at 0.033 ml L^{-1} (Policar et al., 2011). Females were examined for ovulation by abdominal palpation every 12 h from 48 h post injection, and all were stripped by 96 h post-injection. Egg weight (EW) was calculated gravimetrically from the weight of three samples of 200 unfertilized fresh dry eggs from each female. A small sieve and fine filter paper was used to remove the remaining ovarian fluid to ensure accurate measurements. Egg diameter (ED) was measured stereoscopically using a digital camera (Olympus Camedia C5060WZ) and measuring 150 eggs from each female. Digital images (Olympus Micro Image 4.0.1. for Windows) were used for measurement of ED.

Immediately before fertilization, 30 g of eggs from each female were mixed in a 3 L plastic dish. In total, 150 g of eggs (approximately 24 550 eggs) were used for fertilization and incubation. Stripped sperm samples from 15 fish were used for fertilization. Sperm was collected separately in 5 ml syringes from each fish (Hulak et al., 2008) with an average of 1 ml uncontaminated sperm collected from each male. Syringes were immediately placed on ice till their use for the fertilization process. Combined sperm mixture from all males was checked for motility before use as described by Raat (1988). Following Lahnsteiner (2000), approximately 5 x 10^6 spermatozoa per egg were used for fertilisation. Fertilized eggs were treated with cow milk (3.5% fat) for 45 min to prevent stickiness (Jørgensen et al., 2010). Subsequently eggs were washed with hatchery water, and samples of 300 ± 10 eggs were placed into 25 separate transparent 2.5 L plastic incubators for incubation as described by Svinger et al. (2013).

Incubators were equipped with independent controllable water inflows. The five egg groups A, B, C, D, and E were incubated at 3, 6, 10, 14, and 18 °C, respectively. A separate temperature-controlled refrigeration (DELTON H120, Sinop CB a.s., Czech Republic, precision 0.1 °C) or heating (T-Computer Set, AB Aqua Medic, Germany, precision 0.1 °C) recirculation system was installed for each temperature. Dissolved oxygen concentration, pH, and temperature were measured twice daily (Table 1). Environmental variables (NH_3 < 0.015 mg L^{-1}, NO_2 < 0.01 mg L^{-1}) were the same in all systems and optimal for northern pike egg incubation (Billard, 1996).

Fertilization (FR, %) and hatching (HR, %) rates were calculated for all egg samples in gastrula stage and at the end of the hatching period using the formulae:

$$FR = (SE/TE) \times 100$$

where SE is the number of surviving embryos at gastrula stage, and TE is total number of stocked eggs at the beginning of incubation

$$HR = (HL/TE) \times 100$$

where HL is number of fresh hatch larvae.

Time of incubation period (TIP) (time from egg fertilization to hatching of larvae) is time within which two key events were distinguished: start of hatching (point of hatching for 5% of individuals [H5]) – and the end of hatching (point of hatching of 95% of individuals [H95]), Kamler (2002). Total hatching period (THP) was counted as interval between the start (H5) and the end (H95) of hatching period. TIP and THP were expressed in days and day-degree (°d).

Mortality of eggs was recorded daily at each water temperature treatment. White eggs were identified as dead and were separated from live eggs by using forceps. Yolk sac volume (YsV in μm^3) was measured when 95% of eggs hatched according to the formula for the prolate spheroid:

$$YsV = (\pi/6) \times YsL \times YsD^2$$

where YsL and YsD are yolk sac length and depth, respectively (measured as for ED) (Blaxter and Hempel, 1963).

The relationship between development rate (V_H) (time between key events during ontogenesis) and temperature (°C) during TIP was calculated as a linear model of development rate ($V_H = 1/\tau_H$ day^{-1}) vs. temperature (°C) during TIP in accordance with Kamler (2002). Two biological parameters were derived from this model: temperature of biological zero (t_0), i.e. temperature at which development is hypothetically arrested, and number of effective degree days ($°d_{eff}$), i.e. the number of degree-days above t_0.

Quality of newly hatched larvae was evaluated at each temperature based on weight (BW), total length (TL), frequency of normal morphologically developed larvae (FNL), and resistance of newly hatched larvae to osmotic stress (OS) after 95% hatching of eggs. Body weight and TL of dry larvae were measured as for EW and ED in 150 newly hatched larvae in each temperature treatment. Frequency of normal larvae was evaluated according to Pittman et al. (1990) by assessing percentage of morphologically normal larvae in 200 larvae from each treatment. The body malformations such as lordosis and colour abnormality were recorded. Resistance to osmotic stress was determined through exposure to a solution of 2% Na Cl in hatchery water. Thirty-three newly hatched larvae from each treatment were placed in 1 L of the saline solution. The assay was performed in triplicate (99 larvae from each treatment). The survival rate of larvae was determined after 90 min of exposure (Policar et al., 2010).

All data (ED, EW, FR, TIP, THP, HR, TL, BW, OS, FNL, YsV) are presented as mean ± S.D., and statistical assessment was performed by use of Statistica 10 (StatSoft, Tulsa, USA).

Measured parameters were tested for normality by the Kolmogorov-Smirnov test and for homoscedasticity by the Levene test. The values with homogeneous variances (ED, EW, TL, BW, YsV, FNL) were compared by ANOVA ($P < 0.05$). The non-parametric Kruskal–Wallis test

was used for those values where homogeneity of variance was interrupted (TIP, THP, OS). The tests were followed by Tukey post-hoc multiple comparison for all parameters. The *t*-test was used for comparison of FR and HR.

Results

No differences in size were found among eggs stripped from different females; for this reason eggs were combined. Eggs diameter varied from 2.54 to 2.86 mm (mean 2.7 ± 0.16 mm) and eggs weight ranged from 5.76 to 6.46 mg (mean 6.11 ± 0.35 mg). Diameter of eggs was positively correlated with eggs weight.

The highest value of FR was observed for group B (71.3 ± 4.3%), the lowest for group A (44.6 ± 3.2). No significant differences in FR were found among groups C, D, and E. Groups B and C did not differ significantly (Table 2). The TIP was inversely proportional to temperature and varied from 38 ± 0.33 days (120 ± 1.03 °d) (H_5) to 46 ± 0.42 days (144 ± 1.31 °d) (H_{95}) for group A and from 2.5 ± 0.08 days (44.67 ± 1.42 °d) (H_5) to 3.42 ± 0.06 days (61.11 ± 1.07 °d) (H_{95}) for group E. The lowest HR was found in group A (18.26 ± 2.25%) while values of HR in groups B, C, D, and E were 56.2 ± 3.21%, 65.5 ± 5.4%, 52.3 ± 4.47%, and 47.4 ± 2.55%, respectively. The THP was higher for group A (8 ± 0.3 days; 25.12 ± 0.92 °d) and decreased with increasing temperature to 0.92 ± 0.06 days (16.4 ± 1.07 °d) in group E.

The larval TL was lowest for group B (9.33 ± 0.28 mm) followed by group E (9.21 ± 0.29 mm). The highest larvae TL was observed in group C (10.61 ± 0.37 mm) (Table 3). Body weight of fresh larvae varied from 0.74 ± 0.09 mg in group E to 0.83 ± 0.05 mg in group B. Body weight of larvae did not differ significantly among larvae incubated in groups A, B, C, and D. Frequency of normal larvae was the lowest in group A (FNL=23.8%). High FNL was without significant differences between groups B and C (89.7%, 93.8%). Yolk sac volume was highest for group E (3.89 ± 0.45 μl^3) and this value did not differ significantly from that of group D (3.53 ± 0.4 μl^3). The lowest YsV value was observed for group C (3.04 ± 0.42 μl^3). The resistance of larvae to OS was significantly lower for group A (54 ± 3%). Higher OS values were found in groups C (80 ± 4%), D (76 ± 3%), and E (72 ± 3%), and the highest value was observed for group B (92 ± 3%) (Table 3).

Fertilization and hatching rates as well as quality of larvae indicate that the optimal temperature for successful incubation and production of high quality larvae ranges from 6 to 10 °C.

According to the linear model (Penaz, 2001; Schiemer et al., 2003), pike embryonic development is predicted to cease at approximately 3.3 °C (t_0 = 3.28 °C). Hatching was reached after approximately 70 effective degree-days ($°d_{eff}$ = 69.68) at temperatures above t_0 (Figure 1).

Discussion

The natural spawning period of northern pike extends from mid-March to mid-May in Europe (Kouřil and Hamáčková, 1975; Dubský, 1998). Duration of spawning can range from a few days to a month or more, depending on photoperiod and temperature (Ivanova and Svirskay, 2009). Water temperature of 4–7 °C is sufficient to initiate pike spawning (Lindroth, 1946; Franklin and Smith, 1963; Westers and Stickney, 1993).

Average values of egg weight (6.11 mg) and diameter (2.7 mm) observed in our study were higher than those reported by Murry et al. (2008) for weight and by Benzer et al. (2010) for

diameter. We observed 20–25% heavier and 7–12% larger eggs compared to eggs in above-mentioned studies. This might be due to the origins of the fish. In our study, we used wild broodstock from the south of the Czech Republic. The authors above cited used broodstock from Jefferson County, NY, USA (Murry et al., 2008) and Kapulukaya Dam Lake in Turkey (Benzer et al., 2010).

The highest fertilization rates were found in groups B (6 °C) and C (10 °C). Previous studies have indicated that optimal fertilization rate and larvae development occurs at water temperatures below 12 °C (Cooper, 2000; Farrell et al., 2006). Although there are reports claiming that embryogenesis may occur over a large range of temperatures, 3.7 – 24 °C (Lindroth, 1946; Lillelund, 1957; 1967) and 6.3 – 19.9 °C (Hokanson et al., 1973), most authors suggest an incubation temperature in the range of 7 – 15 °C (Braum, 1964; Huet, 1976; Hassler, 1982). Blaxter (1981) and Mihelakakis and Yoshimatsu (1998) point out that optimal temperatures for incubation and larval development should reflect that process occurring in the natural environment. Our finding of 6 – 10 °C optimal temperature for incubation of northern pike eggs corresponds to the natural water temperature during the spawning season. Incubation of northern pike eggs at temperatures outside this range can allow for shifting of the hatching period and for prolongation or reduction of the incubation period while maintaining an acceptable hatching rate and larvae quality in intensive culture.

The TIP decreased from 46 days at 3 °C to 3.4 days at 18 °C, consistent with the widely observed phenomena that low temperatures retard and high temperatures accelerate embryonic development (Lasker, 1964; Blaxter, 1981; Pepin, 1991; Drozd et al. 2009). The TIP at 10 °C was 13 ± 0.15 days (136 ± 1.56 °d) in our study. Bonisławska et al. (2011) obtained shorter incubation time under the same temperature with 100°d. Our present results lead to TIP of 6 ± 0.08 days (85 ± 1.13 °d) at 14 °C., in agreement with other authors at 14.5 °C (Swift, 1965; Lillelund, 1967 and Hokanson et al., 1973). Both the duration of the incubation period and the THP were inversely proportional to the incubation temperature (Billard, 1996). These observations are in agreement with findings in other fish species: Penaz et al. (1983) for common carp (*Cyprinus carpio*), Ojanguren and Braña (2003) for brown trout (*Salmo trutta* L.) and Drozd et al. (2009) for weatherfish – (*Misgurnus fossilis* L.).

Hassler (1982) reported an optimal hatching range of 6.2 °C to 20.9 °C while below 3.1 °C and above 24 °C are lethal temperatures. According to our data, ontogenesis hypothetically ceased in northern pike at approximately 3.3 °C, which may explain the significantly lower hatching rate and egg survival for group A (18%). This group also produced the highest number of abnormal larvae, up to 76%. Lillelund (1967) and Hokanson et al. (1973) reported that all abnormal larvae incubated below 3 °C died soon after hatching. However, both biological zero and the number of effective degree days obtained in the present study are in accordance with the values for northern pike obtained by meta-analysis of primary data from literature, as shown by Kamler (2005).

Size of newly hatched larvae and yolk sac size are related to the temperature (Blaxter, 1992; Trabelsi et al., 2013). Thus, it is suggested that yolk sac utilisation is optimal at the temperature that produces the largest larvae. Larval size is generally considered an important quality indicator, as larger larvae are supposed to be stronger, better swimmers, and less susceptible to damage (Blaxter, 1969). In the present study, largest larvae size and smallest yolk sac volume were found for group C (10.61 ± 0.37 mm; 3.04 ± 0.42 μm^3 respectively). Larvae from group B showed high resistance to OS and low numbers of abnormal larvae (10.3%). However, higher tolerance to OS was observed for larvae from group B (92 ± 3%) after 90 min.

The present study showed that the optimal temperature range for northern pike embryo development, based on egg incubation and hatching time, fertilization and hatching rates, quality of larvae including larva size, development, and resistance to osmotic stress, under controlled conditions is about 6–10 °C. This is lower than the 12 °C found previously, and is in accordance with temperatures experienced during the natural spawning season.

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Table 1. *Main water variables measured during egg incubation in northern pike (Esox lucius L.). O_2 – concentration of dissolved oxygen (expressed in mg O_2 L^{-1}); T_{water} – water temperature (°C).*

Group	A			B			C			D			E		
Parameter	O_2	T_{water}	pH	O_2	T_{water}	pH	O_2	T_{water}	pH	O_2	T_{water}	pH	O_2	T_{water}	pH
Minimum	13.7	2.3	7.32	10.5	4.6	7.41	7.65	9.5	7.39	6.85	13.8	7.43	6.6	17.6	7.48
Maximum	15.9	3.9	7.62	12.6	7.4	7.57	11.05	11.8	7.61	9.45	14.4	7.63	8.7	18.2	7.66
Mean	14.3	3.14	7.41	11.3	6.2	7.49	9.51	10.45	7.5	8.4	14.17	7.52	7.6	17.87	7.55
S.D.	0.63	0.73	0.17	0.58	0.51	0.14	1.12	0.43	0.1	0.59	0.17	0.12	10.2	0.13	0.11

The presented data were measured during whole experiment twice a day. The concentration of dissolved oxygen and pH were measured in effluent of the incubators**.** The water temperature was measured inside the incubators.

Table 2. *Fertilization rate (FR, %), hatching rate (HR, %), time of incubation period (interval from start H_5 to finish H_{95} of hatching; day, °d) and duration of total hatching period (day, °d) dependence on water temperature in northern pike (Esox lucius L.).*

Groups	FR, %	HR, %	Time of incubation period				Total hatching period	
			Start (H_5)		End (H_{95})			
			day	°d	day	°d	day	°d
A	$44.6^{a} \pm 3.2$	$18.26^{a} \pm 2.25$	38 ± 0.33	120 ± 1.03	46 ± 0.42	144 ± 1.31	8 ± 0.3	25.12 ± 0.92
B	$71.3^{c} \pm 4.3$	$56.2^{bc} \pm 3.21$	19 ± 0.25	117 ± 1.55	23 ± 0.2	142.6 ± 1.24	4 ± 0.23	24.8 ± 1.42
C	$65.6^{bc} \pm 3.1$	$65.5^{c} \pm 5.41$	11 ± 0.17	115 ± 1.77	13 ± 0.15	136 ± 1.56	2 ± 0.15	21 ± 0.15
D	$61.2^{b} \pm 3.3$	$52.3^{b} \pm 4.47$	4 ± 0.15	56.7 ± 2.12	6 ± 0.08	85 ± 1.13	2 ± 0.13	28.34 ± 1.8
E	$53.5^{b} \pm 2.7$	$47.4^{b} \pm 2.55$	2.5 ± 0.08	44.7 ± 1.42	3.42 ± 0.06	61.11 ± 1.07	0.92 ± 0.06	16.4 ± 1.07

All data presented as mean ± S.D. The different letters within columns indicate significant differences between the groups, $P<0.05$. In each group were 300 ± 10 eggs, all values corresponding to five replicates.

