Induced spawning in bream (*Abramis brama* L.) using pellets containing GnRH

D. Kucharczyk[1], R. Kujawa[1], A. Mamcarz[1], K. Targońska-Dietrich[1], E. Wyszomirska[2], J. Glogowski[3], I. Babiak[1], T. Szabó[4]

[1]Department of Lake and River Fisheries, Department of Animal Biochemistry, Warmia and Mazury University in Olsztyn, Olsztyn-Kortowo, Poland
[2]Institute of Public Health, Warsaw, Poland
[3]Institute of Animal Reproduction and Food Research of Polish Academy of Sciences, Olsztyn, Poland
[4]Institute of Animal Husbandry, Gödöllö University of Agricultural Sciences, Gödöllö, Hungary

ABSTRACT: Wild spawners of common bream, *Abramis brama*, were caught in the Kortowskie Lake (north Poland) and transported to a hatchery for artificial spawning. Fish were hormonally induced using GnRH analogue combined with metoclopramide (ovopel). The results of bream reproduction in captivity were compared with fish treated with the combination of hCG and CPE and with control group injected saline. Males from treated groups produced significantly more milt (over 4.3 ml/kg vs. 2.1) of better qualities: spermatozoon concentration (over 9.3×10^9 vs. 6.8) and motility (over 85% vs. 62). Females from the control group did not spawn whereas those from hormonally induced groups ovulated: 62% after CPE treatment and 100% after GnRHa treatment. Generally, the fish after ovopel stimulation showed the best hatchery parameters.

Keywords: controlled reproduction; spermiation; ovulation; incubation process

One of the most important problems in cyprinid aquaculture is to obtain good-quality gametes (Kulikovsky *et al.*, 1996; Horvath *et al.*, 1997). For this reason many hormonal treatments are used to stimulate gamete maturation in commercial cyprinids. One of the most commonly applied spawning agents is carp pituitary extract (CPE) (Yaron *et al.*, 1984; Thalathiah *et al.*, 1988), in same cases with addition of human chorionic gonadotropin (hCG) (Kucharczyk *et al.*, 1997a). The good results of induced ovulation in cyprinid fish were also obtained after hormonal stimulation by a synthetic analogue of gonadotropin-releasing hormone (GnRH), frequently with strong dopamine antagonists (Yaron, 1995; Barth *et al.*, 1997). Recently, Horvath *et al.* (1997) proposed a new technique of ovulation stimulation in commercial cyprinids using pellets containing GnRH analogue (ovopel). One ovopel pellet (average weight about 25 mg) contains mammalian GnRH analogue (D-Ala^6, Pro^{9}Net-mGnRH at a dose of 18–20 μg) and dopamine antagonist: metoclopramide (dose 8–10 mg).

The above-mentioned problem of artificial spawning is much better visible in the case of wild cyprinids, mostly captured from natural populations. Since many cyprinid wild stocks become extinct, there is a need for fast development of techniques of controlled propagation of these fish. Generally, the papers describing methods of artificial spawning of wild cyprinids are very scarce as well as data on reproductive biology of wild cyprinids and hatchery techniques (Penaz and Gajdusek, 1979; Kucharczyk *et al.*, 1997c; Babiak *et al.*, 1998). Some of the presented methods of artificial propagation are finished at the moment

of ovulation (Glubokov *et al*., 1991). Our earlier researches on wild cyprinids showed that the problem of the biological quality of gametes was also very important (Kucharczyk *et al*., 1997b). On the other hand, the development of controlled reproduction of wild cyprinids is still needed as an integral component of ongoing conservation efforts (Wildt *et al*., 1993).

Bream (*Abramis brama* L.) is one of the most important freshwater species in Poland and some other European countries (Glubokov *et al*., 1991); average annual bream catch in Poland ranged from 20 (Szczerbowski, 1985) to 50 kg/ha (Brylińska and Tadajewska, 1986). Artificial spawning is necessary to develop techniques of genome engineering and banking in bream (Kucharczyk, 2002). The aim of the present study was to test ovopel as a spawning agent in artificial reproduction of wild matured common bream (*Abramis brama* L.), in comparison with CPE and hCG stimulation which worked for common bream very well (Kucharczyk *et al*., 1997a).

