Arch. Pol. Fish.	Archives of Polish Fisheries	Vol. 16	Fasc. 4	341-354	2008

A REVIEW OF THE ARTIFICIAL REPRODUCTION OF ASP, *ASPIUS ASPIUS* (L.), AND NASE, *CHONDROSTOMA NASUS* (L.)

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ABSTRACT. This paper presents the results of studies of the reproductive biotechnology of asp, *Aspius aspius* (L.), and nase, *Chondrostoma nasus* (L.), supplemented with unpublished materials of the authors. Reproducing these species under controlled conditions without hormonal preparations is not possible. The most frequently used preparations are carp pituitary homogenate (CPH) and gonadotropin releasing hormone analogue (GnRH) combined with a dopamine receptor antagonist. In both asp and nase, the highest percentage of ovulating females, the highest fecundity, and the highest embryo survival were attained after stimulation with Ovopel and Ovaprim. However, the results obtained with CPH were not much worse. The semen of both species was cryopreserved, which permits the long-term safeguarding of sperm genetic material.

Key words: RHEOPHILIC CYPRINIDS, ENDANGERED SPECIES, SPAWNING, HORMONAL STIMULATION, CRYOPRESERVATION

INTRODUCTION

Among the inland freshwater fish species of Europe, rheophilic cyprinids are one of the most sensitive to changes in the environment that stem primarily from constructions on rivers and pollution. Systematic studies of the rivers of central Poland indicate there are permanent access limitations for many rheophilic fish including ide, *Leuciscus idus* (L.), dace, *Leuciscus leuciscus* (L.), chub, *Leuciscus cephalus* (L.), barbel, *Barbus barbus* (L.), nase, *Chondrostoma nasus* (L.), or gudgeon, *Gobio gobio* (L.) (Penczak et al. 1998, 2004, Penczak and Kruk 2000). Negative changes also effect roach, *Rutilus rutilus* (L.), which is one species that is widely recognized as being resistant to anthropogenic stress. Transformations in the natural environment mean that many local populations of both nase, *Chondrostoma nasus* (L.), and asp, *Aspius aspius* (L.), are currently endangered.

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DOI 10.2478/s10086-008-0022-4

Nase were once abundant in rivers inhabiting them from the lower grayling zone to the upper sections of the bream zone (Witkowski 1992, Lusk and Halačka 1995, Peňáz 1996, Keckeis et al. 1997). However, transverse constructions and river regulation, changing aquatic relationships, habitat degradation, and overexploitation by recreational fisheries have caused declines in the abundance of local populations (Peňáz 1996, Penczak and Kruk 2000, Schiemer et al. 2003, Penczak et al. 2004). Currently, nase is one of the most threatened of rheophilic cyprinids in Poland (Przybylski et al. 2004). Increasing demand for stocking material of this species (Kujawa 2004, Spurny et al. 2004, Wojda 2004, Wolnicki 2005, Mickiewicz et al. 2007) has generated the need for developing reproduction biotechnology under controlled conditions for this species (Keckeis 2001, Szabó et al. 2002, Schiemer et al. 2003, Żarski et al. 2008a).

The abundance of asp in Polish waters have also decreased, and like nase, there is increasing demand for stocking material of this species (Kujawa 2004, Wojda 2004, Wolnicki 2005, Mickiewicz et al. 2007). This rendered it necessary to optimize the propagation and rearing of rheophilic cyprinid species (Kucharczyk 2002, Kujawa 2004, Wolnicki 2005). The production of asp spawning material, including reproduction under controlled conditions as an element of active protection, has become an issue of interest to both scientists and those who exploit the waters (Cieśla 1998, Kujawa et al. 1998, Śliwiński 1998, Cejko et al. 2008, Żarski et al. 2008b). From one perspective, this stems from progressing environmental changes, limited abundance, and even the endangered status of rheophilic fish populations and species (McClure et al. 2008), including cyprinids (Penczak and Kruk 2000, Penczak et al. 2004), and, from the other perspective, the relatively high profitability of producing stocking material of asp and nase (Kupren et al. 2008).

