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RESEARCH ARTICLE

Influence of temperature on the effectiveness of the hormonal stimulation of male ide, *Leuciscus idus* (L.)

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Abstract. Increasing the temperature of water (by 1-2°C) in which spawners of rheophilous fish are held following hormonal stimulation until gametes are obtained is a common procedure in hatching practice. The aim of the study presented here is to determine the influence of water temperature following stimulation with Ovopel on the investigated quantitative and qualitative characteristics of ide, *Leuciscus idus* (L.), milt. The experiment comprised subjecting the fish to hormonal stimulation using a single intraperitoneal injection of Ovopel at a dose of 1 pellet kg^{-1} of body weight. After injection, the water temperature of group I was maintained at a constant level of 10°C, while for the other two groups it was increased to 12°C (group II) and 14°C (group III) within 4 hours. Changes of temperature after hormonal injection had no statistically significant effect either on milt quantity or its quality parameters. The values of motility were low in all groups (29, 30, 35% for groups I, II, and III, respectively). The mean values of seminal plasma osmotic pressure were 129, 139, and 154 mOsm kg^{-1} for groups I, II, and III, respectively. The mean values of spermatozoa concentration in the milt of the males held after hormonal stimulation in water at 10, 12, and 14°C were 10.68, 10.71, and 9.92 $\times 10^9$ ml^{-1}, respectively. Following the injections of Ovopel, total number of spermatozoa produced by the males (TSP, $\times 10^9$) was the highest in the group held at 12°C (3.03 $\times 10^9$); however, it was not statistically different from the other groups. No significant differences in either TSP or the number of spermatozoa per kg of their body weight (TNS, $\times 10^9$ kg^{-1} b.w.) among the studied groups were noted ($P > 0.05$). In contrast to females, there is no need to change the water thermal conditions for ide males after hormonal stimulation.

Keywords: *Leuciscus idus*, Ovopel, temperature, milt, spermatozoa

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Introduction

Temperature is the main environmental factor influencing the life processes of fish and other heterothermal aquatic organisms. Every stage of life, starting with the embryonic, to larval and stock development, through the development of mature individuals and their reproduction, requires appropriate thermal conditions depending on species biology. Temperature determines not only the achievement of sexual maturity, but also the period during which fish spawn (Bromage et al. 2001, Hilder and Pankhurst 2003, Anguis and Canavate 2005). In the case of cyprinids, temperature determines the quality

of gametes produced by the fish, and its influence is apparent as early as in the egg incubation stage in the development rate, embryo hatching, and their early ontogenesis (Kucharczyk et al. 1997, 1998, Kujawa et al. 1999).

The artificial reproduction of rheophilous cyprinid fish of the genus Leuciscus is impossible without hormonal or thermal stimulation (Vanderplank 1938, Cieśla 1998, Jamróz et al. 2008, Krejszeff et al. 2008, Cejko et al. 2010), with the few documented exceptions of totally domesticated fish (Krejszeff et al. 2009). Therefore, hormonal substances that act upon the pituitary gland or the gonads are applied to achieve artificial reproduction (Kucharczyk et al. 2008). Ovopel [(D-Ala^{6}Pro^{9}NEt)-mGnRH and metoclopramide] is popular in current fishery practice because of its low price (in comparison with other hormonal substances available commercially), easy preparation, simple spawner application procedure, and high effectiveness (ovulation/spermiation) (Hakuć-Błażowska et al. 2009).

Increasing the water temperature (by 1-2°C) at which spawners of rheophilous fish are held following hormonal stimulation until gametes are obtained from them is a frequent procedure applied in hatching practice (Jamróz et al. 2008, Cejko et al. 2010). The aim is to accelerate the last stage of the maturation of spermatozoa in the spermatic ducts and the ovulation of eggs. Of all the rheophilous cyprinid fish, the reproduction of ide under controlled conditions has been studied most widely to date (Kucharczyk et al. 1999, 2008, Jamróz et al. 2008, Cejko et al. 2010), which is reflected in increasing stocking levels of this species from year to year (Turkowski et al. 2008). However limited information is available regarding the detailed characteristics of ide milt (Kowalski et al. 2003, Cejko et al. 2010). The aim of the present study was to determine the influence water temperature following stimulation with Ovopel had on the quantitative and qualitative characteristics of ide milt.