MATERIAL AND METHODS

Fish

Bream spawners were obtained in the spring season from Kortowskie Lake (Olsztyn District, Poland). Fish were selected according to the following criteria: the belly of females had to be fully distended and bulging, soft and resilient to touch; males had to be little spermiating. The size of spawners ranged from 0.8 to 1.2 kg. The water temperature in a hatchery was gradually raised from 15 to 20°C. Selected males and females were kept in separate 1 000-litre tanks in a hatchery with controlled temperature (20°C) and photoperiod (18 hrs light and 6 hrs dark).

Checking the oocyte maturation

All fish were individually marked using floy tags, weighed, and oocytes were taken from females using the method described by Kujawa and Kucharczyk (1996). Oocytes were sampled *in vivo* and placed in Serra's solution for clarification of the cytoplasm. After 5 minutes, the position of oocyte nucleus was determined using a 4–stage scale:

stage 1 – germinal vesicle in the central position (CGV)

stage 2 – early migration of germinal vesicle (less than a half of the radius – MGV)

stage 3 – late migration of germinal vesicle (more than a half of the radius – PGV)

stage 4 – peripheral germinal vesicle or germinal vesicle breakdown (GVBD)

Only those females were used for further experiments in which oocyte maturation was between 2–3 and 3 division, which is the best moment for hormonal stimulation in cyprinids (Kozlowski, 1994).

Hormonal treatment

Fish were divided into three groups: two experimental groups and control one. After five days of acclimation to 20°C, the fish were treated with respective hormonal injections of common carp pituitary (Argent, USA) extract with the addition of hCG (Biomed, Poland) or ovopel (Table 1). All spawning agents were prepared with 0.9% NaCl: the pituitary extract was homogenized, hCG dissolved and ovopel pellets were pulverised in a mortar and then dissolved. Injections of hCG were intramuscular in the dorsal region of the body (Thalathiah *et al*., 1988). Injections of pituitary (Kucharczyk *et al*., 1997a) and ovopel (Horvath *et al*., 1997) extracts were intraperitoneal at the base of the pelvic fin.

Table 1. The doses of hormones applied in artificial spawning of common bream (*Abramis brama* L.)

Group	Males hormonal dose	Females	
		priming dose	resolving dose
1	2.0 mg CPE	1 000 IU hCG 0.4 mg CPE	3.6 mg CPE
2	1/2 ovopel pellet	1/10 ovopel pellet	1 ovopel pellet
3 (control)	+	+	+

+ – injections from 0.9% NaCl

Before manipulations fish were anaesthetised with 2-phenoxyethanol (0.5 ml per l). Time intervals between respective injections are shown in Table 1. Additionally, thirty males were divided into three groups (Table 2) to obtain hormones every three days to induce spermiation.

Collection of gametes and incubation

Ripe gamete donors were anaesthetized in a solution of 2-phenoxyethanol (0.5 ml/l). Milt was collected with plastic syringes and kept at 4°C until further treatment. Within 30 min after collection spermatozoon motility was estimated subjectively under a microscope (× 500) in 0.5% solution of NaCl; such solution ensured intensive motility of spermatozoa (Kucharczyk *et al*., 1996). Concentration of spermatozoa was assessed by a cytometric method (Bürker chamber) (Glogowski *et al*., 1997).

Females were checked each hour between 12 and 24 hours after resolving injections. Eggs were stripped into a plastic vessel and were fertilized using a "dry method" (Kucharczyk *et al*., 1997a). Only those sperms were taken for egg fertilization that showed the motility of more than 80% of spermatozoa. Two egg samples (250–300 eggs each) from each female were mixed with 0.5 ml of pooled milt sample. Eggs were incubated on Petri dishes at 20–21°C, which was found as an optimal temperature for bream embryonic development (Kucharczyk *et al*., 1997d). The incubation time to mass hatching was 90–100 degree-days (D°).

Milt from males induced to spermiate was collected one day after hormonal stimulation. All treatments with these samples were the same as described above.