One of the basic restoration activities is the development of biotechniques for reproducing and rearing juvenile fish (McClure et al. 2008). In the instances of asp and nase, there is a lack of comprehensive studies of the reproductive biotechnologies of these species. The aim of the current study was to analyze the most important issues linked to the reproduction of asp and nase under controlled conditions. This paper describes hormonal stimulation and its impact on the effectiveness of reproduction, removing adhesiveness from spawn, and the influence semen cryopreservation has on the species under discussion.

OBTAINING SPAWNERS

Initiating programs for restoration or the production of stocking material requires obtaining high quality reproductors. The limited numbers of natural populations has made it increasingly difficult to obtain spawners (Peňáz 1996, Penczak and Kruk 2000, Kujawa et al. 2006). One of the most common methods for obtaining reproductors is to catch fish in the natural environment (Cieśla 1998, Śliwiński 2000, Kujawa et al. 2001). Spawners caught with electric gear are usually in good condition (Cieśla and Konieczny 2000), while those caught in net gear often have external or internal injuries (Kujawa et al. 2001, 2006), which can cause problems during spawning and increase mortality. It has been demonstrated that net fishing does not always reduce the quality of reproductors (Kujawa et al. 2006). When treated gently, the number of females asp spawners caught in gill nets and which later produce eggs can be increased threefold in comparison to the ovulating females obtained by fisherman's using traditional methods, in which more care is taken with the gear than with the condition of the fish. This is significant since most rheophilic cyprinids reproductors are currently still obtained through the traditional method.

Obtaining greater quantities of nase and asp spawners is increasingly difficult, and this is particularly evident in the case of the latter species as it does not occur in large stocks and often undertakes spawning migrations singly or in small groups (Kujawa and Kucharczyk 1994). Jakucewicz and Jakubowski (1990) recommended obtaining asp, ide, chub, and barbel during the spawning period, but not at the spawning grounds. They also suggest that it is possible to catch the fish in fall and then hold them in ponds until spring. These authors also point out, however, that of the rheophhilic cyprinid species, only ide overwinters well. This contradicts with observations from the 1998-2008 period, when the survival of asp reproductors in small earthen ponds with high water flow rates (400 dm^3 min^{-1}) held each time from four to five months was from 80 to 100% (Kucharczyk, unpublished data). It is also possible to keep asp spawners under pond conditions and then reproduce them effectively (Śliwiński 1998). Cieśla (1998) compared the results of reproducing asp spawners obtained from the wild and then held for two years in ponds to that of asp reared from the larval stage in ponds and concluded that the latter were equally as suitable as reproductors and that the percentage of ovulating females in both groups was 100%.

Nase begin their spawning migration upstream when the water reaches a temperature of 4°C (Augustyn 2002). At the temperature range of 8-12°C, they begin to spawn under natural conditions, but spawning activity at the spawning grounds is brief and lasts from one to three days (Cieśla and Konieczny 2000, Keckeis et al. 2000). This makes obtaining fish that are not yet spent problematic (Cieśla and Konieczny 2000). The solution to this difficult problem appears to be rearing a brood stock of nase, which is possible to do in carp ponds. According to Lusk (1997), nase attains greater length growth in ponds than it does in the natural environment, and since in many fish species sexual maturation is linked with body size, pond rearing should shorten the period in which sexual maturity is achieved.