Materials and Methods

Origin and transport of fish

The breeding school of male ide aged three years with body weights of 0.14-0.32 kg originated from the Knieja Fish Farm near Częstochowa (southern Poland), where in April 2008 they were caught from earthen ponds and transported to an aquarium hall at the Department of Lake and River Fisheries, University of Warmia and Mazury in Olsztyn. First, the fish were stocked into one tank with a volume of 1 m^3 with open circulation and full temperature and photoperiod control (Kujawa et al. 1999). The water temperature at which the male ide were held was initially 10°C, which is considered to be the optimum temperature during that period (Kucharczyk et al. 2008).

Hormonal stimulation and manipulations with spawners

After six days of adaptation, 33 fish were subjected to hormonal stimulation with a single intraperitoneal injection of Ovopel, (Unic-trade, Hungary) at a dose 1 pellet kg^{-1} of body weight (Kucharczyk et al. 2008). Fifteen fish were not injected (control group). Next, the fish were separated into three tanks (11 stimulated and 5 non-stimulated fish per group). In group I the water temperature was maintained at a constant level of 10°C, but it was increased to 12°C for group II, and to 14°C for group III over the course of four hours. Thirty-six hours following injection, the fish were caught from the tanks, and the milt was collected into sterile syringes by delicately massaging the abdomens.

All of the males from the control groups remained immature; therefore, no milt samples were obtained from the 15 control fish from the three groups. Before milt collection from the experimental groups, the individuals were weighed. Both body weights and the quantity of milt obtained were recorded. The milt was collected carefully to avoid contamination with urine, feces, or blood. The fish were anesthetized prior to

manipulation with 2-phenoxyethanol at a dose of 0.5 ml dm^{-3} (Sigma-Aldrich, St. Louis, MO, USA). After the manipulations the fish were revived and returned to the tanks.

Determination of the basic parameters of milt

The collected milt samples were transferred from syringes to tubes and transported on ice (+4°C) to the Department of Gamete and Embryo Biology (DG&EB), Institute of Animal Reproduction and Food Research, Polish Academy of Sciences in Olsztyn where the material was analyzed in detail. Immediately after the milt was delivered to the DG&EB, the motility of the spermatozoa (%) was determined with the subjective method using a light microscope under ×400 magnification. The spermatozoa were activated by mixing 1 µl of milt with 30 µl of activation solution containing 86 mM NaCl and 0.5% bovine serum albumin (Sigma-Aldrich, St. Louis, MO). The values determined were accurate to within 10% (Cejko et al. 2010). The spermatozoa concentration in the milt ($\times 10^9$ ml^{-1}) was determined with the spectrophotometric method described by Ciereszko and Dabrowski (1993). The milt was diluted with 0.7% NaCl (Sigma-Aldrich, St. Louis, MO) at 1:1000 and the absorption of the samples was measured using a Beckman DU-640 spectrophotometer (Analytical Instruments, LLS, Golden Valley, MN, USA) at α=530 nm. The seminal plasma was extracted by centrifuging the milt for 10 min at 10.000 × g. The supernatant was transferred to test tubes, and the seminal plasma osmotic pressure (mOsm kg^{-1}) was determined using a Vapor Pressure Osmometer 5520 (WESCOR, Logan, UT, USA).

Determination of milt volume and spermatozoa number

The total volume of milt (TVM, ml) was measured during collection using syringes with a 0.01 ml calibration. The weight of the males (kg) and the TVM were used to calculate the volume of milt obtained in ml per kg^{-1} of spawner body weight (VOM, ml kg^{-1} b.w.). The TVM and the concentration of spermatozoa in the milt were used to calculate the total sperm production in billions (TSP, $\times 10^9$). The TSP and the weight of the males were used to calculate the total number of spermatozoa in billions per kg^{-1} body weight (TNS, $\times 10^9$ kg^{-1} b.w.).

