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Evaluation of Treatments for Induction of Ovulation in Northern Pike (*Esox lucius* L.)

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Abstract

Various induction protocols were compared with the aim to reach ovulation in northern pike (*Esox lucius* L.). The effectiveness of salmon D-Arg^{6}Pro^{9}NEt-GnRHa at two doses in combination with various additives were tested: 50 and 100 µg kg^{-1} (groups GnRHa 1 and 2), GnRHa at 50 and 100 µg kg^{-1} in combination with 8 mg kg^{-1} metoclopramide (groups 3 and 4), GnRHa at 50 and 100 µg kg^{-1} emulsified in Freund´s incomplete adjuvant (FIA) (groups 5 and 6), and GnRHa at 50 and 100 µg kg-1 emulsified with FIA 8 mg kg^{-1} and metoclopramide (groups GnRHa 7 and 8). These treatments were compared to indoor controlled conditions (Group ICC, injected with saline solution, without hormonal treatment) and two traditional treatments: 3 mg kg^{-1} carp pituitary (Group CP) and ambient outdoor conditions (Group AOC, injected with saline solution only, without hormonal treatment).

All fish in Group CP and 70% of those in Group AOC ovulated. The latency periods for Group CP was 96±4 h and 264±58 h for Group AOC. One Group-3 female ovulated spontaneously 108 h post-injection, whereas none of the other GnRHa injected or Group ICC fish ovulated. The pseudogonadosomatic index was 19.3±5.9%; 18.5% and 17.8±7.5% in the AOC, Group 3 and CP, respectively. The fertilization rate reached 88.6±4.5%; 85.5±12.0% and 66.0±13.7% in AOC, Group 3 and CP, respectively. The hatching rate was 68.6±9.9%; 65.5±7.5% and 54.4±8.0% in AOC, Group 3 and CP, respectively. The ovarian fluid pH was significantly higher (8.27±0.03) in AOC compared to CP group (8.11±0.02) (P>0.05). Ovarian fluid of the spontaneously ovulated Group-3 fish had a pH of 8.35. There were no significant differences in egg size and weight among successfully reproduced groups – AOC and CP (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 (Hazman and Gökçek, 2014) and is also a popular food fish and in sport angling (Bondarenko *et al*., 2015). Northern pike have synchronous development of oocytes and they spawn annually in the spring at water temperatures between 5 and 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 (Szabó, 2001). The main drawbacks to this type of production are disparity in female maturation stages and a total reliance on environmental factors. These limitations reduce the possibilities for production of same-age larvae that are needed for successful subsequent culture (Bondarenko *et al*., 2015). 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 a pond environment or captured too early during the spring do not mature in hatchery conditions (Szabó, 2008). A lack of necessary 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 (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, to date, studies have shown that replacement of CP by GnRHa with or without dopamine inhibitors has not been effective for induction of ovulation in pike (Billard and Marcel, 1980; Pecha *et al*., 1992; Szabó, 2001, 2003, 2008). Ineffectiveness of GnRHa for induction of pike ovulation might be 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 enhance efficacy and allow a lower dose and thereby increase the potency of antivenins (Pratanaphon *et al*., 1997) and reduce vaccine costs (Singh and O'Hagan, 1999). The mechanism of action for Freund´s incomplete adjuvant (FIA) is the promotion of the formation of depots of antigen at a site of immunization (Guy, 2007).

Freund´s incomplete adjuvant has been demonstrated to be an efficient carrier of GnRHa in rainbow trout *Oncorhynchus mykiss* (Arabaci *et al*., 2004; Vazirzadeh *et al*., 2008; Svinger *et al*., 2013a) and chum salmon *Oncorhynchus keta* (Park *et al*., 2007).

The objective of the present study was to compare efficacy of traditional induction methods and synchronization of ovulation in northern pike; natural maturation, in or out-of-doors were compared with CP induction and ovulation with using salmon D-Arg^6Pro^9NEt-GnRHa at doses of 50 and 100 μg kg^{-1} with or without FIA and the dopamine inhibitor (metoclopramide).