All spawners were kept one week after the end of the experiment (time of obtaining gametes) to observe their survival.

Statistical analysis

Statistical differences between groups (spermiation success and incubation success) were analysed with Duncan's multiple range test ($P < 0.05$) (Platt, 1977).

RESULTS

Males from the control group produced a smaller volume of milt with lower spermatozoon concentration and motility than those after hormonal stimulation (Table 3). Generally, there were no statistical differences between milt parameters collected from males after hormonal stimulation. Survival of males used in this experiment was excellent.

In the experiment when the fish were induced every three days, the situation was similar as described above. Males from treated groups produced milt during thirteen days (Figure 1), in contrast to them the control ones gave milt only by the 7th day of the experiment. On all days the highest quantity of milt was recorded in the group stimulated with ovopel (group 2); the maximum quantities (about 6 ml/kg) were observed on days 4 and 7. In all collected milt samples the semen obtained from control males (group 3) was characterized by lower spermatozoon motility (Figure 2) and concentration (Figure 3). The highest percentage of motile spermatozoa in treated groups was observed on day 4. Except the last days of the experiment, there were no statistical differences in spermatozoon motility between treated groups. Generally, the highest spermatozoon motility was found out in the sperm obtained from fish treated with ovopel. The highest spermatozoon concentration was noted on day 4 in group 2. From the 7th day statistical differences in spermatozoon concentration between treated groups were observed.

Table 2. The doses of hormones applied to involve spermiation of common bream (*Abramis brama* L.). Numbers of males in each group was 10

Group	Days of applied hormonal stimulation				
	0	3	6	9	12
1	3 mg CPE	3 mg CPE	3 mg CPE	3 mg CPE	3 mg CPE
2	1 ovopel pellet	1 ovopel pellet	1 ovopel pellet	1 ovopel pellet	1 ovopel pellet
3	+	+	+	+	+

+ – injection from 0.9% NaCl

Table 3. The results (± SD) obtained in artificial spawning of common bream (*Abramis brama* L.). Data marked with the same letter do not differ statistically. The data characterizing semen and egg qualities were acquired from all males and from the females that ovulated. Groups are described in Material and Methods

Group of fish	Control	1	2
Nos. of males	8	10	10
Spermiation success (%)	100	100	100
Quantity of milt (ml/kg)	2.1 ± 0.3[b]	4.3 ± 0.4[a]	4.4 ± 0.5[a]
Spermatozoon motility (%)	62 ± 21[b]	86 ± 9[a]	85 ± 11[a]
Spermatozoon concentration (× 10^9)	6.8 ± 1.1[b]	9.3 ± 1.2[a]	10.2 ± 1.4[a]
Mortality of males (%)	0	0	0
Nos. of females	12	16	18
Percentage of ovulation	0	62	100
Oocyte maturation in non-ovulated females	slight	yes	–
Latency time (hrs)	–	14–16	16–20
Embryo survival (%)	–	45.9 ± 21.7[b]	69.3 ± 12.6[a]
Mortality of females (%)	0	12.5	5.5

Generally, the best results of bream milt qualities were obtained when the males were treated with ovopel. There were no problems with fish survival used in this experiment.

Large differences were observed in the percentage of ovulation between groups (Table 3). In group 3 all females ovulated, in group 2 about 60% of them whereas in the control group no ovulation

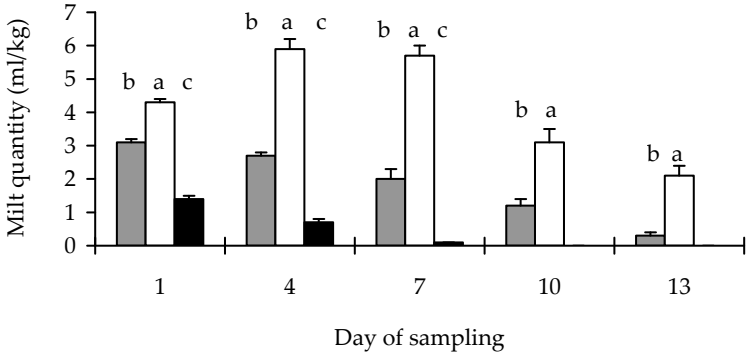

Figure 1. The quantity of milt (ml/kg) obtained from common bream (*Abramis brama* L.) males during 13 days after hormonal stimulation. Vertical bars show SD. Data marked with the same letter do not differ statistically. Groups are described in Table 2

