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Out-of-season artificial reproduction of common dace (*Leuciscus leuciscus* L.) under controlled conditions

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ABSTRACT

This study focused on the artificial reproduction of common dace *Leuciscus leuciscus* (L.) outside the reproductive season. The results indicate there is the possibility of initiating the reproducion in individuals of this before the natural spawning season. There could be induction of spermiation by altering the environmental conditions. For females, hormonal injections were necessary for induction of final oocyte maturation and ovulation. Generally, there were high percentages of spermiation (100%) and ovulation (87%–100%) as well as low mortality (0%–11% and 7%–13% for males and females, respectively) among the induced spawners. The greatest embryo survival occurred when Ovaprim (84.4%) or hCG (89.6%) was administered, although the latency time using hCG was at least twice as long compared to when other spawning agents were used (84–92 hrs and 30–44 hrs). The results from present study could be applicable for optimization of breeding stock numbers in aquaculture enterprises and in providing insights for conservation of *L. leuciscus* endangered populations.

1. Introduction

Common dace *Leuciscus leuciscus* (L.), together with several other fish species, is included in the group of European riverine cyprinids. *L. leuciscus* is present in large areas of Europe most frequently populating the upper and middle river sections. With the climatic conditions of Poland, reproduction occurs most frequently from March until May during midday hours at water temperatures from 10 to 12 °C (Kokurewicz, 1971). Dace, due to its small size, does not have any economic importance. Because of that, for many years, similar to other rheophilic cyprinids, this species was almost entirely unknown to those other than ichthyologists, fishermen and anglers. During recent years, however, the interest in rheophilic species (including *L. leuciscus*) has increased significantly (Kupren et al., 2011; Nowosad et al., 2014a; Targońska et al., 2015) because of the threat of local population loss or the total extinction of the species. The loss of spawning grounds caused by pollution of the aquatic environment or regulation of rivers is the major cause of the loss of numbers of dace. In recent years, wide temperature fluctuations, however, have been observed during the spring period when dace spawning occurs. Nowosad et al. (2014a) reported that temperature fluctuation has a negative impact on *L. leuciscus* spawning efficiency, including ovulation success and embryo survival rate. Besides improving environmental conditions, the production of breeding stock based on reproduction, incubation and initial rearing under controlled conditions is one of the possible

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solutions for protecting riverine fish (Cejko et al., 2012; Harzevili et al., 2013; Cejko and Kucharczyk, 2015; Kujawa et al., 2015; Kupren et al., 2015; Kucharczyk et al., 2016; Nowosad et al., 2016). Artificial reproduction is also an element necessary for the development of genome engineering techniques and cryopreservation of semen (Kucharczyk, 1999, 2001; Nowosad et al., 2014b, 2015) which might be useful for gene pool protection.

A relatively large number of studies on reproduction have been conducted on another representative of the genus Leuciscus, the ide *Leuciscus idus* (Targońska et al., 2011, 2012; Sarosiek et al., 2012; Cieśla et al., 2014; Nowosad et al., 2018) due to its greater economic importance. A limited study on *L. leuciscus* artificial reproduction during the spawning season exists (Cejko et al., 2012; Nowosad et al., 2014a; Targońska et al., 2015), but no attempts were made to achieve artificial reproduction outside (before) the natural spawning time. This was because of the great amount of temperature fluctuation during the dace spawning season (Nowosad et al., 2014a), which provides for an opportunity to artificially reproduce this species before water temperature fluctuations start to occur in the early spring season. For these reason, it is very important to determine if it is possible to have an induced season of reproduction in common dace. Important reproductive variables for this determination are, rate of ovulation, spermiation, and embryo survival when there is out-of-season reproduction. The aim of the present study, therefore, was to induce out-of-season artificial reproduction protocol of *L. leuciscus* under controlled conditions and assess important reproductive variables.

2. Material and methods

2.1. Fish

Dace spawners (weight from 90–240 g) were obtained during early December from flow-through lakes on the Szwaderki Fishery and Janowo Fish Farms (north Poland). The fish were obtained using dragging equipment (drag-net).