HORMONAL PREPARATIONS USED IN CONTROLLED REARING OF ASP AND NASE

Various hormonal preparations have been used in asp and nase reproduction. The most frequently used stimulation method was two injections of carp pituitary homogenate (CPH) (Table 1). Jakucewicz and Jakubowski (1990) recommended the administration of this hormone preparation to asp reproductors in two dosages, with a 24-hour period between injections. The dose of the first injection was from 0.4 to 0.6 mg kg^{-1} body weight (BW), and the second was from 1.2 to 3.5 mg kg^{-1} BW (Table 1). Satisfactory asp reproduction results were obtained using CPH in the first injection and human chorionic gonadotropin (hCG) in the second (Kujawa et al. 1997, Babiak et al. 1998). In subsequent years, the suitability of the preparation Ovopel (Unic-trade, Hungary) for the controlled reproduction of asp was confirmed (Kucharczyk and Szabó 1998, Śliwiński 2000, Kujawa et al. 2006, Żarski et al. 2008b). Ovopel is a granulated preparation containing mammalian analogue GnRH (D-Ala^6 Pro^9Net-mGnRH) and metoclopramide, a dopamine uptake antagonist (Horvath et al. 1997). One granule of Ovopel with an average weight of 25 mg contained 18-20 μg of D-Ala^6 Pro^9Net-mGnRH and 8-10 mg of metoclopramide. In asp reproduction, Ovopel is administered in one or two injections (Table 1). In the past few years the preparation Ovaprim (Syndel, Canada) has also been tested. This preparation contains 20 μg ml^{-1} of salmon analogue GnRH (D-Arg^6 Pro^9Net-sGnRH) and 10 mg ml^{-1} domperidon as the dopamine receptor antagonist. Asp spawners of both sexes were administered a single intra-peritoneal injection under the abdominal fin at a dose of 0.5 ml kg^{-1} BW (Żarski et al. 2008c).

TABLE 1

Dosages of hormonal preparations (recalculated per kg of body weight) and the results of controlled asp, *Aspius aspius* reproduction

First injection	Second injection	Ovulation (%)	Latency time (h)	Survival to eyed-egg stage (%)	Author
0.4-06 mg CPH	1.2-1.4 mg CPH	nd	48	nd	1
4.0 mg CPH		100	36	nd	2
0.5 mg CPH	3.5 mg CPH	100	12-28	nd	2
0.5 mg CPH	1.5 mg CPH	100	12-28	nd	2
0.4 mg CPH	1.6 mg CPH	100	30-38	nd	3*
4.0 mg CPH		50	38	nd	4
0.2 pellet Ovopel	1 pellet Ovopel	85-100	26-50	55-60	4
2 pellet Ovopel		63	42	44.5	5
0.5 ml Ovaprim		100	47	63.1	5

1 – Jakucewicz and Jakubowski (1990), 2 – Śliwiński (1998), 3 – Kucharczyk et al. (1998b), 4 – Śliwiński (2000), 5 – Żarski et al. (2008a)

** out-of-season reproduction, nd – not determined*

CPH and the GnRH analogue with or without a dopamine uptake antagonist were tested in the controlled reproduction of nase (Szabó et al. 2002, Żarski et al. 2008a) (Table 2). In contrast to asp, single injections were administered to this species. CPH was administered in doses of 3 to 6 mg kg^{-1} BW, while mammalian analogue GnRH was given at a dose of 20 μg kg^{-1} BW. Ovaprim was successfully applied in nase reproduction at a dose of 0.5 ml kg^{-1}.

TABLE 2

Dosages of hormonal preparations (recalculated per kg of body weight) and the results of controlled nase, *Chondrostoma nasus* reproduction

Dosage/hormone	Ovulation (%)	Latency time (h)	Fertilization (%)	Author
3 mg CPH	67	nd	69.1	1
6 mg CPH	67	nd	74.7	1
20 μg mGnRHa	0	nd	-	1
20 μg mGnRHa + 10 mg domperidon	83	nd	83.5	1
1 pellet Ovopel	89	51-75	nd	2
0.5 ml Ovaprim	100	51	nd	2

1 – Szabó et al. (2002), 2 – Żarski et al. (2008b)
nd – not determined

RESULTS OF THE HORMONAL STIMULATION OF ASP

There is no information regarding the possibility of obtaining fish ready for spawning at spawning grounds and obtaining gametes from them. Applying hormonal stimulation in asp spawners often resulted in a high percentage of ovulating females (Kucharczyk et al. 1998, Śliwiński 1998, 2000, Żarski et al. 2008a) (Table 1), in contrast to fish subjected only to manipulations of the environment, after which the females produced no eggs. Administering hormonal preparations of any kind resulted in ovulation rates ranging from 50 to 100% of the treated females. The latency time differed, however; with CPH it was from 12 to 38 hours (Kucharczyk et al. 1998, Śliwiński 1998, 2000), while with Ovopel and Ovaprim it was from 26 to 50 hours (Kucharczyk et al. 1998, Żarski et al. 2008a). Asp weighing about 1 kg that were stimulated with pituitary homogenate (with a total dose range of 2.0 to 4.0 mg kg^{-1}) produced eggs in quantities from 11.2-11.7% of female body weight. The relative working fecundity of these females ranged from about 27500 to about 29000 eggs (Śliwiński 1998). The relative fecundity of females of a mean body weight of 4 kg was determined at 50000-58500 eggs kg^{-1} (Żarski et al. 2008c). Differences in the relative fecundity were also noted depending on which hormonal preparation was administered. Lower fecundity was noted with Ovaprim (50000 eggs kg^{-1}), than with Ovopel (58500 eggs kg^{-1}) (Żarski et al. 2008c).