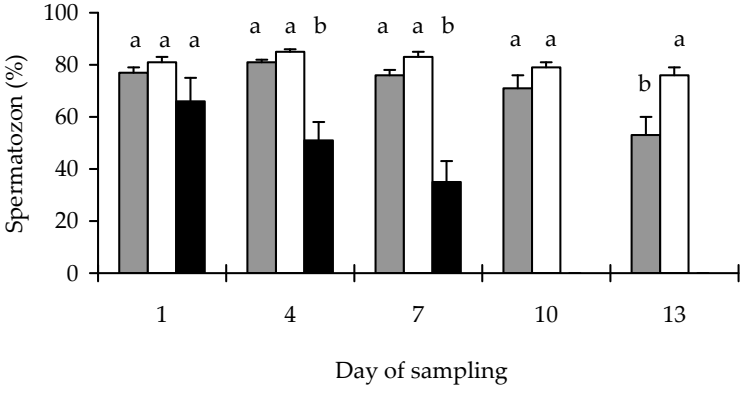

Figure 2. The spermatozoon motility collected from common bream (*Abramis brama* L.) males during 13 days after hormonal stimulation. Vertical bars show SD. Data marked with the same letter do not differ statistically. Groups are described in Table 2

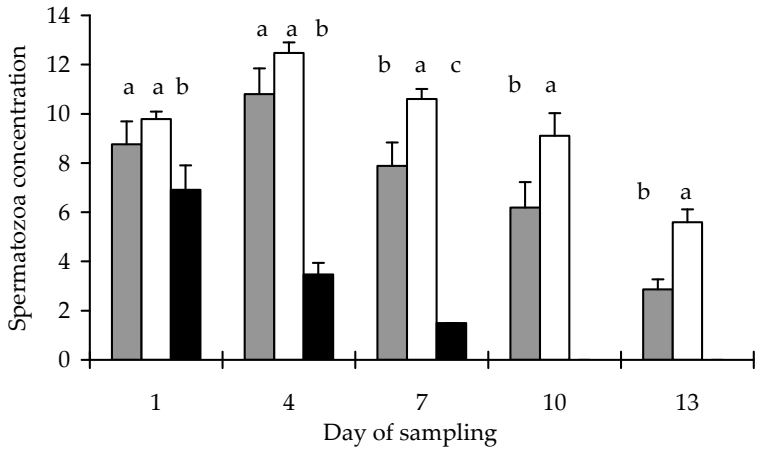

Figure 3. Spermatozoon concentration (± SD) determined in milt obtained from common bream (*Abramis brama* L.) males during 13 days after hormonal stimulation. Data marked with the same letter do not differ statistically. Groups are described in Table 2

was noted. Oocytes collected from non-ovulated females after hormonal stimulation with hCG and CPE showed significant maturation: from stages 2–3 and 3 to 3–4 and 4. In oocytes sampled from control fish only limited (slight) maturation was observed. Fish from group 2 ovulated earlier than those from group 3. Better biological quality of eggs, expressed as an embryo survival to the eyed stage, was observed in group 3. A low level of mortality was generally observed after spawning.

DISSCUSION

In many papers dealing with artificial spawning of cyprinids the problem of spermiation was not the aim of the study (Thalathiah *et al*., 1988; Glubokov *et al*., 1991; Drori *et al*., 1994; Barth *et al*., 1997; Horvath *et al*., 1997; Fornies *et al*., 2001; Dumas *et al*., 2004). Only a limited number of papers studied this problem (Takashima *et al*., 1984; Kucharczyk *et al*., 1997a). It is well known from hatchery practice of cyprinids, especially from wild stocks or populations, that one of the main problems is not ovulation but a small volume of sampled milt, in many cases with low spermatozoon concentration and bad spermatozoon motility (Kucharczyk, 2002).