2.2. Photothermal and hormonal stimulation

Some of the fish were transported directly to the hatchery of the Department of Lake and River Fisheries, University of Warmia and Mazury, where there was placement in 1000-L tanks with aeration and temperature control (Kujawa et al., 1999). Following a 2-week period of housing the fish at 2 °C, the process of increasing the water temperature by 2 °C per week was initiated so that there was a 10 °C temperature in the tanks by mid-January. During the time of temperature adjustment, the males were observed to excrete small quantities of semen and there was the appearance of the spawning rush on the heads and the entire upper part of the male's body. The fish were marked and the maturity of oocytes (1–2 or 2 stadium) was verified using a catheter and the methods described by Brzuska (1979). The fish were divided into five groups (Table 1). Males and females from individual groups were injected with CPH (homogenate from the pituitary gland of carp – Argent, Redmond, WA, USA), Ovopel (Hungarian preparation containing a super-active analogue of mammalian GnRH and dopamine inhibitor metoclopramide, Uni-trade, Budapest, Hungary), Ovaprim (Canadian preparation containing a super-active analogue of salmon GnRH and dopamine inhibitor domperidone, Syndel, Qualicum Beach, BC, Canada) and human chorionic gonadotropin (hCG, Intervet International, Boxmeer, The Netherlands). One Ovopel pellet contains 18 to 20 μg of mGnRHa and 8 to 10 mg metoclopramide (Horvath et al., 1997*)* and 1 mL of Ovaprim contains 20 μg sGnRHa and 10 mg domperidone (Peter et al., 1993). The dose for ovulation induction was divided into two parts for each experimental group. The first injection (priming dose) contained 10% of the amount of the second doses (resolving dose). Males were administered a single injection containing half the dose of that of the second doses administered to females (Table 1). Simultaneously with the second injection, the water temperature was increased to 12 °C, which was found to be the optimal temperature for common dace artificial reproduction (Nowosad et al., 2014a) and embryo development (Kupren et al., 2011). Doses of hormones, numbers of individuals in groups as well as number of injections and the time between the injections are presented in Table 1. The injections were administered intra-peritoneally under the ventral fin. All hormonal preparations (except Ovaprim) were homogenized and diluted in sterile 0.9% NaCl (Nowosad et al., 2014a; Targońska et al., 2015). Before any manipulations were conducted, the fish were anaesthetised in a solution of MS-222 (0.15 mg/kg) (Finquel Argent, Redmond, WA, USA).

Table 1
Hormonal doses applied in artificial reproduction of dace (*Leuciscus leuciscus* L) in out-of-season spawning in controlled conditions; Time between injections in females was 24 h. Number of spawners is presented in Table 2.

Group	Males	Females	
	Hormonal dose	Primary dose	Resolving dose
1 (control)	0.9% NaCl	0.9% NaCl	0.9% NaCl
2 (CPH)	2.0 mg	0.36 mg	3.6 mg
3 (Ovopel)	0.5 pellet	0.1 pellet	1 pellet
4 (Ovaprim)	0.25 m L	0.05 mL	0.5 m L
5 (hCG)	500 IU	100 IU	1000 IU

2.3. Gametes' collection and fertilization

The semen was obtained by conducting a belly massage (Cejko et al., 2012) into syringes calibrated at 0.01 mL. Before determination of sperm motility, the semen was stored in a refrigerator at 4 °C. Sperm motility was determined using a microscope (magnification 500×) by diluting the semen in a 0.5% NaCl solution immediately before observation at 1:1 using the method described by Glogowski et al. (1997) for common bream. Females were assessed every 4 h between hours 24 and 48 after the resolving injection and later every 12 h until 96 h after the injection. The eggs were collected into plastic containers through applying the appropriate amount of pressure on the ventral area of females and eggs remained in the collection containers until fertilization, which was conducted by using the "dry" method modified by Kucharczyk et al. (2016) for the ide and asp species. For this purpose, eggs were mixed with semen and the water was subsequently added. Only sperm having a motility exceeding 60% were used. Egg incubation was conducted in Petri dishes. Eggs from each female were placed separately in three dishes containing 80 to 100 eggs each, fertilized with 0.05 mL of pooled sperm and then appropriately marked samples were placed in a tank with continuous fresh water inflow. The incubation temperature was 12 °C which was determined to be optimal for dace embryonic development (Kupren et al., 2011). At the time of the eyed-egg-stage (appearance of pigment in the embryos eyes) embryonic development, the percentage of live embryos was determined.

