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A REVIEW OF THE REPRODUCTION BIOTECHNOLOGY FOR FISH FROM THE GENUS *LEUCISCUS*

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ABSTRACT. This review presents the results of papers published from 1990 to 2008 on the reproductive biotechnology of rheophilic cyprinid fish of the genus *Leuciscus*: ide, *Leuciscus idus* (L.), dace, *Leuciscus leuciscus* (L.), and chub, *Leuciscus cephalus* (L.). The issues studied included obtaining reproductors both from the wild and through aquaculture. Particular attention was paid to issues linked with the insufficient quality of spawners, which stemmed most frequently from their inappropriate handling. The effects of stimulating rheophilic cyprinid fish with various hormonal preparations was analyzed. Initially, carp pituitary homogenate (CPH) was used, and for a long period this was the only preparation in use. Later, human chorionic gonadotropin (hCG) was used alone or in combination with CPH. It was confirmed that hCG is not suitable for the reproduction of rheophilic cyprinid fish. In the late 1990s, a gonadotropin releasing hormone analogue (GnRH) was introduced. Initially, Ovopel was used, which is a mammalian analogue of GnRH (D-Ala^6 Pro^{9}Net-mGnRH) with a dopamine receptor antagonist (metoclopramide). In recent years, studies have begun on the use of Ovaprim, a salmon analogue of GnRH (D-Arg^6 Pro^{9}Net-sGnRH) with a dopamine receptor antagonist (domperidone), and on GnRHa without a dopamine receptor antagonist. It has been confirmed that hormonal stimulation combined with stimulation through the manipulation of environmental conditions is essential for the reproduction of rheophilic cyprinid fish species of the genus *Leuciscus* under controlled conditions. The effects of reproduction under controlled conditions differ significantly among the species analyzed. The lowest percentage of ovulating females and spermiating males was noted in chub. Genome manipulation performed on the studied species is also described, including gynogenesis and androgenesis. The latter, in combination with semen cryopreservation, creates possibilities for restoring threatened populations and species.

Key words: IDE, DACE, CHUB, CONTROLLED REPRODUCTION, GAMETES, GENOME MANIPULATION

INTRODUCTION

The beginning of the twenty-first century saw breakthroughs in the development and exploitation of aquaculture. In addition to propagation issues, increasing attention has been focused on the impact aquaculture has on the natural environment (Harache

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2000, Horvath 2000, Varadi 2000, Balon 2004, McClure et al. 2008, Ross et al. 2008). It must be assumed that, as in recent years and currently, species and population preservation will remain one of the leading trends in aquaculture and that further development in biotechnologies will serve these goals (Philippart 1995, Foresti 2000, Kucharczyk 2002, Kamiński et al. 2004, Bolland et al. 2008, McClure et al. 2008, Ross et al. 2008).

The first documented attempts to stimulate reproduction in fish from the genus *Leuciscus* under controlled conditions were reported by Vanderplank (1938). Dace, *Leuciscus leuciscus* (L.), was chosen as the model fish. During the experiment, the fish were administered mammilian hormones, and they were noted to react variously to preparations used singly or in combination. The administration of human chorianic gonadtotropin (hCG) and progesterone cause oocyte maturation in comparison to the control group, in which dace received injections of saline solution. Some combinations of hormonal preparations inhibited the maturation of gametes and negated the positive influence of environmental factors. Simultaneously, the preparations administered had an impact on spawner mortality (Vanderplank 1938).

Studies focused on reproductive biotechnologies for rheophilic cyprinid fish were undertaken to increase stocking material production capacity and to protect the gene pool of endangered local populations (Kucharczyk 2002, Wojtczak et al. 2003, Targońska-Dietrich et al. 2003, Kowalski et al. 2004, 2006, Kucharczyk et al. 2004, 2008a, Kujawa 2004, Wolnicki 2005, Kujawa et al. 2006a, b, 2007, 2008, Cejko et al. 2008, Kupren et al. 2008a, Kwiatkowski et al. 2008a, Targońska et al. 2008, Żarski et al. 2008a).

Recent interest in rheophilic cyprinid fish species, including ide, *Leuciscus idus* (L.), dace, and chub, *Leuciscus cephalus* (L.), has been spurred by their declining abundance in running waters and the increased demand for stocking material. An upward trend in the introduction of rheophilic cyprinid species to open waters has been noted in Poland over the past few years, and ide comprises more than 70% of the stocking material of the genus *Leuciscus* produced (Wojda et al. 2000, Wojda 2004, Mickiewicz et al. 2007, Kupren et al. 2008b). This is because this species is relatively resistant to stress and manipulation, and the biotechnologies for its reproduction (Kucharczyk et al. 1999a, Kucharczyk 2002, Targońska-Dietrich et al. 2004, Jamróz et al. 2008a, b, Żarski et al. 2008b), and larval rearing (Wolnicki and Górny 1995, Kujawa 2004, Shiri Harzevili et al. 2004, Wolnicki 2005, Kwiatkowski et al. 2008b, Żarski et al. 2008a)

are well developed. Obtaining good, reproducible results in studies and the availability of broodstocks created the possibility of treating ide as a model fish among the rheophilic cyprinids. The results of research on this species are also used in studies of the reproductive biotechnology of other cyprinid fish; the results of which, however, are not uniformly successful. Chub has recently come under closer scrutiny (Arlinghaus and Wolter 2003, Shiri Harzevili et al. 2003, Kujawa 2004, Bolland et al. 2007, 2008, Krejszeff et al. 2008a, Żarski et al. 2008b). It is a popular species in recreational fishing and is an important component of the ichthyofauna of running waters (Błachuta 1998, Penczak et al. 1998, Bolland et al. 2007, 2008, Krejszeff et al. 2008a). Although dace has never been popular in recreational fisheries or commercially, it is also an important component of aquatic ecosystems.