A few studies also undertook the analysis of the hormonal stimulation of asp males. The administration of either Ovopel or Ovaprim for the controlled reproduction of asp resulted in spermiation in 100% of the males (Żarski et al. 2008c). Cejko et al. (2008) determined the basic quality indicators and selected biochemical parameters of asp semen following spermiation stimulated with Ovaprim and Ovopel. Higher motility, sperm concentrations, and protein content in the seminal plasma were noted in the semen samples obtained after stimulation with Ovaprim. The osmotic pressure of the semen plasma, which was measured following the administration of Ovopel, was higher than when the males had been given Ovaprim. It was also confirmed that the enzyme activity in the seminal plasma of fish stimulated with Ovaprim was higher in comparison to the results obtained after administering Ovopel. The concentration of asp sperm ranged from 6 to 11 mld cm^{-3} (Babiak et al. 1998, Cejko et al. 2008).

RESULTS OF THE HORMONAL STIMULATION OF NASE

The first spawning of nase was conducted using mature spawners caught at spawning grounds, and these specimens were not stimulated to gamete production with hormonal preparations (Halačka and Lusk 1995, Kamler et al. 1998, Keckeis et al. 1996, 2001, Cieśla and Konieczny 2000, Augustyn 2002). In this case, the females were stripped of eggs at the spawning grounds just above the water (Cieśla and Konieczny 2000, Augustyn 2002) or the females were transported to the hatchery and the gametes were obtained there (Cieśla and Konieczny 2000). Halačka et al. (1997) determined the relative fecundity of nase from the eggs obtained from females maturing under natural conditions without the use of hormonal stimulation at 6100-41100 eggs kg^{-1}. The studies conducted by Żarski et al. (2008b) indicated that the relative fecundity of nase in which ovulation had been induced with hormonal preparations is close to the upper limit determined by Halačka et. al. (1997) within the range of 36800 eggs kg^{-1} (after stimulation with Ovaprim) to 40200 eggs kg^{-1} (with Ovopel). These data are concurrent, and the lower fecundity limit determined by Halačka et al. (1997) might result from the loss of some eggs by the females during capture or through spawning acts commenced in the natural environment.

Studies conducted by Szabó et al. (2002) and Żarski et al. (2008a) indicated that stimulating nase with CPH and a GnRH analogue with dopamine inhibitor influences the occurrence of ovulation, which was not noted in the control group of females. No ovulation was noted either after the administration of GnRHa alone without a dopamine inhibitor (Table 2). The comparison of the effects of various preparations indicated that the percentage of females ovulating after the administration of GnRH analogue with a dopamine inhibitor was higher than following treatment with CPH (Szabó et al. 2002). The embryonic survival following controlled reproduction three days after fertilization was 69 to 84%. However, embryonic survival at the moment of hatch of the larvae of the fish caught at the spawning grounds was 75-95% (Augustyn 2002). In this case oocyte selection was performed prior to fertilization. Eggs of poor quality were omitted and not fertilized. This is why it is difficult to compare the results obtained, and far reaching conclusions should not be drawn based on them.

There is very little published information regarding the reproductive biotechnology of male nase. It is possible to obtain milt without hormonal stimulation (Żarski et al.

2008a). The semen concentration of nase is higher than that of asp. Lahnsteiner et al. (2000) determined the sperm concentration of nase at 10-20 mld cm^{-3}.