The research was conducted in accordance with the permission of the local ethics committee for animal experiments (27/2010N for years 2010–2015).

2.4. Statistical analysis

The assessment for normality of the data distribution, expressed as mean ± SD, was ascertained using SPSS software version 18. Differences between the means of the groups for each variable were evaluated using an ANOVA and the Tukey's multiple range test for group comparisons. A $P < 0.05$ was considered to be significant.

3. Results

The results of dace spawning outside the natural spawning season are presented in Table 2. Spermiation was observed in males from all groups, including the controls. The volume of semen produced was similar in all cases, ranging from 0.2 and 0.4 mL/kg bodyweight. There were treatment differences for motility of spermatozoa. For males subjected to hormonal stimulation, sperm motility rates were similar (67%–73%) among groups and greater than in the controls (53%) (Table 2). For females subjected to stimulation with only the environmental factors (light and temperature), there was a small amount of oocyte maturation without ovulation occurring during the experiment. All fish injected with hormones, however, had oocytes that were of high quality. The embryo survival rate from fertilization until the eyed-egg-stage of development was quite similar, ranging from 72% to 90%, when the greatest embryo survival rates in groups where there was stimulation of oocyte development and ovulation with Ovaprim and hCG (Table 2). There were also differences in the values for artificial spawning variables among the groups where there was hormonal stimulation. Those differences appeared in both: embryo survival rates and the time between the last injection and ovulation (latency time) and in the embryo survival rates of spawners in the different treatment groups. Fish stimulated with CPH had ovulations about 3 to 4 h earlier than those treated with Ovopel, 10 h earlier than those treated with Ovaprim and the time to ovulation (30 to 33 h) was about half that of specimens treated with hCG (84 to 92 h). The mortality of embryos derived from breeders was very little and similar for all groups (Table 2). The relationship between latency time and embryo survival indicated that in out-of-season artificial reproduction of common dace, the greatest embryo survival occurred when the eggs were obtained from females with later ovulations (Fig. 1).

Table 2
The Results of out-of-season artificial reproduction of dace (*Leuciscus leuciscus* L) using different hormonal agents under controlled conditions. The data with the same letter in rows did not differ statistically ($P < 0.05$).

Group of fish	1 (Control)	2 (CPH)	3 (Ovopel)	4 (Ovaprim)	5 (hCG)
No. of males	8	9	9	9	9
Spermiation [%]	100	100	100	100	100
Semen quantity [ml*kg^{-1}] (± SD)	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.2 ± 0.1
Spermatozoa motility [%](± SD)	53 ± 6***b***	67 ± 7***a***	68 ± 8***a***	73 ± 8***a***	70 ± 7***a***
Males mortality [%]	0	11	11	11	0
No. of females	10	15	15	15	15
Oocytes maturation stage	1/2-2	1/2-2	1/2-2	1/2-2	1/2-2
Ovulation rate [%]	0	100	100	100	87
Maturity of oocytes in non-ovulated females	Slightly (to 2 stage – 70%) or resorbtion (30%)	–	–	–	High (to 4-stage)
Latency time [hrs]	–	30 – 33	36 – 40	36-44	84-92
Embryos survival [%](± SD)	–	72.5 ± 5.4***b***	75.8 ± 6.2***b***	84.4 ± 6.6***ab***	89.6 ± 5.1***a***
Females mortality [%]	10	13	13	7	7

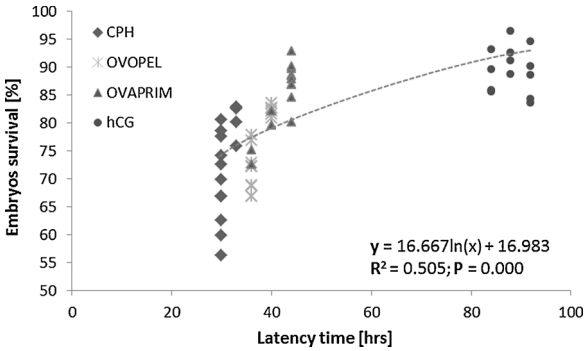

Fig. 1. Relationship between latency time and embryo survival in out-of-season artificial reproduction of common dace (*Leuciscus leuciscus* L) using different hormonal treatments in controlled conditions.