The reproductive biotechnology of rheophilic cyprinid fish includes not only determining the optimal combination of hormonal preparations to be used in controlled reproduction, but also optimizing environmental conditions. One of the basic factors determining the reproductive results is temperature (Bromage et al. 2001, Hilder and Pankhurst 2003, Anguis and Canavate 2005). It is key during embryonic development as it is the main factor influencing the length of the embryonic period, the survival of the embryos, and their state of development at hatch (Kokurewicz 1971, Kupren et al. 2008a). Temperatures noted at dace, ide, and chub spawning grounds fluctuate between 5 and 20°C (Kokurewicz 1971, Kupren 2005), and this range is applied at hatcheries.

Recent developments in biotechnology include the application of genome manipulation, including gynogenesis and androgenesis (Kucharczyk 1999, 2001, 2002, Kucharczyk et al. 2004, 2007b, 2008a, b, c, d, Kwiatkowski et al. 2008a) and the cryopreservation of semen (Babiak et al. 1998, Lahnsteiner et al. 2000, 2003) for genome banks, among other uses.

The aim of the current paper was to analyze the results of studies of the biotechnology of controlled reproduction of fish from the genus *Leuciscus*. The paper presents the most important aspects of reproductive biotechnology including hormonal stimulation and its influence on reproductive effectiveness and the application of genome manipulations such as gynogenesis and androgenesis. The suitability of using genome manipulation to restore endangered local populations of fish from the genus *Leuciscus* is also assessed.

OBTAINING SPAWNERS

The effective reproduction of rheophilic cyprinid fish is only possible when there is an appropriate number of spawners available. In Poland, reproductors are most frequently obtained from the natural environment (Kupren et al. 2003, Targońska-Dietrich et al. 2003, 2004, Krejszeff et al. 2008a). Jakucewicz and Jakubowski (1990) and Cieśla (1998) reported that fish caught with gill-nets were not suitable for reproduction as these fish had both internal and external injuries. Kujawa et al. (2001) noted that individuals obtained, for example, from fish passes might appear to be uninjured externally, but they often have internal injuries that prevent them from reproducing. Cieśla (1998) compared the reproductive effectiveness of asp, *Aspius aspius* (L.), barbel, *Barbus barbus* (L.), vimba, *Vimba vimba* (L.), and chub caught with nets and electrofishing with cultured individuals or those held for several months in ponds, and reported that the reproductive results were better with the spawners that had been reared in ponds than with those caught in the natural environment. How fish are obtained is highly influential in their later suitability as reproductors. Kujawa et al. (2006b) did a comparative study of reproduction results of asp, ide, and chub caught with gill nets. Authors compare the two methods used for removing spawners from the nets and describe the further manipulation they are subjected to aboard boats. The "traditional" method was that used by fishers during commercial catches, while the "experimental" method was a more careful one followed by holding the fish under well oxygenated conditions. It was confirmed that gill nets can be used as gear for catching spawners as long as the fish are removed from the nets gently. The percentage of ovulating spawners obtained when the fish were manipulated appropriately was increased approximately fivefold in asp, threefold in chub, and twofold in ide (Kujawa et al. 2006b).

Appropriate transport conditions to the hatchery are very important. To minimize stress, it is beneficial to add a small amount of anesthetic, such as 2-phenoxythanol, to the water (Kujawa et al. 2001). Regardless of how the spawners are caught, they should be held under conditions that prevent body injury and reduce stress.

Rheophilic cyprinid spawners from the genus *Leuciscus* can also be obtained from ponds. Most frequently these are fish caught in rivers and then held in ponds for longer periods or are the progeny of such spawners (Jakucewicz and Jakubowski 1990, Cieśla

1998, Śliwiński 2000, Jamróz et al. 2008b). Other ways of obtaining spawners is to rear fish in tanks that are part of recirculating systems (Targońska-Dietrich et al. 2004, Kujawa et al. 2006a). Fish cultured under controlled conditions are decidedly more frequently ready for reproduction than fish originating from natural conditions. For example, semen was obtainable from male ide and dace nearly the whole time they were held at a temperature range of 12-15°C, while females spawned as many as several times annually (Targońska-Dietrich et al. 2004, Kujawa et al. 2006a).