Table 3. *Influence of the water temperature on quality of northern pike (Esox lucius L.) larvae. Total lengths (TL, mm), body weight (BW, mg) and yolk sac volume (YsV, µl³) were measured in sample of 150 newly hatched larvae. Survival assays after exposure to osmotic stress (in %) were performed in 99 from each group. Frequency of normal morphologically developed larvae was evaluated on 200 larvae samples from each treatment.*

Group	TL (mm)	BW (mg)	YsV (µl³)	Survival after osmotic stress (%)	Normally developed larvae (%)	Malformed larvae (%)
A	$9.67^{b} \pm 0.36$	$0.78^{ab} \pm 0.06$	$3.41^{b} \pm 0.44$	$54^{a} \pm 3$	$23.8^{a} \pm 4.14$	$76.2^{c} \pm 3.18$
B	$9.33^{a} \pm 0.28$	$0.83^{b} \pm 0.05$	$3.3^{ab} \pm 0.66$	$92^{c} \pm 3$	$89.7^{c} \pm 3.62$	$10.3^{a} \pm 1.93$
C	$10.61^{c} \pm 0.37$	$0.8^{b} \pm 0.06$	$3.04^{a} \pm 0.42$	$80^{b} \pm 4$	$93.8^{c} \pm 3.17$	$6.2^{a} \pm 2.36$
D	$9.83^{b} \pm 0.86$	$0.79^{b} \pm 0.09$	$3.53^{bc} \pm 0.4$	$76^{b} \pm 3$	$81.8^{b} \pm 2.12$	$18.2^{b} \pm 3.47$
E	$9.21^{a} \pm 0.29$	$0.74^{a} \pm 0.09$	$3.89^{c} \pm 0.45$	$72^{b} \pm 3$	$87.1^{b} \pm 2.42$	$12.9^{b} \pm 1.84$

All data are presented as mean ± S.D. The different letters within columns indicate significant differences between the groups, $P<0.05$.

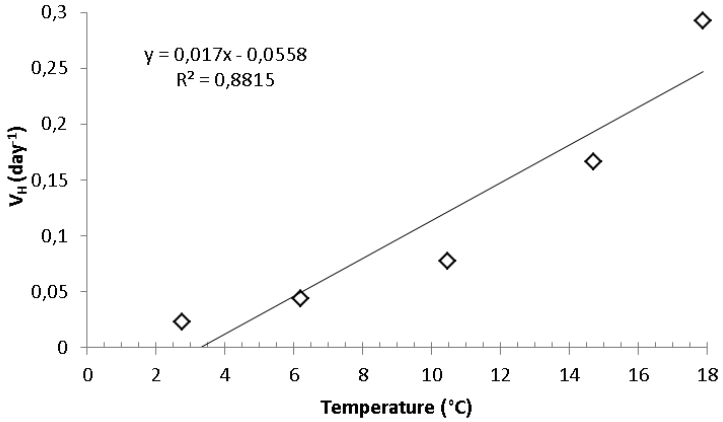

Figure 1. *Linear regression model ($y = a \cdot x+b$) of relationship of ontogenetic rate ($V_H = 1/\tau_H$, day^{-1}) and temperature (°C) during the incubation period (H_5–H_{95}) in northern pike (Esox lucius).*

Those relationship shows two biological parameters: temperature of biological zero (t_0), temperature when ontogeny is theoretically stopped, and number of effective day-degrees ($D°_{eff}$), i.e. number of day-degrees above t_0. Points on graph represent the mean value of the temperature.

CHAPTER 5

REPRODUCTION AND REARING OF ADVANCED NORTHERN PIKE (*ESOX LUCIUS* L.) FRY

Paper IV:

Bondarenko, V., Křišťan, J., Švinger, V., Policar, T., 2013. Reproduction and rearing of advanced northern pike (*Esox lucius* L.) fry. Practical handbook FFWP USB, no. 144, 43 pp. (*in Czech*)

It was allowed by the publisher on 28th April, 2013 to include this handbook in this Ph.D. thesis.

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v Českých Budějovicích
University of South Bohemia
in České Budějovice

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Table of Contents

I. The objective of the methodology

The objective of this certified methodology is to describe and explain, new procedures of controlled reproduction of broodstock pike (*Esox lucius L.*) optimizing synchronization of broodstock spawning, the procedure of artificial fertilization and incubation of eggs and obtaining good-quality and highly viable larvae of this species. Another objective of this specialized methodology is to describe modern and effective production methods of juvenile pike to the advanced fry of a total length (TL) of 30 – 50 mm which are subsequently used for rearing the older age categories of fish until they reach the category of commercial fish with a body weight ranging from 1 to 3 kg. It is supposed that this scientific publication could help Czech production fisheries to increase the efficiency and production of pike in the Czech Republic which may diversity the Czech fishery production.

II. Description of the methodology

2.1. Commercial importance of pike in Europe

Pike (Fig. 1) is a commercially attractive piscivorous fish species occurring in many different freshwater biotopes of the Northern hemisphere (Crossman, 1996). This species is also very popular among sport fishermen (Mann, 1996). The popularity is, first of all, caused by a wide-spread occurrence of pike due to its high adaptability to various natural biotopes (Crossman, 1996). Secondly, it is the fish predator that offers a highly interesting sport fishing experience (Lusk and Krčál, 1982; Mann, 1996). Thirdly, pike is popular owing to its high quality muscle mass that has a little fishy taste, is highly dietary and easy to digest (Lusk and Krčál, 1982). On the other hand, it must be mentioned, that in Europe pike represents a supplementary fish species which is, in no case, caught by sport fishermen on a mass scale. As a matter of fact, pike is a an apex predator in open waters which occurs in given localities in relatively low densities (3–4 $kg.ha^{-1}$) and its density is often even lower than 1 $kg.ha^{-1}$. However, this assertion is not generally valid in Finland, Russia, and Ukraine where the mentioned fish species is abundantly caught by sport fishermen (Mann, 1996). Pike abundance in the above-mentioned countries is given by a large number and the total area of water surface of different lakes or reservoirs (Crossman, 1996).

In addition to sport fishing, pike is frequently used in extensive polyculture fish farming in ponds. Under such conditions, pike adopts the role of a predator whose task is to suppress occurrence of small and less commercially important cyprinid fish species, such as roach (*Rutilus rutilus*), common bream (*Abramis brama*), silver bream (*Abramis bjoerkna),* rudd (*Scardinius erythrophthalmus*), topmouth gudgeon (*Pseudorasbora parva*) and gibel carp (*Carassius gibelio*). Suppression of occurrence of these less valuable fish species in ponds reduces their competition against main, commercially most significant, fish species – common carp (*Cyprinus carpio*) (Adámek et al., 2010). This use of pike in pond farming maintains a high and an effective production of carp in ponds and its second contribution lies in an assessment of biomass of less commercially important fish species in a form of a biomass growth of economically highly valuable species (Lusk and Krčál, 1982; Dubský, 1998).

A strong predation pressure exerted by pike is used in controlled stocks during so-called biomanipulations in water supply reservoirs. The principle of controlled fish stocks is to support populations of piscivorous fish species that are able to control biomass of small planktonophagous fish species. A decreased occurrence and biomass of these small planktonophagous species reduces their grazing pressure on filtering zooplankton. This fact

enables greater development of zooplankton which effectively reduces development of phytoplankton, or more precisely, massive occurrence of so-called water bloom. This measure can ensure a relatively good water quality in water supply reservoirs. The assertion, however, is valid only if a low final fish biomass (lower than 100 $kg.ha^{-1}$) is kept in reservoirs and the aquatic environment is burdened with a low content of phosphorus at a mesotrophic level (Adámek et al., 2010).

Fig. 1. *Broodstock pike (Esox lucius L.).*

2.2. Current methods of a commercial fish production and its amount in Europe and the Czech Republic

At present, commercial pike is produced in Europe mainly in two ways. The first method involves capturing wild fish in large lakes and rivers. In this way, an amount of 17 700–24 500 tonnes of pike was produced per year over the past decade in Europe. The biggest European producers of fish obtained in this way are, Russia (8 000–16 000 t) and Finland (6 500–8 300 t). Other significant producers of pike by means of capturing are the following European countries (data in brackets represent an average annual production of pike in the course of the past decade): Poland (250–325 t), Hungary (170–280 t), Germany (170–210 t), the Czech Republic (140–180 t), Estonia (95–200 t) and Serbia (70–220 t) (FAO, 2013a). In the Czech Republic, pike are primarily caught from open waters by sport fishermen. Annual catches of pike carried out in this way amounted to a level of 120–170 tonnes between 2008–2010 (Ženíšková and Gall, 2011).

In addition to capturing pike from open waters, this fish species has traditionally been produced and reared in the above-mentioned extensive pond farming. Owing to this type of fish farming, an amount of 220–850 tonnes of pike is annually produced for the fish market in Europe which represents only about 3–10% of the production obtained by catching. The most significant producers of pike by means of pond fish farming in the past decade were the following countries: Russia (4–280 t), Poland (0–170 t), the Czech Republic (60–110 t), Belarus (40–120 t) and Hungary (30–80 t) (FAO, 2013b).

The biggest Czech producer of commercial pike is the Třeboň Fishery plc. with the total production of 22 tonnes in 2011. Other significant Czech producers of pike are the following fisheries and the data in brackets provide the annual production in 2011: Kardašova Řečice Fishery Ltd. (8.1 t), Hluboká Fishery Ltd. (4.8 t), Mariánské Lázně Fishery Ltd. (4.6 t) and Chlumec nad Cidlinou Fishery plc. (4.2 t). Advanced pike fry (TL = 30–50 mm) is stocked into production ponds at very low densities around 100–400 pcs.ha^{-1} at the beginning of a 2–4 year production cycle (Hamáčková, 1987). An average final biomass of pike reared in polycultural pond stocks of commercial fish ranges from 0.7 to 16.0 kg.ha^{-1} under the Czech fishery conditions (Kratochvíl, 2012). These very low production densities are influenced by a high predation pressure of pike exerted against fish population in given ponds including a cannibalism among pike (Lusk and Krčál, 1982). On this account, ponds have a very limited production capacity for effective pike farming.

2.3. Factors considerably limiting the current production

The production of commercial pike within the scope of the Czech fishery has been considerably limited both by the biology of the species itself and by insufficient or suboptimal farming conditions (Lusk and Krčál, 1982; Policar, 2012a,b). Biological qualities of pike limiting its rearing comprise: a long spawning period of hormonally untreated broodstock (Policar, 2012a), production of a very low volume of sperm (Linhart, 1984; Billard, 1996; Hulák et al., 2008a), contamination of extracted sperm with urine causing its reduced usability for artificial fertilization of eggs (Berka and Hamáčková, 1980; Billard, 1996; Hulák et al., 2008a), application of testicular sperm causing a loss of broodstock males for further use (Billard, 1996; Lahnsteiner et al., 1998), variable egg quality (Lusk and Krčál, 1982; Policar, 2012a; Švinger et al., 2012), susceptibility of fertilized eggs and embryos to manipulation or unsuccessful incubation (Berka and Hamáčková, 1980), high level of cannibalism (Lusk and Krčál, 1982; Kucska et al., 2005; Szczepkowski, 2009) and high territoriality of fish occurring from juvenile stages (Berka and Hamáčková, 1980; Lusk and Krčál, 1982).

Rearing conditions or interventions limiting pike production are: used suboptimal water temperature for rearing (Szczepkowski, 2009; Policar, 2012b), insufficient amount of suitable food (Berka and Hamáčková, 1980; Hamáčková, 1987) and a low density of reared fish (Lusk and Krčál, 1982; Hamáčková, 1987).

2.4. General reproductive characteristics

In the climatic conditions of Czech Republic, pike reproduce the most often from the end of February until the end of March, or until the beginning of April (Kouřil and Hamáčková, 1975; Dubský, 1998; Bondarenko et al., 2012b). Reproduction takes place at water temperature of 7–10 °C and spawning period is finished when water temperature reaches 14 °C (Lusk and Krčál, 1982; Westers and Stickney, 1993).

Pike belongs to phytophilous species which lay its fertilized eggs onto the submerged macro vegetation, especially fine-leaved aquatic macrophytes. Natural spawning environment of pike is represented by shallow warm sections of ponds and rivers that can even be periodically flooded. Eggs are sticky, therefore, they are firmly stuck to a plant substrate. Natural reproduction of pike is endangered by a low water level or its frequent fluctuation (especially in valley reservoirs) and an insufficient amount of suitable spawning substrate (Lusk and Krčál, 1982).

Sexual maturation of broodstock is dependent on temperature conditions and a food offer available in localities (Billard, 1996). In Spain, where pike was stocked and subsequently

acclimatized, males as well as females mature already at the end of the first year of life (Crossman, 1996). It is also possible to encounter one-year-old sexually mature fish even in the Central European conditions (Kouřil and Hamáčková, 1975; Lusk and Krčál, 1982), in the Czech climatic conditions, pike maturate at the end of the second or third, and sometimes even fourth, year of life (Billard, 1996). Males mature earlier than females when they reach a total length of 180 mm as early as in the first year of life. Females can be sexually mature in the second year of their life when they achieve a TL of 260 mm (Billard, 1996; Hubenova and Zaikov, 2007).

Sexual organs (ovaries and testicles) of both genders are paired, placed in abdominal part along the external side of kidneys (Kouřil et al., 1976). Ovaries are typically pear-shaped and before the spawning period they turn orange which characterizes final stages of oocytes. Testicles are elongated, white or cream-coloured (Lenhard and Cakic, 2002). Male's testicles intensively grow and develop already at the end of summer. Female's oocytes in ovaries intensively develop during the winter period until the beginning of spring (from November till March, or the beginning of April) when the final oocyte stages occupy up to 95% of an ovarian volume. The GSI (the gonadosomatic index expresses a percentage of a gonad weight compare to body weight) in females ranges in summer around 1–2%, in autumn, it is 5% and in winter, it reach up to 10–12%. In spring, before spawning, the female's GSI amounted to 18–20%. In this period, ovaries fill in a considerable part of the abdominal cavity (Billard, 1983; 1996).

Sexual dimorphism, as in other fish species inhabiting the temperate zone, is not too noticeable during the spawning period (Lusk and Krčál, 1982; Dubský, 1998). Females are distinguished only by enlarged abdominal areas. Gender can be recognized according to a shape of genito-urinary papilla (Casselman, 1974; Billard, 1983) which has also been confirmed by our present experience in gender distinguishing in the spawning period. Male's genito-urinary papilla is of a line shape, it is narrow and indistinctive, while female's papilla is fan-shaped, well-perfused with blood and reddish (Fig. 2).