REMOVING ADHESIVENESS AND INCUBATING SPAWN

Although there is not much adhesiveness on the eggs of nase and asp, it is sufficient for the eggs to stick to the walls of the incubation apparatuses and to each other. This leads to the mortality of a large portion of the eggs during incubation. Removing the adhesive from the oocytes of nase is not difficult and is usually accomplished by rinsing them several times with clean water (Augustyn 2002). Halačka and Lusk (1995) conducted a comparative study of this by removing the adhesiveness from nase eggs with seven different methods using water, diatomaceous earth (10 g dm^{-3}), talc (10 g dm^{-3}), talc and table salt (10 g talc and 4.5 g salt in 1 dm^3 water), fresh milk (250 cm^3 dm^{-3}), the Woynarovich method, and a modification of the Woynarovich method. In all instances, the procedure lasted 25 min, with the exception of the last, in which the procedure was extended by a 5 s tannin bath. The highest survival rate at hatch was noted after the adhesive removal procedure with the talc and salt solution (60-80%), while slightly lower survival rates were noted after the application of diatomaceaous earth (50-65%) or water (40-60%). The last method is the most popular in nase reproduction (Cieśla and Konieczny 2000, Augustyn 2002, Żarski et al. 2008c). When the gametes were obtained in the field, the fertilized eggs were transported to the hatchery where they were incubated in Weiss jars at a density of 1-2 dm^3 of swollen eggs per apparatus. The survival of embryos to hatching was high in the range of 75 to 95% (Augustyn 2002). During incubation of nase eggs, problems of indeterminate origin sometimes occur. Between the third and fourth days of incubation, the eggs disintegrate when the egg membrane breaks. According to Cieśla and Konieczny (2000) this process can occur in up to about 90% of the incubating eggs.

Water cannot be used to remove the adhesiveness of asp eggs, so a bath in a solution is required (Kujawa et al. 1997, Cieśla 1998, Kucharczyk et al. 1998, Śliwiński 1998, 2000). A modified version of the Woynarovich solution can be applied (40 g urea and 35 g NaCl dissolved in 10 dm^3 water). The adhesive removal procedure takes about an hour, during which the solution should be changed several times. Next, the eggs should be rinsed quickly twice (for 30 and 20 s) in a tannin solution (6-7 g dis-

solved in 10 dm^3 water), between which it is necessary to rinse them in clean water (Śliwiński 1998, 2000). Another recommended method to remove adhesiveness from asp eggs is to use a solution of talc (40-80 g) and table salt (10-20 g) recalculated for 10 dm^3 of solution. The bathing time should be from 30 min to 1 h (Kujawa et al. 1997, Cieśla 1998, Kucharczyk et al. 1998, Śliwiński 1998, 2000). It is also possible to combine the two methods. Firstly, the eggs are bathed in the Woynarovich fluid, and then in the talc and salt solution. However, when the talc solution is applied, the asp eggs sometimes hatch at a very early in their development even before the eyed-egg stage, which can have a negative impact on survival (Kucharczyk, unpublished data).

THERMAL CONDITIONS DURING REPRODUCTION AND SPAWN INCUBATION

The temperature range in which nase spawn in the natural environment was mimicked during reproduction under controlled conditions. The temperature following hormonal stimulation was maintained at 12°C which resulted in a shortened latency period to ovulation and helped to synchronize it (Szabó et al. 2002, Żarski et al. 2008a). The spawn incubation period is also strictly linked to temperature. The highest survival was noted at 13°C (97%), but the incubation period under these conditions was relatively long at a mean of 17.66 days, and larval hatching lasted for more than five day. Slightly lower embryonic survival (93%) was noted at a temperature of 16°C; however, incubation time was shortened by about a week and larval hatching lasted not much longer than three days. Embryo survival fell to 80% at a temperature of 19°C (Kamler et al. 1998).

Asp spawning in the natural environment is observed at water temperatures of 5 to 12°C (Gąsowska 1962, Gajgalas 1977). Under controlled conditions, hormonal injections are usually administered at a temperature range of 11-14°C (Kujawa et al. 1997, Kucharczyk et al. 1998, Targońska et al., unpublished data). The highest survival noted in embryonic asp during incubation until the hatch stage was confirmed at a temperature of 12.8°C, while at the higher temperature of 19°C or more, much of the spawn dies before reaching the eyed-egg stage (Kujawa et al. 1997). The temperature range that permits incubation and hatching was identified as 7-17°C. Simultaneously, incubation in water with a higher temperature (19°C or more) results not only in sharp declines in survival, but also in increased numbers of deformed individuals (Kujawa et al. 1997).