ENVIRONMENTAL CONDITIONS DURING CONTROLLED REPRODUCTION

Reproductive success depends on the appropriate manipulation of environmental conditions in combination with stimulating the fish with hormonal preparations. Dace, ide, and chub spawn naturally at different times and under different thermal conditions. Dace reproduces at the lowest water temperature (5-12°C), while ide do so at slightly higher temperatures (8-15°C), and chub at a range of 15 to 20°C (Kupren 2005). Appropriate protocols for thermal regimes preceding reproduction under controlled conditions of the species in question have been developed. It was demonstrated that gradually increasing water temperature during the acclimation period and the administration of hormonal preparations has a decided influence on reproduction results (Kucharczyk et al. 1998a, 1999a, 2000, Kupren et al. 2003, Targońska-Dietrich et. 2003, 2004). Prior to the first injection, the water temperature is gradually raised by about 2°C (Fig. 1). It is also recommended to raise water temperature gradually during the period of hormonal injections. The photoperiod applied during the controlled reproduction of ide and dace was 12L:12D (Kucharczyk 2002, Targońska-Dietrich et al. 2003, 2004), while that for chub was 14-16L:8-10D (Kucharczyk et al. 1998a, b, Krejszeff et al. 2008a).

Significantly greater changes in the thermal regime of the water and in photoperiods should be applied during out-of-season reproduction of rheophilic cyprinid fish (Kucharczyk et al. 1999a, Kucharczyk 2002, Targońska-Dietrich et al. 2004, Jamróz et al. 2008a). The thermal regime and photoperiods occurring in the natural environment in fall, winter, and spring should be imitated, albeit within a shorter timespan. These changes in the environment stimulated the maturation of the gametes

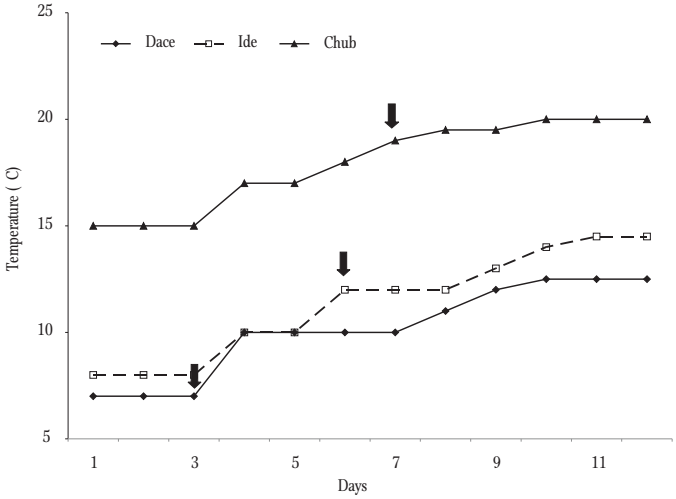

Fig. 1. Thermal regime of water during controlled reproduction of fish of the genus *Leuciscus*. Arrows indicates the initial injection.

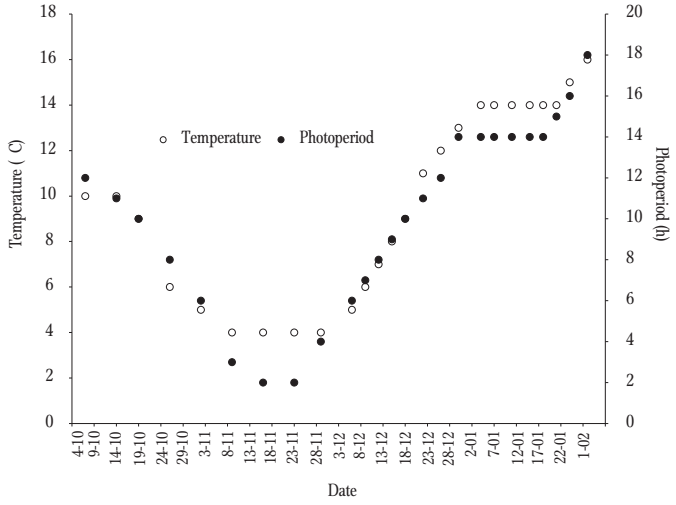

Fig. 2. Environmental conditions during out-of-season fish reproduction based on ide, *Leuciscus idus* (after Targońska-Dietrich et al. 2004, modified).

and, in combination with hormonal stimulation, permitted reproduction to happen. During out-of-season ide reproduction in January, water temperature ranged from 2-12°C . Initially, water temperature in the tanks holding spawners was 10°C, and the photoperiod was 10L:14D (Fig. 2). Over a period of six weeks, the temperature was reduced to 2°C, and the tanks were illuminated for only 4 hours daily. The experiment

proceeded with gradual increases of water temperature in tanks (1.5°C per week) to 12°C. The tank illumination time was simultaneously extended to 12 hours daily.

The maximum temperature during spawning under controlled conditions was 12.5°C for dace, 14.5°C for ide, and 19.5°C for chub (Kucharczyk 2002, Kucharczyk et al. 1998b, 1999a, 2000, 2007a, Kupren et al. 2003, Targońska-Dietrich et al. 2003, 2004, Kujawa et al. 2006a, b, Krejszeff et al. 2008a). Holding fish at higher temperatures can decrease the percentage of ovulating females as well as affect the quality of the gametes expressed in lowered embryo survival. This is especially significant with regard to males since excessive temperatures can lead to halted spermiation (Kujawa et al. 2006b). This is why it is recommended that males are held at slightly lower temperatures (e.g., by 1°C) than those females are held at (Kucharczyk and Targońska, unpublished data).