Statistical analysis

The results obtained were characterized using arithmetic means ($\bar{x}$) and standard deviations (±SD). The percentage data were normalized with arcsin transformation. The data were tested for normal distribution (D'Agostino and Pearson's omnibus normality test) and equal variances (Bartlett's test). Tukey's test (One-way ANOVA, α=0.05) was applied to present the significance of the differences among groups of fish for the quantitative and qualitative characteristics of milt.

Results

As the temperature of water in which the males were held following hormonal stimulation increased, the percentage of motile spermatozoa increased gradually to 35% in the fish held at the highest water temperature of 14°C. The values of motility were slightly lower at 29 and 30% for groups I and II, respectively, for the males held at lower water temperatures. It should be noted that mean motility values did not exceed 40% in any of the groups studied (Table 1), and they did not differ significantly among the groups of fish ($P > 0.05$). The highest mean values of seminal plasma osmotic pressure were recorded for the males held in the water temperature of 14°C following stimulation (154 mOsm kg^{-1}), while at lower temperatures the osmotic pressure values were lower, but they were statistically insignificant (Table 1; $P > 0.05$).The mean values of spermatozoa concentration in the milt of the males held after hormonal stimulation in water at 10 and 12°C were similar at 11 $\times 10^9$ ml^{-1}. The samples of

Table 1
Influence of increased water temperature on the quantity and quality parameters of ide *Leuciscus idus* (L.) milt (N=11) after hormonal stimulation with Ovopel (1 pellet kg^{-1}), total sperm production (TSP), total number of spermatozoa (TNS), total volume of milt (TVM), volume of milt per kg of body weight (VOM), Mean+SD, ($P > 0.05$). Group I – males held after hormonal stimulation at 10°C; group II – males held after hormonal stimulation at 12°C; group III – males held after hormonal stimulation at 14°C

Fish group	Body weight (g)	Motility of spermatozoa (%)	Osmolality (mOsm kg^{-1})	Concentration of spermatozoa ($x10^9$ ml^{-1})	TSP ($x10^9$)	TNS ($x10^9$ kg^{-1})	TVM (ml)	VOM (ml kg^{-1} bw)
I (10°C)	203±21.28	29±17.30	129.62±31.23	10.68±3.89	1.85±0.75	9.23±3.91	0.20±0.23	1.46±1.54
II (12°C)	243±51.37	30±14.99	139.59±22.35	10.71±3.03	3.03±2.10	13.72±6.99	0.30±0.14	1.23±0.84
III (14°C)	209±43.36	35±15.81	153.67±37.37	9.92±3.94	2.97±2.22	13.99±9.83	0.31±0.21	1.48±0.99

milt collected from the fish held at the highest water temperature following stimulation with Ovopel were characterized by lower concentrations (Table 1). The concentration values at each of the temperatures analyzed did not differ significantly ($P > 0.05$). Following the application of Ovopel, the TSP in billions reached the highest values in the group of males held at 12°C (3.03×10^9). The decidedly lowest values of this parameter were recorded at the water temperature of 10°C (1.85×10^9), but no significant differences were noted (Table 1, $P > 0.05$). The number of spermatozoa per kg^{-1} body weight (TNS) increased gradually as the water temperature at which the spawners were held increased. The lowest TNS values were recorded for the males held at 10°C following treatment with Ovopel (9.23×10^9 kg^{-1} b.w.), while the highest values were recorded for males held at 14°C following injection (13.99×10^9 kg^{-1} b.w.). No significant differences in either TSP or TNS among the groups of fish were noted ($P > 0.05$).

As the water temperature at which ide males were held following stimulation with Ovopel increased, a gradual increase in the TVM obtained from the fish was observed, and the highest increase was noted between 10°C (0.20 ml) and 12°C (0.30 ml) in groups I and II (Table 1). The analysis of the VOM indicated that the values for the fish held at the lowest and highest temperatures after hormonal stimulation were similar (group I – 1.46 ml kg^{-1} b.w.; group II – 1.48 ml kg^{-1} b.w.; Table 1). In spite of the increased TVM and VOM values noted as water temperature increased, they did not differ significantly among the groups of fish ($P > 0.05$).