Materials and Methods

Sexually mature northern pike females (age 3 years, body weight [BW] = 2852±856 g and total length [TL] = 695±91 mm) were collected from production ponds of Fishery Nove Hrady Ltd. (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) were selected based on the germinal vesicle migratory position (Szabó, 2003) and they were randomly divided into eleven groups; each group contained ten females. Nine hormone - treatment groups (CP; GnRHa Groups 1-8) and one group - indoor controlled conditions (ICC) was injected with physiological solution with dose 1 ml kg^{-1} (negative control). Each group was 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, the temperature was increased to 10.5±0.2°C; oxygen conditions remained the same. An eleventh experimental group of females were injected with physiological saline (dose 1 ml kg^{-1}) and kept under ambient outdoor conditions (AOC group) in a 500 m^2 pond; littoral vegetation covered approximately 100 m^2 of pond area. Water temperature fluctuated during the day from 6°C to 12°C. A single group of 110 matured males (age 3 years, BW = 1250±250 g and TL = 531±52 mm) was maintained in a single 10,000 L plastic tank under controlled conditions similar to the females. Fish from all groups were kept under a natural photoperiod regime for geographic location of Central Europe.

All fish in all experimental groups were intraperitoneally injected with total volume (1 ml kg^{-1}) of solution to induce of egg 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 a dose of 3 mg kg^{-1}.

GnRHa groups 1 and 2 were injected with salmon D-Arg^6Pro^9NEt-GnRHa (Bachem AG, Germany) at a dose of 50 and 100 μg kg^{-1}, respectively. GnRHa groups 3 and 4 were injected with salmon D-Arg^6Pro^9NEt-GnRHa at a dose of 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^6Pro^9NEt-GnRHa emulsified in FIA at a dose of 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 (Svinger *et al*., 2013a). Groups GnRHa 7 and 8 were treated with salmon D-Arg^6Pro^9NEt-GnRHa at a dose of 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 over 14 days (336 h) post-injection. Success was defined as the percentage of females that ovulated within 336 h post-injection and latency period as time from injection to ovulation.

When ovulation was detected, eggs were manually stripped. The pseudogonadosomatic index (pGSI) was calculated according to the formula (weight of stripped egg/female BW before stripping) × 100 (Svinger *et al*., 2013b). 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 with inoLAB 720 pH meter (WTW, 823 62 Weilheim, Germany) in five areas of the freshly stripped egg mass; average pH was calculated from these five independent measures.

Three 5-g samples of eggs (approximately 800 eggs) were collected from each ovulated female and immediately fertilized with pooled sperm stripped from three males. Sperm was collected according to Bondarenko *et al*. (2015). 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 (Bondarenko *et al*., 2015), a sample of 200 eggs was collected from each batch. Three egg samples from each female were placed into separate transparent 2.5 l incubators (Svinger *et al*., 2013c) for incubation and determination of fertilization and hatching rate (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 of 13.0±0.2°C.

All data related to reproduction of two successfully reproduced groups (AOC and CP groups) were analysed with Statistica 9 (StatSoft, Tulsa, USA). Differences in ovulation success, latency time, 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 in two mentioned experimental groups. A significance level (α) of 0.05 was applied to all tests. The data are presented as the mean±SEM (standard error of mean). Beside groups AOC and CP, mean value of ovulation success, latency period, pGSI, fertilization and hatching rate, egg size and weight and pH of ovarian fluid were found in one spontaneous female from group 3. However, these limited data were not statistically compared with successfully treated and ovulated females from mentioned groups.

Results

Response to different experimental induction of ovulation, latency period, pseudogonadosomatic index, fertilization rate at the gastrula stage, hatching rate, size and weight of eggs, and pH of ovarian fluid are summarized in Table 1.