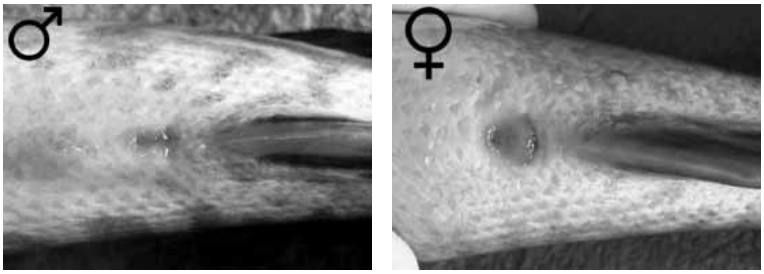

Fig. 2. *Distinguishing gender of pike (Esox lucius L.) by means of a shape and a state of a genito-urinary papilla, male (left) and female (right) (photo: V. Bondarenko).*

2.5. Marking and evidence of broodstock

In order to individually mark particular broodstock, either passive integrated transponders (PIT), sometimes called only "chips" or "microchips" (Rodina and Flajšhans, 2008), or metal fin clips are used (Fig. 3). Individual marking of fish is very important for fishery purposes in order to properly record spawning activities, fertility and survival of fish after the spawning period and, subsequently, also for evidenceof repeated spawnings in the following years. Microchips with a numerical code are the most often implanted in dorsal muscles on the left-hand side of a fish, approximately at a level of the first hard dorsal fin ray in a cranial direction 1 to 1.5 cm

deep under an angle of 30° by means of a sterile single-use or a reusable implanting device. The second mentioned method of fish marking is often employed in French fish hatcheries where certain broodstock are marked with a fin clips with a number of fish attached to dorsal or ventral fin.

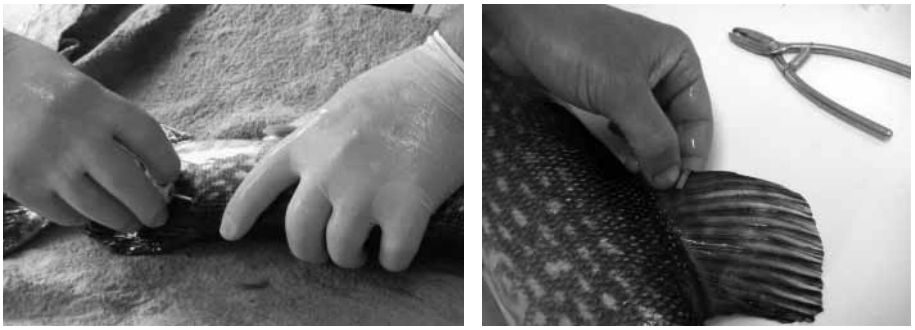

Fig. 3. *Marking of broodstock pike (Esox lucius L.) with a PIT microchip (left) and a fin clips with a number (right) (photo: J. Křišťan).*

It is important to calm fish down before the marking itself with the use of anaesthetics (Fig. 4). Clove oil at a dose of 0.04 $ml.l^{-1}$ is frequently used for pike as well as other fish species (Švinger et al., 2012). After marking, it is advisable to place a given fish into a preventive anti-fungal bath containing a potassium permanganate solution in a concentration of 0.1 $g.l^{-1}$ with an exposure time of 10–15 minutes (Fig. 5). This bath serves as prevention against secondary fungal infection before subsequent stocking of fish into troughs in a hatchery just before the fish spawning period itself (Policar et al., 2011a).

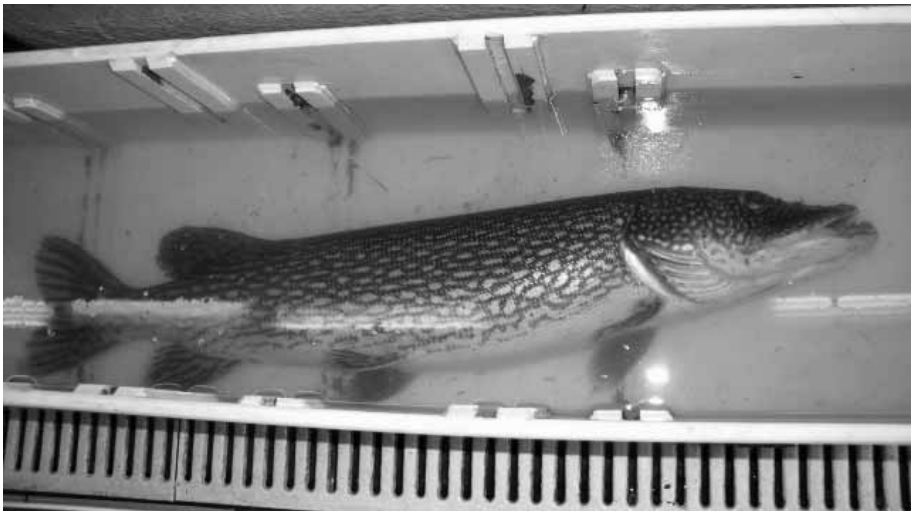

Fig. 4. *Broodstock female pike (Esox lucius L.) in anaesthesia (photo: T. Policar).*

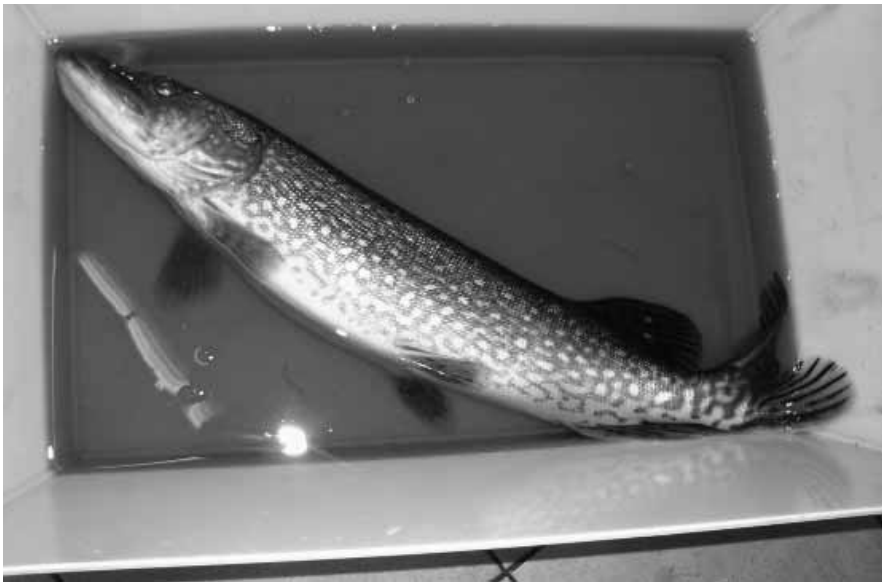

Fig. 5. *Broodstock female pike (Esox lucius L.) in a potassium permanganate bath that serves as prevention against surface fungal infections (photo: T. Policar).*

2.6. Controlled reproduction of pike by thermal and hormonal stimulation

Broodstock designated for stripping are kept in wintering ponds from autumn to spring with a sufficient amount of prey fish (smaller cyprinid fish species). It is recommended to stock one kilogram of prey fish per one kilogram of stocked pike. It is important to remove broodstock from ponds in spring period when the temperature gradually rises to 4–6 °C and a natural spawning period is approaching (Dubský, 1998). After that, broodstock must be transported either into smaller and shallow ditch ponds (surface area around 100 m^2) close to a hatchery or stocked directly into rearing troughs in a hatchery where it is possible to regulate a water temperature and thus influence the time of stripping (Policar, 2012a).

The use of the above-mentioned methods of broodstock stocking is dependent on a stripping method. If fish are stripped without a hormonal treatment, fish of both sex are stocked into ponds that are largely overgrown with macrovegetation and contain a sufficient amount of prey fish (0.5 kg of prey fish per 1 kg of pike). It is important to measure a water temperature twice a day (in the morning and in the afternoon) and to monitor behaviour of broodstock. In spring period, water gradually warms up from 4 °C (in the morning) up to 8–10 °C (afternoon) and fish behaviour changes. When temperature rises, fish stay at pond brims and they intensively swim into littoral macrovegetation. This behaviour suggests that some broodstock females are ready for spawning. After that, it is necessary to remove fish from a trench pond, select ovulating females and catch approximately the same quantity of males. Subsequently, fish ready to spawning are transferred to a fish hatchery where eggs are stripped from female by abdominal massage. After that, eggs are artificially fertilized, desticked and incubated (Policar, 2012a). Individual rearing operations are described in detail in the following chapters.

In case that broodstock are stocked under controlled conditions into fish hatchery reservoirs, hormonal preparations (Fig. 6) are applied to broodstock after initial thermal stimulation (water temperature 9–11 °C) and these preparations ensure final oocyte maturation and their

subsequent ovulation (Bondarenko et al., 2012b). This intervention must be implemented in order to prevent reproductive dysfunction in pike. (Hamáčková et al., 1975; Kouřil and Hamáčková, 1975, 1977; Mylonas and Zohar, 2001).

Fig. 6. *Hormonal injection of broodstock female pike (Esox lucius L.) (photo: T. Policar).*

2.7. Hormonal stimulation of final oocyte maturation and ovulation of eggs

As far as pike is concerned, hormonal stimulation of ovulation under controlled conditions in fish hatcheries was solved in the past mainly by hypophysation by means of dried carp pituitaries at a dose of 3–4 mg.kg^{-1} of live weight for females and 2–4 mg.kg^{-1} of live weight for males (Billard, 1996; Policar, 2012a; Švinger et al., 2012; Bondarenko et al., 2013b). This method has remained the only reliable method used in fishery practice for mass induction of ovulation for this species (Szabó, 2001, 2003, 2008). For some fish species where it was possible to replace hypophysation with application of synthetic GnRHa (Gonadotropin – Releasing Hormone analogue) or a combination of GnRHa with dopaminergic inhibitors (metoclopramide, pimozide and domperidone). In case of pike, this hormonal induction of oocyte ovulation failed either completely or its efficiency was incomparable with a success rate of hypophysation (Szabó, 2003). Billard (1996) also described a successful application of partially purified salmon gonadotropin (PPSG) in order to induce oocyte ovulation with the application of 100 μg PPSG.kg^{-1} of live weight of females. However, the above-mentioned author revealed that the efficiency of this hormonal treatment was considerably decreased in rearing of broodstock in captivity. Fish were caught and immediately hormonally treated with PPSG, 100% ovulation was achieved. In fish kept under controlled conditions for 3 days that were hormonally stimulated with PPSG after that, the ovulation rate decreased to 40%.

Problems connected with an effective induction of oocyte ovulation in pike kept under controlled conditions have recently been connected with the use of low doses of GnRHa (doses under a level of 50 μg.kg^{-1}) and a temporary non-use of a possibility offered by the

GnRHa to apply it with a prolonged effect by means of GnRHa emulsification in adjuvants (e.g., FIA – Freund's incomplete adjuvant). Therefore, in 2012, en experiment testing effectiveness of the following hormonal preparations was designed:

- sGnRHa (synthetic analogue of salmon GnRH) D- Arg^6Pro^9NEt (40–50 μg sGnRHa.kg^{-1} of live weight) in combination with the dopaminergic inhibitor metoclopramide (8–10 mg.kg^{-1}) with a subsequent emulsification in FIA,
- sGnRHa D-Arg^6Pro^9NEt (40–50 μg sGnRHa.kg^{-1} of live weight) in combination with the dopaminergic inhibitor metoclopramide (8–10 mg.kg^{-1}),
- sGnRHa D-Arg^6Pro^9NEt (40–50 μg sGnRHa.kg^{-1} of live weight) with a subsequent emulsification in FIA,
- carp pituitary dissolved in 0.9% physiological saline solution at a dose of 4 mg.kg^{-1} of live weight,
- carp pituitary dissolved in 0.9% physiological saline solution and subsequently homogenized in FIA in the rate of 1:1 at a dose of 4 mg.kg^{-1} of live weight (Policar, 2012a).

An effective hormonal intervention that induced final maturation of oocytes and ovulation of eggs in 100% of fish was carp pituitary dissolved in a physiological saline solution. Homogenized carp pituitary with FIA induced ovulation of eggs in 86.5% of fish (Bondarenko et al., 2013b). The results suggests that homogenized carp pituitary with FIA was not effective in a form of a percentage of ovulated fish in comparison to application of the carp pituitary itself. On that account, this more expensive method of hormonal stimulation of pike ovulation cannot replace in practice the used carp pituitary dissolved only in a physiological saline solution. Other hormonal treatments were ineffective (ovulation of eggs in 0% of fish) with the exception of the use of sGnRHa D-Arg^6Pro^9NEt (40–50 μg.kg^{-1} of live weight) in combination with the dopamine inhibitor metoclopramide (8–10 mg.kg^{-1}). This hormonal treatment induced ovulation in 14% of females. Nevertheless, the effectiveness of this hormonal treatment was very low and probably connected with a spontaneous ovulation. Therefore, this method of hormonal treatment cannot be recommended to be used in fishery practice either (Policar, 2012a). In addition to this experiment, the effectiveness of high doses of mGnRHa (mammalian GnRHa; doses up to 150 μg mGnRHa of Lecirelin – the Supergestran preparation.kg^{-1} of live weight) was also tested but this method did not induce egg ovulation either (Švinger, personal information, 2013).

The above-mentioned results have confirmed that the carp pituitary has remained the only effective hormonal treatment inducing oocyte ovulation. Hypophysation has been apply in hormonally controlled reproduction of fish since the 1930s when it was applied to rainbow trout (*Oncorhynchus mykiss*) for the first time (Hasler et al., 1939). A classic technique of application of dried carp pituitary is their dissolution in 0.7–0.9% physiological saline solution (NaCl) and an intramuscular or intraperitoneal single application at a dose of 3–4 mg.kg^{-1} of live weight of females or 2 mg.kg^{-1} of live weight of males (Pecha et al., 1992; Szabó, 2001, 2008).

Weighing of the required amount of pituitaries must be carried out on a corresponding laboratory weight with accuracy of at least ± 0.001 g. The pituitary is diluted with the above-mentioned physiological saline solution in such a way that a hormonal preparation is given to fish in a volume of 1 ml.kg^{-1}, i.e., with respect to females, 3–4 mg of pituitary is dissolved in 1 ml of a physiological saline solution and, as far as males are concerned, 2 mg of pituitary are dissolved in 1 ml of a physiological saline solution. In order to achieve a thorough homogenization of pituitaries and their mixing with a physiological saline solution, it is advisable to use a laboratory ceramic grinding mortar with a pestle.

2.8. Hormonal treatment of males

Smaller-sized males (0.5–1 kg) are used for artificial stripping of pike (Dubský, 1998). If pike males are hormonally stimulated, dried carp pituitary at a dose of 2–4 $mg.kg^{-1}$ of live weight is used (Policar 2012a; Švinger et al., 2012). Males are injected intramuscularly after anaesthesia (see chapter 2.5) in a period when females are hormonally treated (usually 4 days before the fish stripping itself). The decision whether to apply a hormonal treatment of males depends mainly on a method of sperm collection and on the decision of a responsible employee at a given hatchery. If it is planned to use extracted released sperm, it is advisable that males are hormonally stimulated (Policar 2012a; Švinger et al., 2012). If males are not hormonally treated, it is probable that only a very little amount of sperm around 0.5–2 ml per one male will be obtained (Billard, 1996; Dubský, 1998; Hulák et al., 2008a,b). Such small amount of sperm can cause great difficulties at artificial fertilization of obtained eggs. If so-called testicular sperm is used (sperm obtained from dead males when testicles are removed from male bodies and dissected) it is not necessary to use hormonal stimulation (Hulák et al., 2008a,b). Nevertheless, hormonal stimulation of males is recommended even for this method of sperm extraction. Application of a hormonal treatment increases production of sperm in testis. This fact facilitates the work and positively influences artificial fertilization of eggs. A sufficient amount of sperm for egg fertilization is consequently manifested in a higher rate of egg fertilization (Policar, 2012a).