HORMONAL PREPARATIONS FOR INDUCING REPRODUCTION IN FISH FROM THE GENUS *LEUCISCUS*

Long-term studies have indicated that it is impossible to conduct the controlled reproduction of dace, ide, or chub without hormonal stimulation (Cieśla 1998, Mizieliński et al. 2000, Kucharczyk 2002, Targońska-Dietrich et al. 2004, Krejszeff et al. 2008a). Initially, gamete maturation, ovulation and spermiation were induced with carp pituitary homogenate (CPH) (Cieśla 1998, Kucharczyk et al. 1998a, Kucharczyk 2002). Later, human chorionic gonadotropin (hCG) or a combination of hCG and CPH were administered. By the end of the twentieth century, a gonadotropic releasing hormone (GnRH) analogue had come into use; the first was Ovopel (Unic-trade, Hungary), a mammilian analogue GnRH (D-Ala^6 Pro^{9}Net-mGnRH) with a dopamine receptor antagonist (metoclopramide). In recent years, studies have focused on the use of Ovaprim (Syndel International Inc., Canada), a salmon analogue GnRH (D-Arg^6 Pro^{9}Net-sGnRH) with a dopamine receptor antagonist (domperidone), as well as GnRHa without dopamine receptor antagonists.

TABLE 1

Dosages of hormonal preparations recalculated per kg of body weight and the period between injections in the reproduction of female fish of the genus *Leuciscus*

First injection	Second injection	Time (h)	Author
Dace			
0.4 mg CPH	3.6 mg CPH	24	6
0.1 pellet Ovopel	1 pellet Ovopel	24	6
1000 IU hCG		-	6
1000 IU hCG	3.6 mg CPE	24	6*
0.4 mg CPE			
0.1 pellet Ovopel	1 pellet Ovopel	24	6*
Ide			
0.4-0.6 mg CPH	1.2-1.4 mg CPH	24	1
0.4 mg CPH	1.6 mg CPH	24	3*
1000 IU hCG	3.6 mg CPE	24	4
0.4 mg CPE			
500 IU hCG	2000 IU hCG	24	4
1000 IU hCG	3.6 mg CPE	24	4*, 6*
0.4 mg CPE			
0.1 pellet Ovopel	1 pellet Ovopel	24	4*
2 pellet Ovopel		-	8,10
1000 IU hCG	3.6 mg CPE	12	6
0.4 mg CPE			
500 IU hCG	2000 IU hCG	12	6
0.1 pellet Ovopel	1 pellet Ovopel	12	6, 7
0.1 pellet Ovopel	1 pellet Ovopel	24	4, 6, 7*
0.2 pellet Ovopel	1 pellet Ovopel	24	10
2 pellet Ovopel		-	10
0.5 ml Ovaprim		-	8,10
0.2 pellet Ovopel	0.5 ml Ovaprim	24	10
Chub			
0.4-0.6 mg CPH	1.2-1.4 mg CPH	24	1
0.4 mg CPH	1.6 mg CPH	24	2
0.4 mg CPH	1.6 mg CPH	24	3*
0.2 pellet Ovopel	1 pellet Ovopel	12	5
0.4 mg CPH	3.6 mg CPH	24	9
200 IU hCG	1800 IU hCG	24	9
0.1 pellet Ovopel	1 pellet Ovopel	24	9

1 – Jakucewicz and Jakubowski (1990), 2 – Kucharczyk et al. (1998a), 3 – Kucharczyk et al. (1998b), 4 – Kucharczyk et al. (1999a), 5 – Mizieliński et al. (2000), 6 – Kucharczyk (2002), 7 – Targońska-Dietrich et al. (2004), 8 – Kucharczyk et al. (2007a), 9 – Krejszeff et al. (2008a), 10 – Jamróz et al. (2008a)

** out-of-season reproduction*

Not all of the hormonal preparations tested were effective during the reproduction of rheophilic cyprinid fish. It was confirmed that hCG is not suitable for stimulating

reproduction in rheophilic cyprinid species (Kucharczyk et al. 1999a, Targońska-Dietrich et al. 2003, Krejszeff et al. 2008a); however, it turned out to be effective with cyprinid batch spawners such as rudd, *Scardinius erythrophthalmus* (L.) (Kucharczyk et al. 1997a), or crucian carp, *Carassius auratus auratus* (L.) (Krejszeff et al. 2008b) as well as with other percids (Kucharczyk et al. 1996, 1998c). Not until hCG and CPH were administered together were satisfactory results achieved (Kucharczyk 2002, Targońska-Dietrich et al. 2003). The first attempts to administer Ovopel to rheophilic cyprinid fish were in 1997 (Horvath et al. 1997). Typically, two injections of Ovopel were administered to female fish from the genus *Leuciscus* to stimulate reproduction (Table 1). The initial injection dose was 0.1-0.2 pellet kg^{-1} of body weight, while that of the resolving injection was 1 pellet kg^{-1}. Single injections at a dose of 2 pellet kg^{-1} were also used with ide (Jamróz et al. 2008a). Ovaprim has been used to stimulate fish from the genus *Leuciscus* since 2007. Intra-peritoneal injections of 0.5 ml kg^{-1} were applied (Kucharczyk et al. 2007a, Jamróz et al. 2008a). Recently, effective reproduction has been achieved using Ovopel for the initial injection (0.2 pellet kg^{-1}), and Ovaprim (0.5 ml kg^{-1}) for the resolving injection (Jamróz 2008b).