No ICC fish ovulated, nor in any of the GnRHa treatment groups 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}). This female ovulated spontaneously, and possibly not as a result of the treatment. All females in Group CP and 70% in Group AOC ovulated. The latency period in Group AOC was significantly longer at 264±58 h (mean±SD) than in Group 3 (108 h) and Group CP (96±4 h). The pGSI did not significantly differ between the two groups (AOC = 19.3±5.9 % and CP = 17.8±7.5 %). The pGSI for the spontaneously ovulated female was 18.5%. The mean fertilization rate (FR) and hatching rate (HR) was significantly higher in Group 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%). Fertilization and hatching rates in one female of group 3 were 88.6±4.5% and 65.5±7.5%, respectively. There was no significant difference in egg size (2.7±0.16 – 0.21 mm) and weight (6.11±0.35 – 6.15±0.26 mg) between group AOC and CP, respectively. Similar size (2.8±0.25 mm) and weight (6.4±0.38 mg) were found in eggs of female from Group 3. The ovarian fluid pH for groups CP and AOC were 8.11±0.02 and 8.27±0.03, respectively. The ovarian fluid pH in one spontaneously ovulated female from group 3 was 8.35. Positive effect between the pH of ovarian fluid and fertilization rate correlated in northern pike (correlation coefficient $R^2 = 0.9593$; Figure 1).

Discussion

Single injections (10-50 µg kg^{-1}) with mammalian GnRH analogues have been able to overcome reproductive limitations related to captivity and induce ovulation in various fish species (Mikolajczyk et al., 2008). However, neither mGnRHa or sGnRHa have been effective in ovulating northern pike (Billard and Marcel, 1980; Szabó, 2003) and these observations were verified in the present study. Northern pike is a cold water spawning species and may require a prolonged time with elevated luteinising hormone (LH) levels to complete the final stages of gametogenesis. Similar ineffectiveness has been reported in another cold water species such as 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; Podhorec and Kouril, 2009) that is necessary to complete final oocyte maturation and ovulation in northern pike. Other factors that can influence the progression of gametogenesis are handling stress (De Montalembert *et al*., 1978) and stage of gonad development (Billard and Marcel, 1980). One solution to this inadequate release profile of GnRHa could be the utilisation of GnRHa delivery systems that stimulates sustained elevation of plasma LH and therefore induces the natural progression of plasma steroid increase that is associated with FOM and ovulation (Mylonas and Zohar, 2001).

Amplification of GnRHa potency with a dopamine inhibitor is commonly used in artificial propagation of some fish such as Cyprinidae, but these benefits were not evident in northern pike (Szabó, 2003). Our results confirmed this information. In the CP group, all females ovulated on day 4 post-

Table 1. Effectiveness of treatments [ambient and controlled environment, carp pituitary at 3 mg kg^{-1}, and sGnRHa ($DArg^6Pro^9Net$) 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 (%)	Latency period (h)	pGSI (%)	Fertilization rate in gastrula stage (%)	Hatching rate (%)	Size of eggs (mm)	Weight of eggs (mg)	pH of ovarian fluid
ICC	Saline solution 1 ml kg^{-1}	0							
AOC	Saline solution 1 ml kg^{-1}	70	264^b± 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 ($DArg^6Pro^9Net$) 50 µg kg^{-1}	0							
GnRHa group 2	sGnRHa ($DArg^6Pro^9Net$) 100 µg kg^{-1}	0							
GnRHa group 3	sGnRHa ($DArg^6Pro^9Net$) 50 µg kg^{-1} + Met 8mg kg^{-1}	10	108	18.5	88.6±4.5	65.5±7.5	2.8±0.25	6.4±0.38	8.35±0.03
GnRHa group 4	sGnRHa ($DArg^6Pro^9Net$) 100 µg kg^{-1}+ Met 8mg kg^{-1}	0							
GnRHa group 5	sGnRHa ($DArg^6Pro^9Net$) 50 µg. kg^{-1} + FIA	0							
GnRHa group 6	sGnRHa ($DArg^6Pro^9Net$) 100 µg. kg^{-1} + FIA	0							
GnRHa group 7	sGnRHa ($DArg^6Pro^9Net$) 50 µg. kg^{-1} + Met 8mg kg^{-1} + FIA	0							
GnRHa group 8	sGnRHa ($DArg^6Pro^9Net$) 100 µg. kg^{-1}+ Met 8mg kg^{-1}+ FIA	0							