2.9. Length of latency period, synchronization and a success rate of female stripping

Latency period (latency interval) is a period between hormonal stimulation and stripping of ovulated eggs (Policar et al., 2011a). Latency period of females that are treated with a dried carp pituitary at the above-mentioned dose ranges from 96.0 ± 14.4 and 98.2 ± 2.5 hours from the performed hormonal injection (Policar 2012a; Švinger et al., 2012). In some cases it has been revealed that all females treated with carp pituitary stripped at the same moment, i.e., 96 hours after the hormonal stimulation (Bondarenko et al., 2013b). When a different experiment was carried out, identically treated females were stripped at the same latency period (96 hours), however, the stripping period from the first until the last stripped female took 12 hours (Policar, 2012a). Length of latency period in day degrees ranges around 42.0 ± 6.3 °d which means that fish were kept at a temperature of 10.5 °C for 4 days between the injection and stripping (Policar, 2012a). The success rate of stripped females treated with carp pituitary amounted to 95–100% (Policar, 2012a; Švinger et al., 2012). With regard to pike females which were treated with carp pituitary mixed together with Freund's incomplete adjuvant, latency length of 107.9 ± 10.3 hours was revealed when during 12 hours all females were stripped (Bondarenko et al., 2013b). With regard to females treated with the sGnRHa in combination with metoclopramide, only 14% of treated females stripped at a latency period of 97.5 hours (Policar, 2012a).

Females stimulated only with a rising water temperature have a stripping period much longer than hormonally treated fish. In the course of several stripping periods it was discovered that broodstock stocked into the trench pond close to the fish hatchery in Nové Hrady Fisheries Ltd stripped gradually within approximately one month. For example, in 2012, these stripped fish ovulated during 30 days from 22nd March until 20th April. The success rate of the stripping, that is characterized as an amount of stripped individuals, amounted in these fish to 95% (Fig. 7 and 8). In total, 66% of fish was stripped during the first 8 days of a given stripping period (Policar, 2012a).

Fig. 7. *Ovulating broodstock female pike (Esox lucius L.) prepared for strip (photo: T. Policar).*

Fig. 8. *Stripping of broodstock female pike (Esox lucius L.) (photo: T. Policar).*

2.10. Fecundity of females

An absolute fecundity of females largely fluctuates in dependence on their size, age and a locality where pike occurs (Nikolsky, 1963; Kouřil and Hamáčková, 1975; Billard, 1996; Hubenova and Zaikov, 2007). With an increasing body weight and length, an absolute fecundity of females also increases (Billard, 1996; Hochman, 1964). Hochman (1964) presented a dependence of a number of eggs on a female size in South Moravian ponds (Tab. 1). Dependence of an absolute fecundity on age, size and weight of females was published by Billard (1996) and these values are provided in Tab. 2. Similar results were also presented by Křišťan et al. (2013) and he stated that an absolute fecundity had ranged from 65 000 to 141 000 fish eggs.

Tab. 1. *Number of eggs in individual size categories of female pike (Esox lucius L.) (Hochman, 1964).*

Body length (mm)	300-350	350-400	400-450	450-500	500-550	550-600	600-650	650-700	700-750	750-800	800-850
Absolute fecundity (thousands. pcs of eggs)	10.2	16.5	25.2	36.9	52.5	71.0	94.6	127.2	159.5	190.8	248.0

Tab. 2. *Number of eggs in individual age, size and weight categories of female pike (Esox lucius L.) (Billard, 1996).*

Age (years)	Total length (mm)	Weight (g)	Absolute fecundity (pcs x 1000)	Number of fish
2	325–560	330–2 100	6.0–42.0	23
3	410–720	700–2 900	13.0–80.0	203
4	445–830	1 040–5 340	9.0–127.0	246
5	475–850	1 151–6 500	16.0–167.0	301
6	550–900	1 700–7 200	41.0–250.0	194
7	530–910	1 700–7 600	58.0–165.0	52
8	540–890	2 100–6 200	64.0–203.0	24
9	680–1000	3 000–10 560	71.8–232.6	25
10	750–1020	4 200–10 000	99.8–233.0	13
11	870–960	6 500–9 400	147.2–188.3	4
12	920–940	7 170–7 300	178.2–178.8	2
13	900	7 700	168.8	1
14	950	7 800	126.1	1

Křišťan et al. (2013) indicated a relative fecundity (a number of eggs per a unit of weight) ranging from 20 857 to 31 887 eggs per 1 kg of live weight of fish with a mean value amounting to 26 372 pcs.kg^{-1}. Similar results were also published by Hochman (1964) who determined a relative fecundity within the range of 19 712–49 901 pcs.kg^{-1} with a mean value of 28 652 pcs.kg^{-1}.

2.11. Impact of selected factors on egg fertilization and survival of embryos during their incubation

In general, there is a great problem with regard to pike eggs and embryos that is connected with a high mortality rate both of fertilized eggs and incubated embryos. The mentioned mortality rate is manifested by a lower percentage of egg fertilization and by a low percentage of hatched larvae (Horvát, 1983; Billard, 1996; Policar, 2012a). Fertilization of eggs and hatching of larvae are influenced by several factors. The most important ones involve: stripping period of broodstock, hormonal treatment of females, ovarian plasma pH level, age of broodstock, manipulation with gametes, physiological quality of oocytes and sperm, well-conducted and optimized process of artificial fertilization and incubation of eggs (Billard, 1996; Policar, 2012a; Švinger et al., 2012). Impact of the first five mentioned factors is explained in this chapter. Quality of gametes and a process of artificial fertilization of eggs are explained in the following chapters.

With respect to broodstock that were not hormonally treated, the highest hatching rate of larvae ranging from 52 to 69% was discovered during a month-long stripping period at the beginning of the period. The lower hatching rate of larvae (12–42%) was found in the last decade of the stripping period (Policar, 2012a).

If females are hormonally treated with a dried carp pituitary, fertilization of eggs ranged from 40 to 63% and hatching of larvae was lower approximately by 7–10% than egg fertilization (Horváth, 1983; Policar, 2012a). Billard and Marcel (1980) stimulated a final maturation of oocytes and ovulation of eggs with single doses of dried salmon and carp pituitaries and the values of egg fertilization achieved very different values ranging from 8 to 63%. Szabó (2001, 2008) managed to improve egg fertilization values by means of preparations ensuring a gradual release of gonadotropin during a hormonal treatment of fish. If carp pituitary in 8% solution of sodium salt of carboxymethyl cellulose (CMC-Na) was applied, fertilization of eggs amounted to 66% as opposed to 41% achieved when a classic method of hypophysation in a physiological saline solution was employed. Similar improvement in fertilization of eggs was achieved due to application of the 2% aqueous dispersion of synthetic resin Carbopol 971 P.

A value of an ovarian plasma pH level is considered to represent one of the main indicators determining quality of obtained eggs, or more precisely, of their fertilization (Wojtczak et al., 2007; Lahnsteiner et al., 1999). A decrease in an ovarian plasma pH level can occur if more acidic content of eggs (6.47) penetrates the ovarian plasma. Such phenomenon was noticed at degradation of egg membranes during over-maturation of eggs (Lahnsteiner, 2000) or if eggs are mechanically damaged (Dietrich et al., 2007). In case of pike, the value of ovarian plasma pH was used for the first time by Švinger et al. (2012) as a factor influencing the quality of eggs before fertilization and a survival of embryos in the course of incubation up to the stage of so-called eyed eggs. In the above-mentioned experiment, average values of the ovarian plasma pH level in injected females varied between 7.68–8.39 and evidently higher values were recorded in fish where hypophyseal gonadotropin was provided in an emulsified with Freund's incomplete adjuvant (FIA). Regression analysis confirmed a slightly positive dependence of survival of embryos to the stage of eyed eggs on a higher value of an ovarian plasma pH level. Nevertheless, this relationship must be further verified as the above-mentioned experiment employed a limited number of broodstock (Policar, 2012a; Švinger et al., 2012).

Our experiments also suggested that if older (4–5 year-old) and larger females with an average weight of 4 600 ± 1 450 g are used, survival of embryos to the stage of eyed eggs can be very low (25–28%) (Švinger et al., 2012; Policar, 2012a). These values were obtained after

hormonal treatment of broodstock with a field-tested carp pituitary or without a hormonal treatment. Manipulation with gametes was careful and minimal. Artificial fertilization of eggs was conducted by a certified method. On that account, a very low survival of embryos is attributed to the old age of used broodstock. According to fishery practitioners, it is possible to observe a lower viability of pike embryos after stripping of large and old broodstock females or if collection of ovulated eggs into a grinding mortar is not conducted carefully. In case that eggs are extracted from female bodies by palpation of abdominal parts during stripping, ovulated gametes must not be placed in the bowl from great height (20 cm) (Zvonař, personal information, 2012).

We discovered that a high mortality rate in fertilized eggs and embryos during their incubation (up to 60–70%) occurred in eggs that originated from the first or last parts of an egg harvest. Therefore, during stripping of females, it is recommended to remove and not fertilize those eggs that are obtained as the first or last eggs from cranial or caudal part of ovary.

2.12. Egg size and number of eggs in 1 gram

A size of unfertilized eggs obtained from fish of various sizes and different environments ranges from 2.3 to 3 mm (Toner and Lawler, 1969) and eggs are globular in shape (Frost and Kipling, 1967). In general, younger females produce smaller eggs than older fish. One gram contains from 96 to 155 pieces of unfertilized eggs (Krupauer and Pekař, 1965; Křišťan et al., 2013). A size of eggs increases after fertilization and hydration. Eggs reach a size of 2.6–3.6 mm three hours after fertilization (Forst and Kipling, 1967).

2.13. Methods of sperm collection

As it was already mentioned in chapter 2.8, there are two methods of sperm collection that are generally used for pike (Billard, 1996). The first method involves the use of sperm released from vas deferens by palpation of abdominal region after thermal or hormonal stimulation (Billard et al., 1980; Dubský, 1998). Extracted sperm is applied either directly onto stripped eggs or, more often, is drawn into syringes or pipettes in a volume of 5–10 ml (Berka and Hamáčková, 1980; Hamáčková, 1987; Dubský, 1998; Hulák et al., 2008a). Collection of extracted sperm has an advantage consisting in preservation of sperm for further using. A disadvantage of this method is that sperm gets often contaminated with urine or blood during extracting sperm from male bodies which causes a sperm activation itself and a limited usability for artificial fertilization of eggs (Billard, 1978; Hulák et al., 2008a,b). To prevent sperm contamination with urine, Berka and Hamáčková (1980) recommended to place a male on the side during sperm collection. After that, they suggested to take a catheter that had been adapted from Pasteur capillary pipette and insert it into male genitourinary papilla. This released and discharged urine. Another disadvantage of this sperm collection method can be a very limited amount of sperm from single male. This fact can limit a successful artificial fertilization of eggs or it can require utilisation of a great amount of males which comprises a very demanding organization of work at fish hatcheries (Koldras and Moczarski, 1983; Linhart, 1984; Billard, 1996).

A second method of sperm collection is a use of testicular sperm that can be obtained from a dead male after testicles were removed from its body (Fig. 9) (Billard, 1996; Lahnsteiner et al., 1998; Hulák et al., 2008a). It is important to dry and disrupt testicles after their removal (Fig. 10). Sperm is sieved through a fine polyamide fabric called uhelon (size of meshes of 300 μm) directly onto obtained ovulated eggs (Fig. 11 and 12) (Dubský, 1998). The advantage

of this method of sperm collection in comparison to the previous method lies in obtaining a larger volume of sperm with a higher concentration of sperm per 1 ml, and, above all, the collection ensures clean uncontaminated sperm with urine or blood. The disadvantage of this collection method consists in killing a male, thus in a loss of a broodstock for further rearing (Hulák et al., 2008a). Collected testicular sperm or directly obtained whole testicles can be preserved for 24–48 hours at a temperature of 2–4 °C after drying and blood removal from their surface (Křišťan, personal information, 2013).

Fig. 9. *Removal of testes from a euthanased male pike (Esox lucius L.) (photo: J. Křišťan).*

Fig. 10. *Disruption of testes in order to obtain testicular sperm for artificial fertilization of eggs of pike (Esox lucius L.) (photo: J. Křišťan).*

Fig. 11. *Transfer of crushed testicles of pike (Esox lucius L.) onto a fine uhelon fabric (mesh size 300 μm) (photo: J. Křišťan).*

Fig. 12. *Squeezing of sperm from crushed testicles of pike (Esox lucius L.) through a fine uhelon fabric onto eggs (photo: J. Křišťan).*

2.14. Male reproductive ability and characteristics of their sperm

Concentration of sperm in testicles before stripping period varies from 2.4 to 4.1 x 10^{10} of sperm.g^{-1} in testicles. Total possible production of sperm in one male ranges from 4.4–7.9 x 10^{11} of sperm per 1 kg of live weight of fish (Billard et al., 1983). Sexually mature males release sperm from November until May and a percentage of males producing sperm in this period considerably fluctuates. In November and December, it is less than 15% of males, in January and February, a percentage of males releasing sperm rises to 25–30%. In March and April, more than 60% of males release sperm spontaneously. Contrariwise, in June, there is only a several percent of males which release sperm spontaneously (Billard, 1996).

An actual production of sperm of males which release sperm is determined mainly by a volume of obtained sperm and a concentration of sperm in 1 ml of obtained sperm. Male reproductive ability considerably fluctuates depending on a size and age of a male and also on time when sperm is collected from male. At the beginning of a stripping period, males produce a little amount of sperm ranging from 0.3 to 0.4 ml.kg^{-1} of live weight of fish. The sperm production increases to a value of 1.35 ml.kg^{-1}. At the end of a stripping period, sperm production quickly decreases (Billard, 1996). According to Krupauer and Pekař (1965), an amount of collected sperm is very low and it reaches a volume of 3 ml at maximum. In practice, a lower volume of sperm ranging from 0.5 to 2.5 ml is collected, on average, from one male (Koldras and Moczarski, 1983; Linhart, 1984; Hulák et al., 2008a).

At the beginning of a stripping period, sperm is thick and cream- to snowy white-coloured. Towards the end of this period, sperm is much thinner or even watery (Kouřil and Hamáčková, 1975). An average sperm thickness in a stripping period achieves a value of 22.26 x 10^9 of sperm.ml^{-1} in sperm, with a minimum amount of 2.5 x 10^9 and a maximum amount of 68.0 x 10^9 of sperm.ml^{-1} (Kouřil and Hamáčková, 1975; Linhart, 1984; Hulák et al., 2008a).