RESULTS OF HORMONAL STIMULATION OF MALES FROM THE GENUS *LEUCISCUS*

Semen quality was initially omitted from studies of controlled reproduction of rheophilic cyprinid fish (Cieśla 1998, Śliwiński 1998, 2000, Mizieliński et al. 2000). Interest in this issue was raised by the low survival rates of embryos that was not always linked to egg quality. Studies conducted on other fish species indicated that parameters such as sperm concentration (Rurangwa et al. 2004), sperm motility, or the length of the sperm motility period (Glogowski et al. 1997) had a significant influence on embryo survival. Studies indicated that the fertilization capabilities of individual sperm from individual fish were also of similar significance (Babiak et al. 1998, Casselman et al. 2006). This was confirmed for rheophilic cyprinid fish in the study by Targońska et al. (2008) of four freshwater fish species. In the case of ide, it was demonstrated that embryonic survival was higher when a higher percentage of motile sperm were active for a longer period of time. The fertilization ability of the semen collected from 10 males was also determined. Significant differences in embryo survival (from 24 to 86%) were

TABLE 2

Results of controlled reproduction of male fish of the genus *Leuciscus* with the application of different hormonal preparations. Data refer to hormone dosages recalculated per kg of body weight

Hormone	Total dosage	Spermiation (%)	Sperm volume (ml kg^{-1} body weight)	Sperm motility (%)	Author
Dace					
-	-	100	0.6	62	4
CPH	2.0 mg	100	0.7	71	4
Ovopel	0.5 pellet	100	0.7	70	4
hCG	1000 IU	100	0.6	67	4
CPH	2.0 mg	100	0.2	52	4*
Ovopel	0.5 pellet	100	0.3	67	4*
Ide					
hCG	3700 IU	nd	nd	nd	1*
-	-	100	2.0	49	2
CPH	2.0 mg	100	4.0	76	2
hCG	1000 IU	100	2.1	74	2
Ovopel	0.5 pellet	100	4.5	72	2
-	-	100	1.0	43	2*
CPH	2.0 mg	100	3.2	65	2*
Ovopel	0.5 pellet	100	1.7	68	2*
Ovopel	0.5 pellet	100	3.2	80	3
-	-	100	2.1	52	4
CPH	2.0 mg	100	4.3	76	4
Ovopel	0.5 pellet	100	2.2	77	4
hCG	1000 IU	100	4.4	75	4
-	-	100	1.1	41	4*
CPH	2.0 mg	100	3.4	66	4*
Ovopel	0.5 pellet	100	1.9	67	4*
-	-	100	1.1	60	5*
Ovopel	0.5 pellet	100	1.3	65	5*
Ovopel	1 pellet	100	nd	67	8
Ovaprim	0.5 ml	100	nd	80	8
LH-RH	20 μg	100	nd	81	8
-	-	100	nd	66	8
Ovaprim	0.5 ml	100	nd	73-75	6
Ovopel	1 pellet	100	nd	72-77	6
Chub					
-	-	53	2.4	60-70	7
CPH	2.0 mg	100	3.7	80-90	7
hCG	1000 IU	68	2.5	80-90	7
Ovopel	0.5 pellet	100	3.6	80-90	7

*1 – Kucharczyk et al. (1998b), 2 – Kucharczyk et al. (1999a), 3 – Kucharczyk et al. (2000), 4 – Kucharczyk (2002), 5- Targońska-Dietrich et al. (2004), 6 – Kucharczyk et al. (2007a), 7 – Krejszeff et al. (2008a), 8 – Jamróz et al. (2008a), * out-of-season reproduction, nd – not determined, – without hormonal stimulation*

noted in samples that were fertilized with the semen of different males. Survival in the control group, which was fertilized by mixed semen, was over 85% and did not differ statistically from the highest result. This indicates that it is necessary to use mixed semen from many males for fertilization in the controlled reproduction of rheophilic cyprinid species.