4. Discussion

The results of dace reproduction outside the natural spawning season under controlled conditions were very similar to those observed with other species of the genus Leuciscus, ide (Targońska - Dietrich et al., 2004). For both species, stimulation by changing the environment conditions (mainly temperature) only resulted in semen production but there were no treatment effects detected in females. The fact that males subjected to hormonal stimulation produced semen of greater quality, although in similar volumes, than the controls was an obvious positive effect of hormonal administrations. Comparing the data obtained in the present study with the results of the dace spawning study conducted during the spawning season (Cejko et al., 2012; Nowosad et al., 2014a; Targońska et al., 2015) males certainly produced less semen (less than half) outside the spawning season. The sperm motility in the present study was similar to that of sperm collected during the spawning season in the previous studies. Similar observations also apply to ide (Targońska-Dietrich et al., 2004; Targońska et al., 2011; Sarosiek et al., 2012) and common bream *Abramis brama* (L.) (Kucharczyk et al., 1997, 2005). In some other cyprinids (e.g., domesticated zebrafish; *Danio rerio*), the spawners can reproduce spontaneously under controlled conditions without exogenous hormonal stimulation (Nowosad et al., 2017).

In contrast to the males, female treatment with hormones was an absolute requirement for ovulation, similar to what has been ascertained during the spawning season (Targońska et al., 2015). Treatments with the two tested preparations (i.e., CPH and Ovopel) similar to what occurred with ide (Targońska-Dietrich et al., 2004) ensured obtaining eggs from all females. The other two preparations used in the present study: Ovaprim and hCG, which were not assessed in studies of out-of-season spawning with ide, also resulted in high ovulation and embryo survival rates. Generally, greater survival rates at the eyed-egg-stage of development observed during the incubation of eggs obtained both before and during the spawning season indicates the gametes are of high quality. The time calculated from administration of the resolving injection until obtaining the eggs (ovulation) was slightly longer with use of Ovopel and Ovaprim in comparison to CPH. These results are similar to those for cyprinids, and Eurasian perch *Perca fluviatilis* (L.) (Kucharczyk et al., 1996, 1998). This is a consequence of the fact that the extract from pituitary gland functions directly on the gametes to effect the maturation, while the isolated gonadoliberins or the artificial analogues (Yaron, 1995), including Ovopel, have an indirect effect on endocrine glands of females inducing the excretion of reproductive hormones causing gamete maturation. Comparing the time between the last injection and ovulation of female dace during (Targońska et al., 2015) and outside the spawning season, those times were similar in the present and previous study, although ovulation outside the season occurred somewhat later. There was a very long latency time in groups treated with hCG – three times longer than with CPH treatment. With artificial reproduction of single spawning cyprinids (e.g., ide, bream), administration of hCG, usually did not result in oocyte development and ovulation (Kucharczyk et al., 1997, 2005). In the present study, the latency time after treatment with hCG was very long and with this treatment there was the greatest embryo survival rate. There was a similar previous finding with northern pike (*Esox lucius* L.), where there were longer latency times after administration of hCG to ovulation with greater fertilization rates as a result of the use of hCG compared with other hormonal treatments (Szabo, 2008).

The mortality of dace spawners (males and females) as compared to the results achieved during the spawning season was similar (Targońska et al., 2015) and was low due to optimal conditions for common dace spawners kept under controlled conditions. It is concluded that by applying appropriate hormonal preparations, common dace can be successfully reproduced in hatchery conditions outside the spawning season with ovulations occurring at a highly acceptable rate for artificial reproduction and the biological quality of the gametes being very good. The approach used in the present study provides for the possibility of enhancing the populations of dace and consequently avoiding the threat of loss of local populations or the total extinction of the species

Conflict of interest statement

The authors declare that they have no conflict of interest.

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