An absolute and relative fecundity was assessed by Linhart (1984) who revealed an absolute fecundity ranging from 3.2 to 25.2 x 10^9 of sperm per one male in 38 males (TL = 355–540 mm). A relative fecundity of males varied from 1.4 to 36 x 10^9 of sperm.kg^{-1} of live weight of a male.

Osmolality of released sperm achieves values of 204–314 mOsmol.kg^{-1}. The duration of sperm motility period is dependent on an activation solution. If sperm is activated with distilled water, the period of sperm motility will be shorter in comparison to sperm activated with urine. In general, it applies that a percentage of moving sperm quickly decreases with time. Only 60% of sperm are motile 15 seconds after activation and only 35% or 10% after 30 or 40 seconds after activation. The velocity of sperm motility 15 seconds after activation is 163 ± 40 μm (Hulák et al., 2008a,b)

Hulák et al. (2008a,b) engaged in comparison of composition of stripped and testicular sperm. The above-mentioned team of authors revealed that testicular sperm had higher concentration of sodium (Na^+ = 123 ± 9 mM), chloride (Cl^- = 127 ± 7 mM) and potassium ions (K^+ = 35 ± 5 mM), and next, it had higher osmolality (358 ± 77 mOsmol.kg^{-1}) and a higher concentration of sperm in 1 ml (34 ± 5 x 10^9.ml^{-1}) as opposed to stripped sperm where the following average ion concentration was discovered: Na^+ = 116 ± 9 mM, Cl^- = 116 ± 7 mMa K^+ = 25 ± 4 mM, osmolality: 273 ± 21 mOsmol.kg^{-1} and a concentration of sperm in 1 ml: 23 ± 4 x 10^9.ml^{-1}.

2.15. Sperm morphology and characteristics

Sperm from pike, as well as from all the other fish species, consists of three main morphological parts – head, midpiece and tail that has a typical flagellate shape. Pike sperm belong to primitive so-called "aqua" sperm (Alavi et al., 2009a,b). The size of head ranges from 1.2 μm to 1.6 μm. The shape and size of a sperm head is very important since sperm head penetrates the egg membranes of eggs, especially through the microphyle opening. The whole structure of flagellum consists of two central and nine peripheral tubules. Sperm flagellum can be divided into a proximal, central and tail part. Sperm flagellum of pike has a so-called "fin" (Alavi et al., 2009a) (Fig. 13).

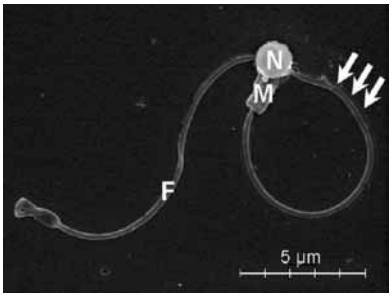

Fig. 13. *Morphology of pike (Esox lucius L.) sperm under an electron microscope (Alavi et al., 2009a); sperm head with a cell nucleus (N), midpiece (M)) and flagellum (F) with a peripheral fin (arrows) (photo: S.M.H. Alavi).*

2.16. Artificial fertilization of eggs with using of activation medium

Fertilization of eggs in a proportion of 3–4 ml of sperm to 1 kg of eggs is carried out immediately after the stripped sperm is collected. Sperm should be collected separately at least from 3 males (Billard, 1996; Křišťan et al., 2013). Křišťan et al. (2013) optimized the proportion of sperm per 1 fertilized egg and they determined the most suitable proportion of ensuring a high rate of egg fertilization (67%) and larval hatching (65%) at a level of 500 000 sperm per 1 egg, which approximately corresponds to the above-mentioned proportion of sperm and eggs during fertilization. Billard (1996) recommended the proportion to be a bit lower – 400 000 sperm per 1 egg, with the rate of egg fertilization ranging around 60–70%.

After sperm is applied onto eggs, it is advisable to gently mix sperm and eggs together. It is necessary to be very careful because eggs are highly sensitive to an excessive and thoughtless treatment at this time. Consequently, it is important to use activation medium (Fig. 14) in a volume of 0.5 l of activation medium per 1 kg of eggs over the mixture of eggs and sperm. The simplest activation medium is fresh water from a given fish hatchery. However, if artificial fertilization of eggs is carried out, it is recommended to use a activation medium of salt (NaCl) in a concentration of 7 $g.l^{-1}$ in order to achieve better and longer sperm motility. Scientific literature (Dyk, 1940; Berka and Hamáčková, 1980; Alavi et al., 2009a) also recommended using various activation medium that can increase the percentage of egg fertilization:

- Ringer's activation medium containing: 6 $g.l^{-1}$ NaCl, 0.075 $g.l^{-1}$ KCl, 0.15 $g.l^{-1}$ $CaCl_2.2H_2O$ and 0.1 $g.l^{-1}$ $NaHCO_3$ (Dyk, 1940),
- solution prepared by mixing of 15 g of urea in 1 litre of water (Berka and Hamáčková, 1980) and

- NaCl activation medium adapted by 20mM Tris to an osmotic pressure of 288 mOsmol and pH 8.5 (Alavi et al., 2009a).

An average fertilization of pike eggs in fishery facilities after artificial stripping and egg fertilization ranges around 40–70%. Egg fertilization can be considerably increased by using some of the above-mentioned activation solution to a level of up to 75–80%.

After sperm activation, the mixture of sperm, eggs and activation medium is gently mixed (Fig. 15) and is left to stand for 5–10 minutes (Billard, 1996; Policar 2012a). Then, the mixture of eggs and sperm is rinsed several times with water and fertilized eggs freed of sperm residues are thus prepared to eliminate the stickiness (so-called desticking) of eggs.

Fig. 14. *Activation of sperm with an activation medium in pike (Esox lucius L.) (photo: J. Křišťan).*

Fig. 15. *Gentle mixing of eggs, sperm and an activation medium during artificial fertilization of pike eggs (Esox lucius L.) (photo: J. Křišťan).*

2.17. Elimination of egg stickiness before incubation

Surface of fertilized eggs of pike becomes sticky in several minutes (4–5 min) after water is added. It is necessary to mention again that elimination of egg stickiness must be carried out very gently since fertilized eggs are highly sensitive to manipulation. For desticking of pike eggs, it is recommended to use several following methods that our team successfully tested in practice. Individual methods of egg desticking are classified in the text according to the easiest obtained media or chemicals used in this process. However, the easiest obtained

media or chemicals generally require a longer application use that leads to completion of egg desticking:

- application of cow milk containing 3.5% of fat that must be used for 60–90 minutes (Hamáčková, 1987),
- application of pond clay or clay used for production of ceramics (Fig. 16) for 30–40 minutes (Dubský, 1998; Policar, 2012a),
- application of a solution obtained by mixing of 100 g of talc and 20–25 g of salt (NaCl) in 1 litre of water for 20–30 minutes (Hamáčková, 1987) and
- application of a solution obtained by mixing of 5.52 g of NaCl; 3.75 g of glycine and 2.42 g of Tris in 1 litre of water for 15 minutes
- Berka and Hamáčková (1980) also recommended eliminating the egg stickiness with the use of 5% solution of powdered starch with the exposure time of 10 minutes.

The above-mentioned desticking solutions are used usually in the volume proportion of 1 : 2 (eggs : solution). After desticking of egg surface, individual eggs start to separate from each other and they do not form a mass stuck together any longer. At this moment, desticked eggs are rinsed several times with clean water from a hatchery and they are placed in incubation bottles.

Fig. 16. *Elimination of stickiness of eggs of pike (Esox lucius L.) by means of clay (photo: J. Křišťan).*

2.18. Incubation of eggs and hatching of embryos

In the majority of cases, incubation of pike eggs in Europe is conducted in Chase bottles (Fig. 17). It is necessary to keep eggs in permanent, however, only a subtle movement in incubation bottles by means of inflowing water. A "subtle movement" must be emphasised because it has been confirmed that too sharp or strong flow rate can cause an excessive movement of eggs which thus results in an increased mortality of incubating eggs and embryos. In addition to Chase bottles, it is also recommended to use modified Zug bottles in fishery practice. In adapted Zug bottles, a perforated funnel is installed at the bottom of bottles through which water inflowing from the bottom of a bottle is gently flowing through. It ensures a weak water flow rate and a subtle movement of incubated eggs. The advantage of modified Zug bottles is that a fish hatchery does not have to purchase other special types of incubation bottles and it can use common Zug bottles for egg incubation. For incubation of pike eggs, it is also recommended to use McDonnald or Kannengieter bottles that are, however, mostly used in the USA or Western Europe (Hochleithner, 2004). Water flow rate in incubation Chase bottles in a volume of 5 l is set to a level of 3–6 $l.min^{-1}$ and 4–8 $l.min^{-1}$ in ten-litre Zug bottles. A lower water flow rate in bottles is always employed at the beginning of incubation and a higher rate of water flow rate at the end of incubation.

Fig. 17. *Artificial incubation of eggs in Chase bottles (photo: T. Policar).*

In general, 2 litres of eggs are placed into ten-litre modified Zug bottles and eggs consequently become swollen and increase their volume (up to three times). After swelling up, eggs can represent up to 2/3 of volume of a incubation bottle. Oxygen content in water flowing into incubation bottles should range from 7 to 9 mg $O_2.l^{-1}$. Water used for incubation of pike eggs should be filtered through sieves of a mechanical filter and it should thus get rid of coarse impurities. Water used for pike incubation should be of a similar quality as water used in salmonid hatcheries. Billard (1996) and Hochleithner (2004) shared the same opinion.

Optimal water temperature for egg development varies between 6–10 °C (Bondarenko et al., 2013a; submitted). At the beginning of egg incubation, fishery facilities usually use a water temperature ranging from 8 to 10 °C that is gradually increased to a temperature of 12–14 °C towards the end of incubation. Hamáčková (1987) reported a high mortality rate of eggs and embryos during incubation with water temperature from 4 °C to 22 °C. Lillelund (1967) and Hokanson et al. (1973) monitored experimentally the development of pike eggs at a temperature range between 3.7–24 °C. Survival of embryos higher than 80% was achieved at a water temperature varying between 6.4–17.7 °C. When a water temperature achieved 3 °C, only 9% of larvae hatched. According to Swift (1965), the optimal incubation temperature is 9 °C. This water temperature ensured the highest hatching rate of pike embryos (60–80%). Lillelund (1967) incubated pike eggs also at temperature of 5.8 °C with a good hatching rate of larvae at a level of 70%. Nevertheless, he stated that with regard to such incubated eggs, there was a high mortality of larvae one day after hatching. The author noticed that if hatched larvae were immediately transferred after hatching to temperature of 9–18 °C, a considerably decreased mortality of larvae was achieved. It is also necessary to avoid great water temperature fluctuation during egg and embryo incubation. Daily temperature changes by 5 °C (from 15 °C to 20 °C) caused a decrease in a hatching rate by 12% (Lillelund, 1967). For the first 30–40 °d, any manipulation with eggs must be avoided (mixing or releasing eggs stuck together). Such excessive manipulation causes increased losses by 20–25%. Dead and live eggs at the stage of eyed eggs can be separated by means of so-called separating

solution of NaCl prepared by dissolving of 136 g of salt in one litre of water. Separation of dead eggs from one incubation bottle takes about 1 minute. Live embryos float on the surface which must be transferred back to fresh water in a newly prepared incubation bottle as soon as possible. Removal of dead eggs is advisable to be carried out to protect live incubated eggs against fungal infection (most frequently the *Saprolegnia* and *Achlya* genera). In addition to removal of dead eggs, it is also possible to carry out a short immersion antifungal bath in salt (NaCl) in a concentration of 20 $g.l^{-1}$ for 20 minutes without a water flow at the half of the egg incubation period (approximately 5–8 days after fertilization). This bath can be carried out directly in an incubation bottle or outside the bottle.

At the eye-stage or at the beginning of the hatching period, it is advisable to transfer embryos into flat hatchery apparatuses or cradles with a mesh size of 2–5 mm at their bottoms or walls for so-called final hatching (Fig. 18). At this time, it is necessary to stop the inflow of water into incubation bottles. Subsequently, embryos are gently removed by suction without strong shaking movements into buckets by means of which the removed embryos are placed into the above-mentioned apparatuses or cradles.

Total incubation period of pike is dependent on a water temperature. If a water temperature varies between 8–10 °C, incubation lasts 110–140 °d which is between 11 to 17.5 days. If the temperature is 14 °C, it is 85 °d (6 days) and if the temperature reaches 18 °C, the sum of daily degrees required for larval hatching is 61 °d (3.42 days) (Bondarenko et al., 2013a, submitted). The incubation period of pike with the use of various water temperatures was published by Lillelund (1967) and individual values are provided in Tab. 3.

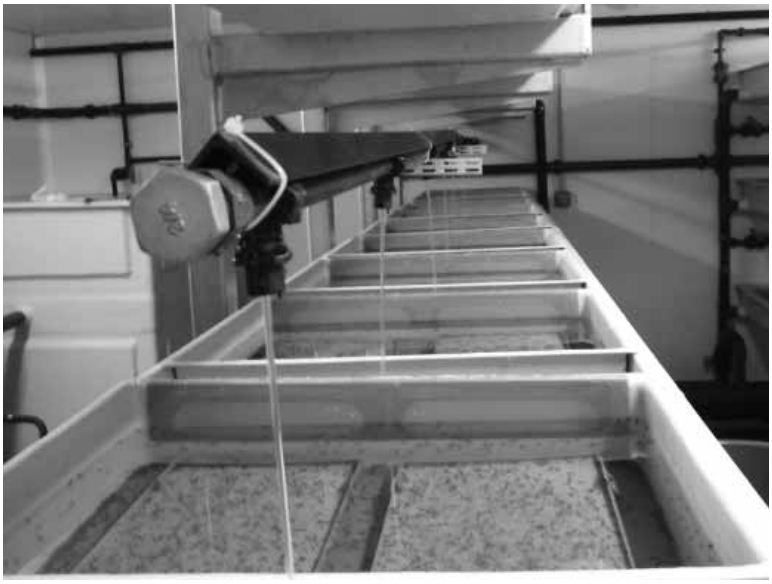

Fig. 18. *Final hatching of pike larvae (Esox lucius L.) on flat hatching apparatuses (photo: J. Křišťan).*

Tab. 3. *Length of incubation period depending on a water temperature during incubation of eggs and embryos of pike (Esox lucius L.) (Lillelund, 1967).*

Water temperature (°C)	Length of incubation	
	days	°d
5.8	30.9	179
9	15.2	137
12	9.4	113
15	6.3	95
18	4.7	85

The length of the process of larval hatching itself is also largely dependent on a water temperature. Based on our experience, this period can last 4 days at a water temperature of 6 °C and less than a day (0.92 of a day) at a water temperature of 18 °C (Bondarenko et al., submitted). Larval hatching can be accelerated by a steep increase in a water temperature by 5–7 °C which decreases the concentration of dissolved oxygen in water. Such increase in a water temperature is advisable to be carried out at a time when approximately 10% of larvae have already hatched. In the course of the intensive hatching of embryos, it is highly important to remove egg skin and dead embryos by suction at regular intervals of 2 to 6 hours.