As in the previous study (Kucharczyk *et al*., 1997a) common bream males after hormonal stimulation produced significantly more milt than those from the control group with better spermatozoon motility. The volumes of collected sperm in Eurasian perch (*Perca fluvaitilis* L.) (Kucharczyk *et al*., 1998), yellow perch (Dabrowski *et al*., 1994) and common carp (Takashima *et al*., 1984) treated with hormones were also significantly higher in contrast with control groups. In gudgeon (*Gobio gobio* L.) the injection of LHRH with addition of pimozide did not result in more milt than from "control" males (Kestemont, 1989).

Males used for continuous sampling of milt (every three days) initially produced a higher volume of sperm than those hormonally stimulated. On the other hand, the dose of applied hormones was also higher. It is very interesting that common bream males produced high quality milt for thirteen days. Probably, this phenomenon is strictly connected with spawning behaviour of this species. Brylinska and Tadajewska (1986) informed that bream males were the first to be present on spawning grounds and were kept there by the end of the spawning season. In Kortowskie lakes, from which spawners were taken, bream can spawn from April to July, which is in contrast with other neighbouring populations (Kucharczyk, 1996). The time of spawning depends on water temperatures. The results obtained in this study show that hormonal stimulation increases production of semen as well as spermatozoon concentration and motility. It is important not only for artificial propagation of this species under hatchery conditions but also for any restoration programs, i.e. gene bank or cryopreservation (Wildt *et al*., 1993; Glogowski *et al*., 1997; Babiak *et al*., 1998).

The GnRH analogues, combined with strong dopamine antagonists, were generally very good in wild and cultured species stimulation (Barth *et al*., 1997; Horvath *et al*., 1997; Brzuska, 1999, 2000). However, discussion about these data is rather difficult because different forms of GnRH analogues have usually been used, and also in different doses. Different forms of GnRH-a, sometimes from different sources, e.g. mammalian, fish, chicken, etc., have different activity. Important differences were observed in latency time after the application of different spawning media. The shortest time between

injections and ovulation was noted when CPE with hCG was used as a spawning agent, in contrast to the fish stimulated with ovopel. The differences in latency in females treated with CPE and GnRH were reported in many papers. It may be explained by the fact that GnRH release from the pituitary and the ovarian response to the released hormones is a sequential process while in fish injected with carp pituitary extract the ovarian response to the exogenous GtH was a single process.

The application of ovopel (GnRHa with dopamine antagonist) influenced high spawning success. All females ovulated, which is in contrast with the results of the other groups. The percentage of ovulation using hCG and CPE was much lower than in a previous study (Kucharczyk *et al*., 1996). Probably this difference is a result of high water temperature fluctuation in the Kortowskie Lake before the spawning season. Temperature is the main environmental factor that strongly influenced gamete development, maturation and spawning success (Tveiten and Johnsen, 1999). The differences in tolerated temperature in spawning success and survival rate of embryos and larvae and their development were observed not only in different fish species but also in different populations (Leskalä and Kucharczyk, 1995). Kokurewicz (1971) reported that the range of spawning temperatures experienced by bream throughout its geographic distribution area is quite wide (between 10 and 27°C). This range of temperatures is very similar to the temperatures observed in May and June on bream spawning grounds in the Kortowskie Lake, inhabited by the studied bream population. Our five years observations showed that the temperatures during the spawning time (May and June) of bream fluctuated on spawning grounds between 13.5 and 26.5°C (Kucharczyk, 2002). The changes in water temperature strongly influenced the spawning success of bream using CPE and hCG. But ovopel, a spawning agent containing GnRHa and metoclopramide works very well, significantly better. It is concluded that this kind of hormonal stimulation might be successfully used for artificial propagation of wild cyprinid fish.

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Corresponding Author

Dariusz Kucharczyk, Department of Lake and River Fisheries, Warmia and Mazury University in Olsztyn, ul. Oczapowskiego 2, 10-718 Olsztyn-Kortowo, Poland
Tel. +48 89 523 42 15, e-mail: darekk@moskit.uwm.edu.pl