CRYOPRESERVATION OF SEMEN

Storing fish semen for long periods of time, even for many years, is essential for current propagation techniques, and is key in protecting the genotypes of endangered species. Asp semen was frozen in small balls on dry ice (Babiak et al. 1998). Among of six cryopreservation techniques applied, the highest embryonic survival to the eyed-egg stage was noted using 10% dimethyl sulfoxide (DMSO) with 0.3 and 0.6 M saccharose. Nase semen was cryopreserved using 10% DMSO with the addition of 0.5% glycine (Lahnsteiner et al. 2000, 2003). The semen was frozen with liquid nitrogen vapor, and then sperm motility was tested. It decreased to 49% in comparison to 77% in semen that had not been frozen. Decreases in the speed of sperm movement were also noted from 91 to 49 $\mu m\ s^{-1}$. The degree to which the concentration and freezing of sperm influenced embryonic survival was also determined (Lahnsteiner et al. 2003). In groups fertilized with unfrozen semen in which there were 0.6, 1.1, or 2.2 mln sperm per oocyte, the survival of embryos was 65, 73, or 71%, respectively. With cryopreserved semen, the decreased quantity of sperm per oocyte significantly lowered the percentage of hatched embryos to 29, 37, and 66%, respectively, at the same concentrations. In choosing the most appropriate variant, it was not noted that the semen freezing and thawing process had an impact on the survival of embryos at the hatch stage, while using semen with a lower sperm concentration resulted in an approximate twofold decrease in survival.

CONCLUSIONS

In theory, transferring biotechnologies developed at scientific facilities to the users of open waters who exploit spawning stocks of endangered species of rheophilic cyprinid fish is a unique opportunity to halt and/or reverse the negative impact human activities have had on the state of the ichthyofauna in rivers. The aspects of aquaculture presented in the current paper are some of the most important since they set forth the conditions for realizing the principles of active preservation. The published results of experiments from this field are already being applied in fisheries practice. However, there remains a lack of data regarding, among other aspects, the possibility of conducting out-of-season reproduction and the impact of temperature and fluctuations in it have on the results of hormonal stimulation.

ACKNOWLEDGMENTS

This study was financed within the framework of the project "Optimizing the production of stocking material of rheophilic cyprinid species under controlled conditions" of the Sectoral Operational Programme "Fisheries and Fish Processing 2004-2006" (00040-61535-OR1400009/07).

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Received – 01 December 2008

Accepted – 28 December 2008

STRESZCZENIE

PRZEGLĄD KONTROLOWANEGO ROZRODU BOLENIA, *ASPIUS ASPIUS* (L.) I ŚWINKI, *CHONDROSTOMA NASUS* (L.)

W pracy zaprezentowano wyniki badań z zakresu biotechniki rozrodu bolenia i świnki, uzupełnione o niepublikowane materiały autorów. Zawarto w pracy dane dotyczące sposobu pozyskiwania tarlaków, stosowanych środków hormonalnych, warunki termiczne rozrodu i inkubacji ikry oraz kriokonserwację nasienia bolenia i świnki. Rozmnażanie tych gatunków ryb w warunkach kontrolowanych bez zastosowania środków hormonalnych jest niemożliwe. Najczęściej stosowane dotychczas były: homogenat z przysadki mózgowej karpia (CPH) oraz analogi GnRH połączone z inhibitorem dopaminy (Ovopel oraz Ovaprim). Niezależnie od gatunku najwyższy odsetek owulujących samic (dochodzący nawet do 100%), płodność, a także przeżywalność embrionów osiągnięto po zastosowaniu stymulacji preparatami Ovopel i Ovaprim. Należy jednak podkreślić, że użycie CPH przynosi niewiele gorsze rezultaty. W przypadku obu gatunków opracowano techniki kriokonserwacji nasienia, pozwalające na skuteczne długotrwałe przetrzymywanie materiału genetycznego zawartego w plemnikach. Co istotne, zadawalająco wypadły próby dotyczące możliwości zapładniania ikry rozmrożonymi plemnikami.