2.19. Rearing of larvae until the yolk-sac resorption

An average size of newly hatched larvae of pike ranges from 8.5–9 mm with an average weight of 10–11 mg (Billard, 1996). It is advisable to provide pike larvae in hatching apparatuses, cradles or flat troughs with a possibility to hang on a substrate in a form of straw, branches of conifers, birches or a plastic netting (Fig. 19) with a mesh size of 1–5 mm immediately after hatching (Hamáčková, 1987; Billard, 1996). Larvae start hanging vertically by means of an adhesive papilla that develops on their head within several hours after hatching. Larvae stay in this position for 8–16 days depending on a water temperature which can vary in ponds between 8–16 °C. Hanging of pike larvae on a substrate takes approximately 130 °d. An adhesive papilla starts to be absorbed usually on the 9th day after hatching at a water temperature of 14 °C (Billard, 1996). A substrate enabling larval hanging is not necessary for maintaining the hatched larvae, however, it is suitable because it ensures a very good larval survival (Westers, 1986). After larvae start swimming, they swim towards the water surface and their swim bladder fills in. After that, larvae swim on the water surface or in water column and they digest a yolk-sac. Larvae have a fully functional mouth the second to fourth day after hatching and anus is fully developed within fourth to fifth day after hatching (Balvay, 1983). A complete resorption of a yolk-sac is terminated within 160–180 °d after larval hatching. Larvae start to take in the first exogenous food even before a complete digestion of a yolk-sac approximately 150–160 °d after their hatching. At this time, their size is around 12–15 mm and weight is 12 mg. At this stage, pike larvae swim actively and they quickly start foraging for food (Luquet and Luquet, 1983). Pike larvae are recommended to be stocked into further rearing optimally at the period when more than 50% of larvae keep hanging and other fish swim actively and hang only occasionally. Transport and stocking of larvae into rearing can still be carried out the following day when the majority of fish has already started to swim and only 25% of fish keep hanging. Later larvae stocking is not advisable because larvae starve which results in high losses within several days.

Fig. 19. *Larvae of pike (Esox lucius L.) after they started to swim in a vertical apparatus with a plastic netting used for their hanging (photo: T. Policar).*

2.20. Transport of larvae and juvenile pike intended for further rearing

Larvae intended for further rearing must usually be transported to far distances and the transport duration may last up to 12 hours. In such a case, it is recommended to transport larvae in polyethylene bags with oxygen atmosphere. In a bag with a volume of 20 litres containing 10 litres of water and 10 litres of oxygen atmosphere, up to 50 000 pcs of hanging larvae or of those that have partially started to swim can be transported at a water temperature of 10–12 °C. In bags without oxygen atmosphere, only 30–800 pcs larvae.l^{-1} at a water temperature of 12 °C should be transported and transport duration should not exceed 3 hours.

A similar method of transportation can also be applied to reared advanced pike fry (Fig. 20). It is recommended to transport 300–500 pcs of pike in one polyethylene bag in a volume of 10 litres of water and 20 litres of oxygen atmosphere for the duration of 6 hours at maximum. Since severe pike cannibalism occurs during the transport, it is advised to transport pike in total darkness. Even more effective transport method of a greater amount of pike (20–30 thousand pieces) is a packing transport box of a usable volume of 1 000 litres that is placed on a vehicle.

Fig. 20. *Advanced pike fry (Esox lucius L.) prepared for transport – packed in polyethylene bags with water and oxygen atmosphere (photo: T. Policar).*

2.21. Possibilities of rearing of larvae and juvenile pike to the advanced fry

After larvae have successfully started swimming and have partially digested a yolk-sac, it is necessary to initiate immediately their rearing. In the Czech climatic conditions, natural rearing of pike larvae starts around mid-March and lasts until mid-April. In connection with an intensive rearing of pike in recirculating aquaculture systems (RAS), off-season stripping of broodstock has currently also started to be carried out. It is targeted at continuous production of gametes and subsequently larvae during the entire year when pike is often stripped outside its natural reproduction period. This rearing procedure enables a better use of a rearing space of a given RAS (Muscalu-Nagy et al., 2011).

At present, there are, in total, three methods of effective rearing of pike larvae and juvenile fish to the stage of so-called advanced fry of a total length of 30–50 mm at the age of 14–25 days. Afterwards, fish are stocked into open waters or other production rearing that either uses production ponds with polycultural fish stocks or RAS for intensive rearing. All three methods of rearing of advanced fry employ rearing of pike in monoculture stocks. The first method is rearing in suitable stock-ponds, ditch ponds or ground ponds (Hamáčková et al., 1977; Berka and Hamáčková, 1980; Lusk and Krčál, 1982; Hamáčková, 1987; Dubský, 1998; Policar, 2012a). Other method is pike rearing in special rearing facilities (various cradles, storage ponds, troughs and tanks) although this rearing must be supplied with artificially caught live feed – mostly zooplankton on a daily basis (Hamáčková et al., 1977; Berka and Hamáčková, 1980; Lusk and Krčál, 1982; Hamáčková, 1987; Dubský, 1998). The last method is rearing of pike under controlled conditions of RAS where, at first, larvae are adapted to an intake of artificial pellet feed, established artificial light regime and ensured optimal water temperature for pike growth (25–28 °C) (Szczepkowski, 2009; Policar 2012b; Dušek, 2013).

2.21.1. Classic pond rearing of larvae and juvenile fish to the advanced fry

Pond rearing of larvae employs, above all, stocking into trench and ground ponds (Fig. 21) of a small area from 100 m^2 (Lusk and Krčál, 1982; Hamáčková, 1987), however, ponds of larger areas (up to 1.5 ha) can be used as well (Policar, 2012a). The ideal conditions are if it is possible to use also wintering ponds for this type of rearing that were drained over winter period before pike rearing. In general, it applies that it must be easy to remove fish from a pond and, if possible, ponds must have flat and well-sloped bottoms. It is optimal when fish can be caught at the end of rearing under the dam which enables careful removal of reared juvenile fish from a pond. Inflow and outflow from a pond must be secured against fish escape as well as against penetration of piscivorous fish species into the pond. At the beginning of pike rearing, the water surface is maintained at a level of 50 to 70 cm and after one week, it is elevated to 100–120 cm. Lower water surface at the beginning of rearing enables faster heating of pond water which positively influences the development of a pond food base in a form of zooplankton.

Ponds selected for rearing must be properly prepared for stocking of larvae itself. It is advisable to fertilize ponds of a lower trophy (ponds with a sandy bottom, a low content of phosphorus and nitrogen in their sediment and with an oligotrophic inflow) with manure (dung or compost) at a dose of 300–500 $kg.ha^{-1}$ already several days (14–21 days) before the stocking of larvae itself. When fertilization is carried out, it is very effective to spread manure on the pond bottom in a littoral zone in a form of small heaps, so-called planktonic nests (Dubský, 1998). Such fertilized ponds are subsequently filled up with water at least 12–14 days before stocking of larvae itself. This time is important to ensure a sufficient development of

zooplankton in ponds before larval stocking. Larvae which have started to swim containing the rest of a yolk-sac should be stocked into ponds with an adequate representation of moderately large zooplankton (*Diaphanosoma, Eurycercus, Daphnia, Cyclops*, etc.) at a density of 150–300 individuals.l^{-1} (Hamáčková, 1987).

Fig. 21. *Pond used for rearing of advanced pike fry (Esox lucius L.) (photo: T. Policar).*

Initial density of stocked larvae is determined according to an amount of natural food in a pond, according to a division and a length of a bank line and occurrence of aquatic vegetation in a given pond (Lusk and Krčál, 1982). In general, it applies that the higher the zooplankton density, the more indented bank parts and larger representation of aquatic macrophytes in a pond, the larger the the density of larvae can be used for stocking (Berka and Hamáčková, 1980). Based on our experience of one month's rearing, we recommend carrying out the stocking at an initial larval density ranging from 8 to 30 pcs.m^{-2} which represents a density of 80–300 thousands of larvae.ha^{-1} depending on a trophy and a size of used ponds. Smaller ponds of an area of 0.1–0.2 ha can be stocked at an initial density of up to 300 thousands of larvae.ha^{-1}. Lower density of larvae (80–100 thousands of larvae.ha^{-1}) is used in larger ponds with an area of 1.2–1.5 ha. Other pike breeders recommend the following different initial larval densities. Huet (1976) recommended to apply an initial larval density at a level of 10 000–20 000 pcs.ha^{-1} for a 6–8 week-long rearing. Steffens (1976) described rearing of advanced fry at initial larval densities of 30 000–800 000 pcs.ha^{-1}. Lepič (personal information, 2012) used small ponds of an area of 0.08–0.3 ha with an initial density of 230–250 thousands of larvae per 1 hectare. Lepič (personal information, 2012) further added that at the end of rearing, it was necessary to provide pike in ponds with food in a quantity of approximately 3–5 kg of caught zooplankton on a daily basis. Berka and Hamáčková (1980) documented the development of food in reared larvae and juvenile fish in the course of pike rearing in detail. Based on our knowledge, fish of a total length, TL = 10–12 mm eat mainly medium-sized zooplankton (*Diaphanosoma, Eurycerucus, Daphnia* and *Cyclops*), fish of a total length of 12–20 mm specialized in zooplankton and Chironomidae larvae. Fish of a total length of

20–50 mm eat food selectively in such a way that with an increasing body length fish preferred larger food organisms, such as zooplankton (*Daphnia* and benthic organisms, e.g., larvae of *Chironomidae, Trichoptera, Ephemeroptera* and *Diptera*). With regard to fish of a total length of 25–30 mm, the first signs of cannibalism appeared. Pike is reared from its larval stage until the advanced fry in ponds under a water temperature at the beginning of rearing of usually 12–13 °C and at the end of rearing, it is 14–16 °C. It must be stated that this temperature is not optimal for pike growth and it rather represents a limiting factor for the growth. Due to numerous experiments carried out under controlled conditions, it was revealed that an optimal temperature for a growth of larval and juvenile stages of pike was a water temperature ranging between 24–28 °C (Szczepkowski, 2009; Policar, 2012b). Nevertheless, the above-mentioned natural water temperature in ponds ensured a very good and economically advantageous production of advanced fry. With regard to this rearing, if balanced and sufficient density of food organisms was ensured, reared fish achieved a very high growth velocity (a specific growth rate = SGR = 22.5–30.0%.d^{-1}) and the total fish production was not affected by an excessive cannibalism rate (only 10–15 %) (Policar, 2012b).

In order to achieve a successful rearing of advanced pike fry, it is highly important to regularly monitor the density of zooplankton occurring in rearing ponds. If there is an insufficient amount of food when the zooplankton density decreases below 100 individuals per 1 litre of water, ponds can be provided with zooplankton caught somewhere else or occurrence of zooplankton can be supported by regular pond fertilization with artificial fertilizers (urea or ammonium sulphate at a dose of 20 kg.ha^{-1}). However, such fertilization of ponds must be conducted circumspectly and only in exceptional cases if occurrence of food organisms cannot be supported in any other way. Rearing of advanced pike fry must be terminated at the moment when there are fish of catchable sizes (minimum TL = 30 mm), the maximum rearing capacity is used and a decrease in coarse zooplankton density below the above-mentioned density of 100 individuals per 1 litre of water starts to occur. If the harvest of a pond is performed too late (there is a delay of only several days), it is possible to encounter a high cannibalism rate during advanced pike fry rearing and a low survival of reared fish ranging from 5 to 10%. If the rearing of advanced pike fry is well-conducted, the survival rate varies between 20 to 40%.

The following data provide results and efficiency of advanced pike fry rearing revealed by other authors under different specific conditions. If ponds are stocked with an initial density of 80 000 pcs.ha^{-1}, it is possible to achieve a final production of advanced fry in an amount of 28 000–40 000 pcs of fish per 1 ha of an area with a biomass of 23–25 kg.ha^{-1} at a survival rate of 35–50% in 14–17 days of rearing (Berka and Hamáčková, 1980). Survival of juvenile pike with a final total length of 90–100 mm after a six to eight-week long rearing at an initial density of 10 000–20 000 larvae achieved the level of 5 to 50% (Huet, 1976). Pike cannibalism was relatively high (25–70%) during this rearing. During a twenty-day long rearing of advanced fry at an initial larval density of 300 000 pcs.ha^{-1}, only 12% of fish survived (Steffens, 1976; 1986). Their final TL reached 44 mm and individual weight was 1.2 g. Lepič (personal information, 2012) achieved a fish survival ranging from 10 to 30% during a 15–30-day long rearing. Survival was dependent mainly on cannibalism among reared fish and a final length of produced fish. Lepič added that the longer the rearing, the lower the percentage of fish of larger body sizes (TL = 40–60 mm). Policar (2012a) revealed survival of advanced fry at a level of 15–30% after 15-day long rearing of fish at a final TL of 40–45 mm. A higher survival rate was achieved in ponds at a smaller area (0.16 ha) as opposed to ponds at a higher area (1.1–1.5 ha).

The most suitable period for pond harvesting during rearing of advanced pike fry is the moment when advanced pike fry achieve a total length of 30–40 mm. The term of harvest

may be slightly postponed if a sufficient density of zooplankton and macrozoobenthos is established in a given pond. In such a case, however, zooplankton density must be carefully monitored and recorded at regular daily intervals. If a decrease in density of food organisms is discovered, harvest of reared fish must be immediately conducted. In general, it is important to catch advanced fry into underlying nets or various fishing cages under a pond dam in a very careful manner (Fig. 22). In order to catch advanced pike fry, the same general rules as for catching of advanced pikeperch (*Sander lucioperca L.*) fry are valid. The harvest should be carried out quickly (within 6–12 hours) in a suitable cloudy or rainy weather at air temperature of 18–20 °C at maximum. Fish should be regularly removed from nets and carefully sorted (Policar et al., 2011b).

Reared and caught advanced pike fry are subsequently stocked into stock-ponds or main ponds to one or two-year-old carp stock. The objective in these ponds is to minimize the occurrence of less valuable commercial species and increase the production of carp. Next, caught advanced pike fry can also be sold to sport fishermen who stock the pike fry into sport fishing grounds in open waters (Lusk and Krčál, 1982; Hamáčková, 1987; Dubský, 1998).

Fig. 22. *Harvest of pike advanced fry (Esox lucius L.) under a pond dam (photo: T. Policar).*

2.21.2. Rearing of larvae and juvenile fish to the advanced fry in special rearing facilities of a flow-through type

Rearing of advanced pike fry in special rearing flow-through facilities was mainly employed between 1970s–1990s in Germany, the Netherlands, Hungary, France, Switzerland, Austria and the former Czechoslovakia (Hamáčková et al., 1977). At present, based on our information, this system has not been used too often and in the first decade of the 21st century, it was replaced by rearing of pike in RAS (see chapter 2.21.3). Rearing of pike in special rearing facilities of a flow-through type was generally implemented from the larval stage for 2–4 weeks in different facilities, such as cradles, storage ponds, troughs and tanks (Berka and Hamáčková, 1980; Hamáčková, 1987). In these facilities, reared pike was dependent on a food supply in a form of caught zooplankton (Lusk and Krčál, 1982). Main limiting factors of this rearing were: ensuring a food base for reared fish, low water temperature (13–15 °C) and protection of fish against unicellular parasites (Berka and Hamáčková, 1980). Some farms situated in Germany,

Hungary and France employed a partial heating of a rearing system by geothermal water or waste water which enabled a stable temperature ranging between 16 and 20 °C. An increased water temperature had a positive impact on pike growth, however, there was a greater risk of cannibalism that must have been eliminated by a sufficient feeding (Hamáčková et al., 1977).