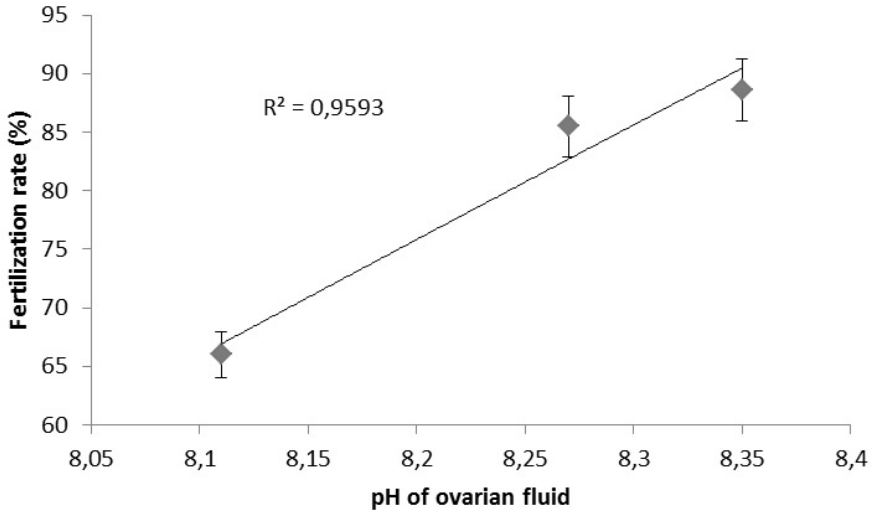

Figure 1. Correlation between pH of ovarian fluid and fertilization rate in northern pike (*Esox lucius* L.).

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).

Szabó *et al*. (2014) found similar ovulation efficacy for silver carp pituitary compared to carp pituitary in northern pike. These authors recommended to wildly apply silver carp pituitary for induction of ovulation in different fish species.

In the AOC group, 70% of females ovulated during the experiment. However, De Montalembert *et al*., (1978) showed that non-stimulated captive females might not ovulate for various reasons: stress, photo-thermal regimes, lack of spawning substrate. The present study also verified that females kept under indoor conditions rarely ovulate. Ivanova (2009) reported that pike females without hormonal treatment can spawn naturally 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 was low when females ovulated during 264±58 hours (11±2.4 days). Duration of spawning period of this group was significantly longer compared to group CP (96±4 hours or 4±0.17 days). The synchronization of ovulation in carnivorous fish species is important for managed reproduction during nursing, since a protracted spawning period may increase cannibalism during larval or juvenile culture because of age and size differences of larvae (Ivanova, 2009). Therefore applications of hormone treatments are widely used in different carnivorous fish species such as pikeperch – *Sander lucioperca* (Křišťan *et al*., 2013) or perch (Policar *et al*., 2008).

The pGSI in our study were similar to those reported by Szabó, (2003) and Szabó *et al*. (2014). We did not find significant differences in pGSI between two treatment groups. Higher fertilization and hatching rates were found in group AOC than in group CP (Table 1). One spontaneously ovulated female from group 3 had higher fertilization and hatching rates, however, these data were limited and not comparable to other groups. Generally, hatching and fertilization rates found in our study were higher than those reported by other authors (De Montalembert *et al*., 1978; Billard and Marcel, 1980; Horváth, 1983; Szabó, 2001; 2003; 2008).

Egg weight and size in groups AOC and CP were same and similar to those published by Murry *et al*. (2008) for weight and by Benzer *et al*. (2010) for diameter.

The pH is considered one of the main indicators of ovarian fluid quality, which affects egg quality (Samarin *et al*., 2015). No information on pH of ovarian fluid has been published in 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). The pH can be lowered from deteriorating eggs being resorbed release material (6.47 according to Dietrich *et al*. (2007)) into 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 pH in the ovarian fluid ranged between 8.11 and 8.35 and was higher in one spontaneous females from group 3. Positive correlation of pH in ovarian fluid on fertilization rate in gastrula stage was found during our study but this is based on a low number of observations. Nevertheless, our results suggest ovarian fluid pH can be important for egg quality in northern pike, as with other fish species (Samarin *et al*., 2015). 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 egg fertilization of northern pike.

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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