The results of studies on the impact of hormonal stimulation on the reproductive results for males of dace, ide, and chub are presented in Table 2. The use of hormones is essential because it increases the volume of semen, and the sperm in it are more motile (Kucharczyk et al. 1999a, Kucharczyk 2002, Kupren et al. 2003, Targońska-Dietrich et al. 2003, 2004, Krejszeff et al. 2008a). The semen obtained during out-of-season reproduction was frequently of lesser quantity and lower quality in comparison to that from the natural spawning period (Kucharczyk 2002, Targońska-Dietrich et al. 2004). In one case of out-of-season ide reproduction, the males produced no semen, and required four injections (Kucharczyk et al. 1998b). It was also confirmed that the period of spermiation in males subjected to out-of-season reproduction was very short, and despite environmental manipulation and hormonal stimulation, it lasted sometimes just 3 to 5 days (Kujawa et al. 2006b). This is linked with the reproductive cycle of wild populations, in which the last phase of testes maturation is probably strictly linked with the season of the year and the dynamics of the natural environment. The greatest quantity of semen and the best sperm motility were noted during the natural breeding period in the wild (Kucharczyk et al. 2000, Krejszeff et al. 2008a).

Studies of sperm motility in dace and ide indicated that there is significant differentiation in the overall motility period of sperm (Lahnsteiner et al. 2004, Kowalski et al. 2006). Ide sperm were active for 3 minutes, ide for 1.5 min, and chub only for about 1 min. Greater similarities among the parameters of sperm motility were confirmed in ide and chub than in dace (Lahnsteiner et al. 2004, Kowalski et al. 2006). The mean initial speed of sperm movement of the first two species oscillated around 60-70 $\mu m\ s^{-1}$, while in dace it was not much higher than 40 $\mu m\ s^{-1}$. The sperm concentration of rheophilic cyprinid fish has been studied very rarely. Kowalski et al. (2004) estimated it at 5.7 mld cm^{-3} for ide, and 15.3 mld cm^{-3} for chub. Lahnsteiner et al. (2000) estimated the chub sperm concentration at 3-5 mld cm^{-3}. The concentration of dace sperm has not yet been determined.

RESULTS OF HORMONAL STIMULATION OF FEMALES FROM THE GENUS *LEUCISCUS*

Hormonal preparations have a slightly different influence on gamete maturation in females from the genus *Leuciscus* (Table 3). Stimulation through manipulations of environmental conditions alone has yet to produce results, and no oocytes were obtained from fish in the control group that were not stimulated hormonally. Nor was spawn obtained from fish that were only stimulated with hCG (Kucharczyk et al. 1999a, Kucharczyk 2002, Targońska-Dietrich et al. 2003, Krejszeff et al. 2008a).

The most variable and unpredictable results were noted in chub. Cieśla (1998) reported results of administering CPH to female chub obtained from the natural environment prior to spawning and to females that had been held in ponds for two years. Spawn was not obtained in either case. However, in another study, stimulating chub females with the same preparation resulted in 100% ovulation (Kucharczyk et al. 1998a, b). These contradictory results were probably influenced by environmental conditions, the origin of the fish, or the method used to obtain the spawners.

High percentages of ovulating females and high embryonic survival rates were achieved when female dace, ide, and chub were stimulated with GnRH analogues in combination with dopamine receptor antagonists (Kucharczyk et al. 1999a, 2007a, Mizieliński et al. 2000, Targońska-Dietrich et al. 2003, 2004, Jamróz et al. 2008a, b, Krejszeff et al. 2008a). Jamróz et al. (2008b) reported that the combination of two preparations containing different GnRH analogues and dopamine receptor antagonists (initial injection – Ovopel, resolving injection – Ovaprim) produced the highest ovulation percentage and the best quality gametes. Similar results were obtained when the two preparations were administered during the controlled reproduction of dace (Żarski, unpublished data), chub (Krejszeff, unpublished data), and asp (Targońska, unpublished data). The excellent, and also reproducible, results obtained with the GnRH analogues and dopamin receptor inhibitors (Ovopel, Ovaprim) has prompted the gradual withdrawal from use of pituitary homogenate from the reproduction of rheophilic cyprinid species. Despite the similar end results of reproduction, these preparations have different effects on fish bodies. CPE has a direct impact on gamete maturation, while GnRH analogues have an indirect impact. GnRH acts on the endocrine

TABLE 3

Results of controlled reproduction of female fish of the genus *Leuciscus* with the application of different hormonal preparations. Data refer to hormone dosages recalculated per kg of body weight

Hormone	Total dosage	Ovulation (%)	Latency time (h)	Survival to eyed-egg stage (%)	Author
Dace					
CPH	4.0 mg	100	30-32	94.1	6
Ovopel	1.1 pellet	100	32-36	94.7	6
hCG+CPH	1000 IU + 4.0 mg	100	30-33	76.7	6*
Ovopel	1.1 pellet	100	36-40	75.7	6*
Ide					
CPH	2.0 mg	100	28-34	nd	2*
hCG + CPH	1000 IU + 0.4 mg	100	30-32	65.8	3
hCG	2500 IU	0		-	3
Ovopel	1.1 pellet	100	36-41	78.2	3
hCG + CPH	1000 IU + 0.4 mg	100	30-32	61.9	3*
Ovopel	1.1 pellet	100	36-41	62.4	3*
Ovopel	2 pellet	90-100	34-36	nd	5
CPH	4.0 mg	100	30-32	65.9	6
Ovopel	1.1 pellet	100	36-40	79.3	6
hCG+CPH	1000 IU + 4.0 mg	100	30-33	66.2	6*
Ovopel	1.1 pellet	100	36-42	65.7	6*
-	-	8		48.2	7*
Ovopel	1.1 pellet	93	34	68.1	7*
Ovopel	1.2 pellet	95	36	66.0	10
Ovaprim	1.0 ml	100	36-44	82.0	10
Ovopel + Ovaprim	0.2 pellet + 0.5 ml	100	38-42	85.0	10
LH-RH	20 μg	20	38-42	85.0	10
-	-	0	-	-	10
Ovaprim	0.5 ml	100	42-47	82.0-92.6	8
Ovopel	2 pellet	90	36-38	68.3-71.0	8
Chub					
CPH	2.0 mg	100	24	nd	1
CPH	2.0 mg	100	26-32	nd	2*
Ovopel	1.2 pellet	60	36-48	nd	4
CPH	4.0 mg	28	16-18	79.0	9
Ovopel	1.1 pellet	36	24-32	87.0	9