Feeding was provided in a form of live and pre-caught zooplankton, e.g., cyclops (Copepoda) and cladocerans (Cladocera). A daily feeding ration of zooplankton amounted to 25–35% of reared fish biomass (Hamáčková et al., 1977; Hamáčková, 1987). Larvae were stocked into rearing systems at an initial density of 6–8 up to 20 larvae.l^{-1} (Dubský, 1998) and they were kept at a light intensity of 250–270 lux (Hamáčková et al., 1977).

Different rearings resulted in different fish production which is summarized in Tab. 4 (Hamáčková et al., 1977).

Tab. 4. *Water temperature and production indicators within pike rearing in so-called special rearing facilities of a flow-through type (Hamáčková et al., 1977).*

Used water temperature (°C)	Duration of rearing (days)	Fish survival rate (%)	TL of reared fish (mm)
16–17	10 - 14	50	22–30
15–20	18 - 23	50	23
11	42	20	25–30

2.21.3. Intensive rearing of larvae and juvenile fish to the advanced fry in RAS

An intensive rearing employing the RAS system (Fig. 23 and 24) has been tested and experimentally used for production of juvenile pike to the advanced fry of a total length of 30–50 mm in the past 10 years mainly in Central Europe (Poland, Hungary and the Czech Republic) (Wolnicki et al., 1997; Myszkowski et al., 1998; Kucska et al., 2005; Szczepkowski, 2009; Policar, 2012b; Dušek, 2013). This system of pike rearing has enabled to use artificial pellet feed, larger densities and high growth rate of reared fish and, at the same time, to eliminate the cannibalism rate (Szczepkowski, 2009; Policar, 2012b). The objective of this pike rearing is to obtain a high-quality pike stocking material within a very limited rearing space without the need to catch and utilize zooplankton. This system of pike rearing has currently experienced the beginnings of its use. However, in Hungary, there are already three commercial fish farms (Aranyponty, Bajcshal and Szegedfish) employing this method of intensive pike rearing to the advanced fry for their production (Kucska, personal information, 2013). However, it can be expected that this system of pike rearing will be used to a greater extent in future. If combined with off-seasonal fish stripping, the system offers a possibility to produce fish continually in the course of the entire year with a high labour productivity (Policar, 2012b). Next, it was revealed that pike adapted to artificial feeding and RAS in older age categories can be effectively and successfully reared in combination with intensive rearing of sturgeons, specifically with Siberian sturgeon (*Acipenser baerii*) (Szczepkowski, personal information, 2010). The advantage of this system is a predictable fish production as opposed to fish rearing in ponds (Policar, 2012b).

Fig. 23. *Recirculating aquaculture system used for intensive rearing of pike (Esox lucius L.) to the advanced fry (photo: T. Policar).*

With regard to intensive rearing of pike, it was discovered that it was possible to successfully use a high initial larval density (20–40 larvae.l^{-1}) (Bondarenko et al., 2012a; Policar, 2012b). An optimal water temperature ensuring a high growth and survival of pike ranges between 24–28 °C, however, it is also possible to use a water temperature of only 19–20 °C (Policar, 2012b). A suitable light regime in the course of one day (24 hours) is either an uninterrupted lighting or a light regime with two eight-hour long lighting sections that are interrupted by two four-hour long sections of dark (L8:D4:L8:D4). The third regime can also be successfully used, that consists of a 16-hour long lighting section and an eight-hour long section of dark. All lighting regimes should be of an intensity of 5–30 lux (Szczepkowski, 2009; Policar, 2012b).

Fig. 24. *Rearing of juvenile pike (Esox lucius L.) to the advanced fry in a tank within the RAS system (photo: T. Policar).*

Feed produced by the BioMar Company – Larviva Wean-Ex 300 and 500 or Inicio Plus G represent a suitable feeding for intensive rearing of pike that was personally tested by our team. Larvae at the beginning of rearing until the advanced fry (Fig. 25) can be fed with granular feed of a particle size of 0.15–0.6 mm with an initial daily feeding ration of 20% of reared pike weight and a final 10% of reared fish biomass (Policar, 2012b).

Fig. 25. *Reared advanced pike fry after a thirteen-day period of rearing within the intensive rearing in RAS system (photo: T. Policar).*

After 13 days of rearing of larvae and juvenile fish in intensive conditions, it is possible to achieve the stage of advanced fry (at a total length of 28.4–30.6 mm and a body weight of 0.095–0.14 g) and a cumulative survival at a level of 54–69%. Under such rearing conditions, reared pike reach the SGR of 18–20%.d^{-1} and the cannibalism rate amounts to 15–18%. It can be stated that such pike rearing under controlled conditions of the RAS system can be interesting and effective from an economical point of view. In 13 days, it is possible to rear up to 25 000 pcs of advanced pike fry under a very small rearing system with a total volume of 8 000 litres (Policar, 2012b).

III. Comparison of the "novelty of procedures"

In the Czech literature sources, it is possible to find a scientific publication describing optimization of reproduction and rearing of pike that originated already in 1987 (Hamáčková, J., 1987. Rearing of pike. Technical standard 46 6836. Prague, The office for Standards and Measurements, 1987, 12p). It means that over the period of approximately 26 years, Czech professionals were not offered any similar scientific publication. However, in the past 26 years, knowledge on reproduction and rearing of this very interesting and commercially used fish species has considerably changed and developed. For that reason, the presented publication provides a summary of a wide range of new information concerning controlled reproduction, sperm biology and physiology, optimization of artificial fertilization, desticking and incubation of eggs and also effective rearing of larvae and juvenile fish to the stage of advanced fry.

IV. Description of application of the certified methodology

The presented methodology is, above all, intended for practical use of described information related to biology, reproduction and rearing of pike in fishery facilities in the Czech Republic. The certified methodology will primarily be applied in the Nové Hrady Fisheries Ltd. It is supposed that this methodology will support an increased production of larvae or advanced fry of pike in the future.

V. Economic aspects

Implementation of the procedures presented in this methodology into the fishery practice is not connected with high costs. The basis lies in implementation of technological measures in practice that are related to reproduction and rearing of pike (e.g., use of hormonal stimulation of broodstock with carp pituitary, optimization of sperm collection and subsequently the whole process of artificial fertilization of obtained ovulated eggs, innovation of incubation of fertilized eggs with the use of Chase bottles or modified Zug bottles, ensuring a high hatching rate and successful final hatching of larvae and subsequent optimization of rearing of larvae and juvenile fish in ponds or RAS). This activity can result in an annual higher synchronization of broodstock stripping in fishery facilities. This can save wage costs of staff (up to CZK 5 000) during the stripping period of pike and it is supposed that savings of up to several tens of hours (20 hours) required for control of broodstock without hormonal stimulation will be achieved. Next, an increase in egg fertilization and larval hatching rate by approximately 10–15% can be achieved. With respect to annual 1–3 million production of pike larvae, the production of larvae in a fishery facility can increase by 100 000–400 000 pcs. This effect can ensure an annual rise in sales by CZK 6 000–36 000 to a fishery facility with the above-mentioned yearly production and a price of pike larvae (CZK 60 for 1 000 pcs). Further application of scientific information contained in this methodology in practice can cause an increase in survival of reared advanced pike fry by 5–20% which can increase sales of a fishery facility producing yearly 100 000 pcs of advanced pike fry of a total length of 30–50 mm from this increased production at a level of CZK 22 500–90 000 every year at a price of an advanced fry of CZK 4.5 per one piece of a total length of 30–50 mm.

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CHAPTER 6

GENERAL DISCUSSION

The northern pike (*Esox lucius* L.) is an economically important freshwater species and a promising species for aquaculture (Margenau et al., 2008). Northern pike are found in most freshwater habitats, from cold deep lakes to warm shallow ponds and muddy rivers. Traditionally, rearing of northern pike is based on pond culture, mostly co-cultured with a variety of other fish species (Milstein, 1992). Intensive northern pike culture has been investigated in Europe in recent years to establish methods of stable and high quality production of juveniles for ongrowing (Kucska et al., 2005).

The overall goal of this study was to investigate effective methods for northern pike reproduction and production of high quality larvae for intensive culture under controlled conditions. Specifically, Chapter 5 focused on the technological aspects of reproduction and rearing of advanced pike fry. Chapter 2 compared efficacy of GnRHa treatments for stimulation of ovulation with that of traditional hormonal (carp pituitary) and non-hormonal (natural environmental stimulation) treatment. Chapter 3 examined flagella swimming characteristics in northern pike spermatozoa, comparing flagellar characteristics of northern pike with those of sterlet (*Acipenser ruthenus* Brandt) using CASA analysis and high speed video microscopy. Finally, the effect of temperature on the egg incubation period, survival of eggs during embryo development, and quality of newly hatched larvae of northern pike under laboratory conditions was examined in Chapter 4. Thus the four chapters, comprising one practical handbook and three empirical studies, on which this thesis is based focus on aspects of controlled reproduction in northern pike.

Paper I. Evaluation of treatments for induction of ovulation in northern pike (*Esox lucius* L.)

An important step in northern pike culture is induction of ovulation under controlled conditions. In Chapter 2, the efficacy of carp pituitary and non-hormone treatment associated with holding mature females in natural ambient conditions for induction and synchronization of ovulation in northern pike was compared to that of sGnRH-a (DArg^{6}Pro^{9}Net) at doses of 50 and 100 μg kg^{-1} with or without Freund´s incomplete adjuvant and dopamine inhibitor (metoclopramide, 8 mg kg^{-1}). Single injections of 10-50 μg kg^{-1} mammalian GnRH analogues have been able to overcome reproductive dysfunction and induce ovulation in various fish species (Mikolajczyk et al., 2008; Svinger et al., 2013). In contrast, single injections of mGnRHa or sGnRHa in northern pike have not been shown effective (Billard and Marcel, 1980; Szabo, 2003).

Ovulation was not observed in northern pike that were injected with any GnRHa formulation. However, all females treated with the carp pituitary and 70 % of those kept under natural conditions ovulated. Other employed hormonal interventions were ineffective with the exception of the use of sGnRHa (DArg^{6}Pro^{9}Net) at 50 μg kg^{-1}+8 mg kg^{-1} Met. This intervention induced ovulation only in 10% of females. This low effect was probably with the result of a spontaneous ovulation. Therefore, this hormone treatment cannot be recommended for use in culture of northern pike.

The results confirm that carp pituitary remains the only effective hormone treatment for inducing ovulation in northern pike (Billard and Marcel, 1980; Pecha et al., 1992; Szabó, 2001, 2003, 2008).

Was found that value of ovarian plasma pH has influence on eggs quality. Were shown that groups with high pH had highest survival rate to gastrula stage. The same relationship were found by other authors for rainbow trout (Dietrich et al., 2007; Wojtczak et al., 2007)

brook char (Svinger et al., 2013). In spite of these statements, our results show that the measurement of the ovary plasma pH could be important factor of the eggs quality in northern pike.

Paper II. Comparison of swimming flagella characteristics in northern pike (*Esox lucius* L.) and sterlet (*Acipenser ruthenus* Brandt) spermatozoa using CASA analysis and high speed video microscopy

The primary goal of the study reported in Chapter 3 was to compare the spermatozoa swimming parameters in northern pike with those of sterlet as a model species, using high speed video microscopy to complement the CASA analysis. A secondary aim was description of a novel means of recognizing and quantifying sperm quality in cultured fish.

Most of the reported data involve events during the initial stages of spermatozoa motility. All swimming parameters including velocity are at their peak during the first seconds of spermatozoa motility (Cosson, 2004; Cosson, 2010).

The method currently used for spermatozoa motility analysis is computer assisted sperm analysis (CASA), which allows obtaining information about head movement, such as number of motile spermatozoa heads and their velocity and linearity (Boryshpolets et al., 2013a; Amann et al., 2014). The description of flagellar parameters has additional potential applications. Fish spermatozoa encounter various physiological situations that result in changes of flagellar shape. An example is exposure to changes in external and internal ion concentration (Cosson, 2004), intracellular ATP level (Perchec, 1995; Cosson, 2012a;) or hypo- or hyper-osmotic conditions (Cosson, 2012b). Detailed analysis of such changes in flagellar morphology constitutes a reservoir of new information that is thus far little exploited (Boryshpolets et al, 2013a).

Physical conditions of surrounding medium, such as the viscosity of ovarian fluid, also affect flagellar morphology and swimming performance (Cosson, 2008). Pollutants may affect fish spermatozoa swimming performance (Alavi et al., 2009), and their effects on flagellar wave characteristics may be quantified in detail using methods of dynamic morphometry from high speed video imagery briefly described in this chapter.

In our stury we found similar results in case of sterlet spermatozoa for flagellar beat frequency – 52 Hz in contrast to 48–42 Hz in Tsvetkova et al., (1996) or 55–60 Hz in Billard et al. (1999). In pike spermatozoa, we observe a beat frequency ranging 30 Hz but no data were found in literature in accordance to this parameter. However, the values of wave amplitude for pike and sterlet are ranging 4.94 ± 0.07 μm and 7.56 ± 0.14 μm respectively. Closely related to the wave amplitude the number of waves along flagella. In our study, the values of 9.43 ± 0.3 μm and 7 ± 0.16 μm are observed for pike and sterlet respectively. These values are to be compared with those published for spermatozoa of other species: 9–12 μm in turbot (Cosson et al., 2008), 10.5 μm in sea bass and 21 μm in European hake (Cosson, 2010).

Ultimately, our results will lead to better understanding of flagellar mechanics (Gibbons, 1983; Inaba, 2007), for example, the generation of three-dimensional movement (rotation of the spermatozoon) specific to spermatozoa of some fish species (Boryshpolet et al., 2013b), such as the helical movement of the eel spermatozoon (Gibbons et al., 1981; Wooley, 1998).

Use of high-speed video allows the recording of successive positions of a flagellum during a single cycle or several full beat cycles. Such data will lead to more accurate modeling of flagellar behavior during the fish spermatozoon motility period and provide a deeper understanding of the basis of sperm motility (Gillies et al., 2013). In addition, these data also allow the development of new approaches for computer simulation of fish spermatozoon flagellum movement. (Boryshpolet et al., 2013b).

Also, use of high-speed video for practical applications could be beneficial as it generates complementary and detailed analysis of cryopreserved sperm, in addition to sperm motility parameters. Flagella damage due to freezing treatment as well as appearances of additional damage during their motility period have been described so far mostly on fixed samples using electron microscope. Instead, the high-speed video records give us opportunity to achieve such analysis in real time, typically during the period when spermatozoa are motile. Therefore, this method can be as well a new field for collaboration between fishermen and laboratory specialist.

Paper III. Effect of water temperature on egg incubation and quality of newly hatched larvae of northern pike (*Esox lucius* L.)

Optimal water temperature for egg development of northern pike ranges from 6–10 °C (Bondarenko at al., 2014). At the beginning of egg incubation, fishery facilities usually use an initial water temperature of 8 to 10 °C that is gradually increased to 12–14 °C toward the end of the incubation period (Lillelund, 1967). According to our data, ontogenesis hypothetically ceased in northern pike at approximately 3.3 °C, which may explain the significantly lower hatching rate (18%) and egg survival in eggs incubated at 3 °C. Lillelund (1967) and Hokanson et al. (1973) monitored the development of northern pike eggs at a temperature range of 3.7–24 °C. A greater than 80% survival rate of embryos was obtained at water temperatures from 6.4–17.7 °C. When water temperature was reduced to 3 °C, the hatching rate was 9%. According to Swift (1965), the optimal incubation temperature is 9 °C which gave a hatching rate of northern pike eggs at 60–80%. Lillelund (1967) incubated northern pike eggs at 5.8 °C and obtained a hatching rate of 70%.

The duration of incubation in northern pike is dependent on water temperature. At 8–10 °C, the incubation period is 110–140°d, which is 11 to 17.5 days. At 14 °C, the period is 85°d (6 days) and at a temperature of 18 °C is 61°d (3.42 days) (Hokanson et al., 1973).

The length of the hatching period is also largely dependent on water temperature (Drozd et al., 2009). Based on our results, the period can extend to 4 days at a water temperature of 6 °C and less than a day (22 hours) at 18 °C. Ontogenesis can be accelerated by a increase in water temperature of 5–7 °C. It is advisable to carry out such an increase in water temperature when approximately 10% of larvae have hatched. In the course of the intensive hatching of embryos, it is very important to remove egg surface membranes and dead embryos at regular intervals of 2 to 6 h (Bonisławska et al., 2011).

Size of newly hatched larvae and yolk sac size are related to water temperature (Blaxter, 1969; Trabelsi et al., 2013). This suggests that yolk sac utilization is optimal at the temperature that produces the largest larvae. Larval size is generally considered an important quality indicator, with larger larvae being stronger, better swimmers, and less susceptible to damage (Blaxter, 1969).

In this study, the highest quality was recorded in larvae incubated at water temperatures of 6 and 10 °C: larva total length (TL) = 9.33 ± 0.28 and 10.61 ± 0.37 mm; resistance to osmotic stress (OS) after 90 min of exposure of saline solution = 92 ± 3% and 80 ± 4 %; frequency of normally developed larvae (FNL) = 89.7 ± 3.62% and 93.8 ± 3.17; yolk sac volume (YsV) = 3.3 ± 0.66 μl^3 and 3.04 ± 0.42 μl^3, respectively. Larvae incubated at water temperatures of 3, 14, and 18 °C were characterized by the lowest values of variables: TL = 9.67 ± 0.36; 9.83 ± 0.86; 9.21 ± 0.29; OS = 54 ± 3; 72 ± 3; 76 ± 3%; FNL = 23.8 ± 4.14; 81.8 ± 2.12; 87.1 ± 2.42% and YsV = 3.41 ± 0.44; 3.53 ± 0.4; 3.89 ± 0.45 μl^3, respectively.

Paper IV. Reproduction and rearing of advanced northern pike (*Esox lucius* L.) fry

The aim of this report was to describe and elucidate new procedures of controlled reproduction of broodstock in northern pike. The procedures of artificial fertilization and egg incubation and methods of obtaining high quality and viable northern pike larvae were described in detail (Bondarenko et al., 2013). A further objective of this handbook was to describe effective methods of production of juvenile northern pike to the stage of advanced fry of total length (TL) 30–50 mm (Balik et al., 2006). Juveniles can be subsequently reared to market-size fish of body weight ranging from 1 to 3 kg (Szczepkowski, 2009). This publication could help Czech commercial fisheries to expand the efficient production of northern pike, which will diversify the Czech fishery production.

The production of marketable northern pike by the Czech fishery as well as in all of Central and Eastern Europe has been considerably limited both its biology and by insufficient or suboptimal farming conditions (Lusk and Krčál, 1982). Biological characteristics of northern pike limiting its rearing include a lengthy spawning period in hormonally untreated broodfish; production of a low volume of sperm (Billard, 1996; Hulák et al., 2008); contamination of stripped sperm with urine or blood (Billard, 1996; Hulák et al., 2008); use of testicular sperm, resulting in a loss of brood males (Lahnsteiner et al., 1998); variable egg quality (Švinger et al., 2012); sensitivity of fertilized eggs and embryos to manipulation and inadequate incubation (Berka and Hamáčková, 1980); extreme cannibalism (Kucska et al., 2005; Szczepkowski, 2009); and high territoriality of the fish beginning in juvenile stages (Berka and Hamáčková, 1980; Lusk and Krčál, 1982).

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ENGLISH SUMMARY

Reproduction and intensive juvenile culture in pike (*Esox lucius* L.)

Volodymyr Bondarenko

Fish hatcheries do not always provide optimal conditions for controlled reproduction and production of high-quality juvenile in northern pike. This study was undertaken to optimize reproduction and intensive juvenile culture of northern pike (*Esox lucius* L.) under controlled conditions.

The efficacy of traditional treatments for induction and synchronization of ovulation in northern pike (carp pituitary or ambient outdoor conditions) was compared to that of sGnRH-a at 50 and 100 μg kg^{-1} with or without Freund´s incomplete adjuvant and dopamine inhibitor (metoclopramide, 8 mg kg^{-1}). Results indicated that GnRH analogues were ineffective under the protocol used in the experiment. Ovulation was observed in females treated with carp pituitary (3 mg kg^{-1}) and in those held in ambient conditions. These results showed that carp pituitary remains the only effective hormone treatment for inducing and synchronizing ovulation in northern pike.

Sperm motility parameters were compared in northern pike and sterlet (*Acipenser ruthenus* Brandt). Analysis was accomplished via high speed video microscopy complemented with CASA analysis. This work described recently developed methods of recognizing and quantifying sperm quality. The data obtained will lead to more accurate modeling of flagellum behavior during the motility period and provide a deeper understanding of basis of spermatozoa motility. In addition, these data also allow the development of new approaches for computer simulation of fish spermatozoa flagellum movement.

The effect of water temperature on duration of embryo ontogenesis and hatching period; fertilization and development to the gastrula stage; hatching rates; and quality of larvae including larval size, development, and resistance to osmotic stress under controlled conditions for northern pike were investigated. Results indicated that the optimal temperature range for northern pike embryo development under controlled conditions is 6–10 °C. Northern pike embryonic development hypothetically ceases at approximately 3.3 °C.

In the certified methodology the basic aspects of controlled reproduction of northern pike was described and explained, including optimization of broodstock management. An objective of the certified methodology was to describe methods for producing high quality juveniles. The aim of this methodology is to provide information to enable European fish farmers to increase the efficiency and expand production of northern pike under their unique production conditions.

The most important outcomes of this study may be summarised as:

1. Manuscript "Evaluation of treatments for induction of ovulation in northern pike (*Esox lucius* L.)," in which results of comparison of hormone treatments for induction and synchronisation of ovulation in northern pike females were reported. The most effective hormone treatment was confirmed as injection with carp pituitary at 3 mg kg^{-1}. We also found that egg quality may be highly dependent on pH of ovarian fluid.
2. Manuscript "Comparison of swimming flagella characteristics in northern pike (*Esox lucius* L.) and sterlet (*Acipenser ruthenus* Brandt) spermatozoa using CASA analysis and high speed video microscopy." Described a novel method of analyses of flagellar movement and spermatozoa motility, which makes possible the description and analysis of spermatozoa movement in higher detail than does the traditional CASA system.

3. Manuscript accepted for publication in *Journal of Applied Ichthyology* "Effect of water temperature on egg incubation time and quality of newly hatched larvae of northern pike (*Esox lucius* L.)" which identified water temperature associated with the highest hatching rate for northern pike egg incubation, the most complete yolk sac resorption, the lowest levels of larva malformation, and the highest resistance to osmotic shock.
4. Publication of the handbook *Reproduction and Rearing of Advanced Fry of Pike (Esox lucius L.)*, which describes successful techniques of artificial reproduction of northern pike, including egg fertilization and incubation, and effective methods of producing high-quality northern pike juveniles to the advanced fry (TL 30–50 mm).

CZECH SUMMARY

Reprodukce a intenzivní chov juvenilních ryb štiky obecné (*Esox lucius* L.)

Volodymyr Bondarenko

Rybí líhně ne vždy nabízí ideální podmínky pro kontrolovanou reprodukci štiky obecné (*Esox lucius* L.) a produkci kvalitních larev ryb. Úkolem této práce bylo optimalizovat reprodukci a intenzivní chov juvenilních ryb štiky v podmínkách řízené akvakultury.

Efektivita tradičních hormonálních přípravků (kapří hypofýza) byla porovnávána s vyššími dávkami GnRH-a (50 a 100 μg kg^{-1}) v kombinaci nebo bez kombinace Freundova inkompletního adjuvancia a dopaminergního inhibitoru (metoclopramide, 8mg kg^{-1}). Tyto přípravky byly použity k indukci a synchronizaci ovulace generačních ryb štiky. Výsledky ukazují, že analogy GnRH byly neefektivní. Ovulace jiker byla pozorována u jikernaček injikovaných kapří hypofýzou (3 mg.kg^{-1}) a u ryb, které nebyly nijak hormonálně stimulovány a byly drženy v přírodních podmínkách výtěrových rybníčků. Tyto výsledky ukazují, že kapří hypofýza je jediným účinným hormonálním přípravkem, pomocí kterého je možné indukovat a synchronizovat ovulaci u štiky obecné.

Byly porovnány parametry pohyblivosti spermií u štiky obecné a jesetera malého (*Acipenser ruthenus* Brandt). Analýzy byly provedeny za pomoci vysokorychlostního video mikroskopování a doplněno o CASA analýzu. Tato práce popisuje a porovnává nové moderní a detailnější způsoby hodnocení a kvantifikace kvality spermatu. Získaná data povedou k přesnějšímu modelování chování bičíku spermie v průběhu období motility spermií a lepšímu porozumění základům pohybu spermie u ryb. Tyto data také umožní vývoj nových počítačových programů sloužících k simulaci pohybu bičíku spermie u ryb.

V průběhu této Ph.D. práce byl v podmínkách kontrolované akvakultury také zkoumán efekt různých teplot vody na efektivitu inkubace jiker štiky zahrnující – embryonální ontogenezi, líhnivost, a kvalitu larev v podobě velikosti larev, vývoj a odolnost larev při osmotických šocích. Výsledky ukazují, že ideální teplotní rozmezí pro kontrolovanou inkubaci a embryonální vývoj štiky je teplota vody mezi 6–10 °C. Dále se ukázalo, že embryonální vývoj štiky je hypoteticky přerušen při 3,3 °C.

V této certifikované metodice jsou popsány a vysvětleny základní aspekty kontrolované reprodukce štiky zahrnující optimalizaci managementu generačních ryb. Další částí této práce bylo popsat metody produkce vysoce kvalitních juvenilních ryb štiky obecné. Cílem této práce je pomoci evropským, zvláště pak českým rybářům, ke zvýšení efektivity produkce štiky v podmínkách kontrolovaného chovu.

Nejvýznamnější výsledky této práce jsou

1. Publikace certifikované metodiky "Reprodukce a chov rychleného plůdku štiky obecné (*Esox lucius* L.)", která popisuje nejvhodnější způsob umělé reprodukce generačních ryb, oplození a inkubace jiker a efektivní metodu produkce vysoce kvalitních juvenilních ryb do stádia rychleného plůdku štiky obecné (celková délka 30 50 mm).
2. V rukopisu "Hodnocení různých způsobů indukce ovulace u štiky obecné (*Esox lucius* L.)" byly popsány různé hormonální přípravky, které mohou být použity k docílení ovulace a její synchronizaci u jikernaček štiky. Jako nejefektivnější se ukázalo použití kapří hypofýzy v dávce 3 mg kg^{-1}. Také jsme zjistily, že kvalita jiker je zřejmě vysoce závislá na pH ovariální tekutiny.

3. Rukopis "Porovnání charakteristik bičíku u spermatu štiky obecné (*Esox lucius* L.) a jesetera malého (*Acipenser ruthenus* Brandt) pomocí CASA analýzy a vysokorychlostního mikroskopování" popisuje nové moderní metody pozorování a analýzy pohybu bičíku spermie. Díky této modení metodě je možné popsat a analyzovat pohyb spermií mnohem detailněji než pomocí klasického CASA systému.
4. Článek akceptovaný k publikaci v Journal of Applied Ichtyology "Efekt teploty vody na inkubaci jiker a kvalitu čerstvě vylíhnutých larev štiky obecné (*Esox lucius* L.)" popisuje optimální teplotu inkubační vody, která vede k nejvyšší líhnivosti larev, resorbci žloutkového váčku, nejnižšímu výskytu malformit a nejvyšší odolnosti k osmotickým šokům u larev štiky.

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LIST OF PUBLICATIONS

PEER-REVIEWED JOURNALS WITH IF

Bondarenko, V., Drozd, B., Policar, T. Effect of water temperature on the egg incubation and quality of newly hatched larvae in northern pike (*Esox lucius* L.). Journal of Applied Ichthyology (accepted)

Bondarenko, V., Podhorec, P., Svinger, V., Policar, T. Evaluation of different treatments for induction of ovulation in Northern pike (*Esox lucius* L.). (manuscript)

Bondarenko, V., Policar, T., Boryshpolets, S., Blecha, M., Cosson., J. Comparison of swimming flagella characteristics in Northern pike (*Esox lucius* L.) and sterlet (*Acipenser ruthenus*) spermatozoa by using of CASA analysis and high speed video microscopy. Cell Biology International. (submitted)

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PEER-REVIEWED JOURNALS WITHOUT IF

Švinger, V.W., **Bondarenko, V.,** Kallert, D.M., Policar, T., 2012. Influence of two ways of pituitary administration on egg quality in Northern pike (*Esox lucius* L.). / Vliv dvou metod hypofyzace na kvalitu jiker u štiky obecné (*Esox lucius* L.). Bulletin VÚRH Vodňany 48: 21–23.

APPLICATION OF METHODOLOGIES, PATENTS, PILOT PLANT, VERIFIED TECHNOLOGY

Bondarenko, V., Křišťan, J., Švinger, V., Policar, T., 2013. Reproduction and rearing of advanced northern pike (*Esox lucius* L.) fry. / Reprodukce a odchov rychleného plůdku štiky obecné (*Esox lucius L.*). Edice Metodik, FROV JU, Vodňany, no. 144, 43 pp.

ABSTRACTS AND CONFERENCE PROCEEDINGS

Bondarenko, V., Drozd, B., Podhorec, P., Kristan, J., Policar, P., 2013. Effect of various temperature regimes on early life history in northern pike (*Esox lucius* L.). Aquaculture Europe 2013, Trondheim, Norway, August 10–12: p. 78–79.

Bondarenko, V., Podhorec, P., Svinger, V., Policar, T., 2013. Evaluation of hormonal preparations for stimulation of ovulation in northern pike (*Esox lucius* L.). Diversification in Inland Finfish Aquaculture II (DIFA II). Vodnany, Czech Republic, September 24–26: p. 102.

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Bondarenko, V., Podhorec, P., Svinger, V., Policar, T., 2012. Effect of different spawning time on fertilization, hatching rate and embryo quality in northern pike, *Esox lucius* (L.). Domestication in Finfish Aquaculture Olsztyn, Poland, October 23–25: p. 68.

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