1 – Kucharczyk et al. (1998a), 2 – Kucharczyk et al. (1998b), 3 – Kucharczyk et al. (1999a), 4 – Mizieliński et al. (2000), 5 – Kucharczyk et al. (2000), 6 – Kucharczyk (2002), 7 – Targońska-Dietrich et al. (2004), 8 – Kucharczyk et al. (2007a), 9 – Krejszeff et al. (2008a), 10 – Jamróz et al. (2008a)

** out-of-season reproduction, nd – not determined, – without hormonal stimulation*

glands of fish that, in turn, produce their own gonadotropic releasing hormones that lead to the maturation of the gametes (Yaron 1995).

One of the negative aspects of reproduction under controlled conditions is spawner mortality, which can be caused by many factors including fish stress, manipulation, the impact of hormonal preparations, or even injuries sustained during transport to the hatchery (Kujawa et al. 2001, 2006b). The highest broodstock mortality (of up to 50%) during the reproduction of ide under controlled conditions was noted after the administration of Ovopel (Kucharczyk et al. 1999a, Kucharczyk 2002). The mortality of reproductors during the natural spawning season was lower than during out-of-season controlled reproduction. Very high spawner mortality was also noted in chub (Krejszeff et al. 2008a). Mortality among females stimulated with saline solution was 53%, while in the groups stimulated with CPE or hCG it was 47 and 49%, respectively. The highest mortality of 68% was noted among females stimulated with Ovopel. The spawners were held for an additional two weeks following spawning, and the mortality described refers to the entire period the fish were held at the hatchery.

GENOME MANIPULATION AND POSSIBILITIES FOR USING IT IN RESTORATION PROGRAMS WITH SEMEN CRYOPRESERVATION

Advances in biotechnology allow for the genome manipulation of gametes and zygotes, the goal of which is to create organisms with appropriate genotype and phenotype characters. Among the many types of procedures that can be used with rheophilic cyprinid fish, two were carried out – gynogenesis (Kucharczyk 1999, Kucharczyk et al. 1999b, 2004, 2007b, 2008a, b, c) and androgenesis (Kucharczyk 2001, 2002, Kucharczyk et al. 2001, 2008d, Kwiatkowski et al. 2008a). Gynogenesis, which is the induction of the development of an organism without paternal DNA, was conducted in several stages. The nuclear DNA in sperm was deactivated with UV radiation (Kucharczyk 1999). Then, thermal shock was used to replicate the nuclear material in order to obtain a diploid organism in which all the genetic material was maternal (Kucharczyk et al. 2004, 2008a, b, c). The effectiveness of this process, understood as the quantity of progeny obtained, was not very high, but the survival of the gynogenic progeny was as high as 10% (Kucharczyk et al. 2004, 2007b, 2008a, b). Lower survival

was noted with the induction of androgenic development. In this process, the nuclear DNA of the oocytes was deactivated without damaging the mtDNA (Kucharczyk 2001, 2002). Then a thermal shock was applied to replicate the nuclear genetic material (Kucharczyk 2002, Kucharczyk et al. 2008d, Kwiatkowski et al. 2008a). The quantity of androgenetic progeny obtained was nearly 2% of the oocytes used in the experiment.

Combining genome engineering methods, particularly androgenesis and semen cryopreservation, makes it possible to recreate populations, even of species that have become extinct (Kucharczyk 2002). The oocytes of related species with inactivated nuclear DNA are fertilized with cryopreserved semen and the genetic material is replicated. The individuals obtained are diploid with nuclear DNA of male origin; however, the mtDNA originates from the oocyte donor (Kucharczyk et al. 2008d, Kwiatkowski et al. 2008a). In rheophilic cyprinid fish, androgenesis was induced in ide using oocytes from dace or chub (Kucharczyk 2002, Kucharczyk et al. 2008d, Kwiatkowski et al. 2008a). Studies on gynogenesis in ide (Kucharczyk 1999, Kucharczyk et al. 1999b) and other cyprinid fish such as bream, *Abramis brama* (L.), (Kucharczyk et al. 1997b) or tench, *Tinca tinca* (L.), (Linhart et al. 1995) confirm that these species are homogametic which means that in androgenetic generation F_1 the females should be homogametic (for example, XX) as should the males (for example, YY). This, in turn, poses difficulties for the reconstruction of a broodstock from these fish since the progeny in the first generation will all be males (XY). This way, XX females can be obtained (immediate androgenic progeny) and XY males (first generation progeny F_2). Thanks to these procedures, it is possible, theoretically, to recreate extinct species, if their frozen semen is available.

These procedures are impossible without the ability to freeze sperm. Semen cryopreservation has not been thoroughly studied yet, and further work on this topic is necessary, including with ide and dace. Lanhsteiner et al. (2000) performed cryopreservation on chub. Semen with a sperm concentration of 3.5-5.0 mld cm^{-3} was diluted immediately following collection and held at a temperature of 4°C for 30 min. The freezing medium selected for chub was 10% DMSO (dimethyl sulfoxide) with 0.5% glycine. The semen was frozen in straws in liquid nitrogen vapor, and after thawing, sperm motility was assessed. In comparison to semen that had not been cryopreserved, lowered rates of motility were observed from 82.3 to 60.6% as well as in

sperm movement speed from 118.0 to 57.7 μm s^{-1}. The results obtained are to be viewed as satisfactory, and permit the effective use of thawed semen for fertilization.

DOMESTICATION PROCESS AND ITS IMPACT ON CONTROLLED REPRODUCTION

The few data there are about the reproduction of fish reared in captivity (Targońska-Dietrich et al. 2004, Kujawa et al. 2006b) or domesticated fish (Krejszeff – unpublished data) indicate that the reproductive behavior of these fish is distinctly different from that displayed under natural conditions. Despite the best efforts to prevent domestication during attempts to improve fish reproduction, it does happen and this elicits, among things, changes in reproductive behavior. In ide that have been cultured for a few generations in ponds, it has been noted that ovulation can be induced by manipulations in the environment or by substantially lower doses of hormonal preparations administered with the first injection (Krejszeff, unpublished data). The latency time was also shorter than in fish from the natural environment. These results are only preliminary and require further study on a larger sample of fish. It is possible that soon a separate protocol will have to be prepared for the artificial reproduction of domesticated fish under controlled conditions.

The controlled reproduction of fish from the genus *Leuciscus* is not without its challenges. Based on the current review of the literature and the authors' own observations, the difficulty of reproducing rheophilic cyprinid fish from the genus *Leuciscus* can be ranked. The fewest number of difficulties are encountered during the artificial reproduction of ide under controlled conditions, followed by dace and, finally, chub, which are the most difficult species to breed.

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STRESZCZENIE

PRZEGLĄD BIOTECHNOLOGII SZTUCZNEGO ROZRODU RYB Z RODZAJU *LEUCISCUS*

Przedstawiono wyniki prac opublikowanych w latach 1990-2008 z zakresu biotechnologii rozrodu karpiowatych ryb reofilnych z rodzaju *Leuciscus*: jazia, *Leuciscus idus* (L.), jelca, *Leuciscus leuciscus* (L.) i klenia, *Leuciscus cephalus* (L.). Przedstawiono zagadnienia związane z pozyskiwaniem reproduktorów, zarówno ze środowiska naturalnego jak i z hodowli. Zwrócono szczególną uwagę na kwestie związane z nieodpowiednią jakością tarlaków, wynikającą najczęściej z nieumiejętnego obchodzenia się z nimi. Przeanalizowano efekty stosowania stymulacji hormonalnej karpiowatych ryb reofilnych różnymi środkami hormonalnymi. Jako pierwszy środek hormonalny używano homogenatu z przysadek mózgowych karpia (CPH) i przez długi okres był to jedyny stosowany środek. Później stosowano ludzką gonadotropinę kosmówkową (hCG) bądź kombinację hCG i CPH. Wykazano, że hCG nie nadaje się do rozrodu karpiowatych ryb reofilnych. Pod koniec XX wieku zaczęto stosować analogi hormonu uwalniającego gonadotropinę (GnRH). Początkowo stosowano Ovopel, ssaczy analog GnRH (D-Ala^6 Pro^{9}Net-mGnRH) z inhibitorem dopaminy (metoclopramide). W ostatnich latach rozpoczęto badania nad zastosowaniem preparatu Ovaprim, łososiowatego analogu GnRH (D-Arg^6 Pro^{9}Net-sGnRH) połączonego z inhibitorem dopaminy (domperidone) oraz GnRHa bez inhibitorów antydopaminowych. Wykazano, że stosowanie stymulacji hormonalnej, połączonej wraz ze stymulacją warunkami środowiskowymi, jest niezbędne w celu przeprowadzenia rozrodu karpiowatych ryb reofilnych z rodzaju *Leuciscus* w warunkach kontrolowanych. Efekty rozrodu w warunkach kontrolowanych różnią się znacznie u analizowanych gatunków. Najniższy odsetek owulujących samic i spermacji u samców odnotowano u klenia. Opisano również prowadzone na badanych gatunkach manipulacje genomowe, w tym gynogenezę i androgenezę. Ta ostatnia metoda w połączeniu z kriokonserwacją nasienia stwarza możliwości odtwarzania zagrożonych wyginięciem populacji i gatunków.