Discussion

Kucharczyk et al. (1999) obtained significant volumes of good quality milt from ide males during the spawning season at 15°C following stimulation with Ovopel (VOM – 4.4 ml kg^{-1} b.w.; motility of spermatozoa – 75%). The authors suggested that the good quality of gametes is correlated with the appropriate water temperature in which the spawners are held until milt collection. In the current experiment, temperature was irrelevant to milt production expressed in TVM or VOM, and no significant differences were noted among the groups (Table 1). Previous studies on the influence of time period following hormonal stimulation on quality parameters of ide milt indicated that at 12°C the TVM value was 0.35 ml 36 hours after Ovopel treatment (Cejko et al. 2010). The value of this parameter in the present study was similar at a mean of 0.30 ml (Table 1). The volume of milt collected from ide males of similar body weights (under 0.20 kg) held at the same thermal conditions and receiving the same hormonal treatment can be projected.

Studies by Kujawa et al. (2006) indicate that keeping rheophilous fish spawners at high temperatures can decrease the percentage of ovulating females, the quality of the eggs obtained, and the

survival rates of the embryos. These authors also suggest that the negative influence of high temperature manifests in decreased milt quality inhibiting its spontaneous release. In studies by Jamróz et al. (2008) at 14°C, milt was obtained from all ide males, and the percentage of motile spermatozoa was similar despite the hormonal substance applied (60-80%). The current results indicate that spermatozoa motility in males held at higher water temperatures does not decrease; however, this parameter was very low (below 40%) in all three groups. This might have resulted from a low initial level of male maturity, or it could have been caused by the milt transportation procedure used. Sample contamination with urine can influence milt parameters, but special care was taken when collecting the milt from the males. The collection of milt 36 h after administering Ovopel produced good results in the percentage of motile spermatozoa in this species (Cejko et al. 2010).

The results of the present study indicate that the seminal plasma osmotic pressure was low regardless of the water temperature in which the males were held (mean values below 150 mOsm kg^{-1}). It is probable that, in contrast to other cyprinid species, the seminal plasma osmolality values do not exceed 200 mOsm kg^{-1}, and this represents a species-specific characteristic of ide (Cejko et al. 2010). However, Kowalski et al. (2003) reported higher values of 240 mOsm kg^{-1}. These authors used golden orfe, which might have caused these differences (Kowalski et al. 2003). The present study indicates that mean ide plasma osmolality values did not exceed 150 mOsm kg^{-1}, while those for golden orfe exceeded this range (220 mOsm kg^{-1}, B.I. Cejko unpublished date). Another explanation for the lower osmolality could be urine contamination of the samples that resulted in lower seminal plasma osmolality and sperm motility as was described for common carp, *Cyprinus carpio* L. (Perchec et al. 1998). Although special care was taken during milt collection to ensure obtaining high quality specimens, it is difficult to avoid contamination completely since minute volumes of urine are not easily detected in milt. Monitoring the lower viscosity and color changes in milt samples containing urine is possible with sea bass, *Dicentrarchus labrax* (L.) (Fauvel et al. 1999), but this is not possible with ide. In the future, a quick test for detecting urine contamination in milt samples should be developed.

The absence of significant differences in the motility of spermatozoa, seminal plasma osmotic pressure, TVM, VOM, TSP, TNS, and the concentration of spermatozoa in milt from males held at three temperatures does not permit concluding which temperature is the most appropriate for ide males held under controlled conditions after hormonal stimulation. These results might indicate that water temperature does not dramatically affect male ide achieving maturity. At this stage of study it can be assumed that the basis for recommending temperatures lower than 14°C for male ide held before spawning is the economic cost involved in heating significant volumes of water in hatcheries. Unlike with females, there is no need to change the water thermal conditions for males after stimulation since this had relatively little influence on each of the parameters of milt quantity and quality analyzed. The results presented in this paper cast new light on current procedures and manipulations in the reproduction of rheophilous cyprinid fish, and the proposed solutions might be of significance for ide reproduction.

In summation, the current results demonstrate that male ide respond similarly to hormonal treatment at water temperatures of 10, 12, and 14°C. Although mean values of TSP, TNS, and TVN were the lowest in the samples collected from the fish held at 10°C, there were no statistically significant differences among the groups. This probably indicates that spermiation in this species is not dramatically affected by water temperature changes within the range of 10 to 14°C.

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Streszczenie

Wpływ temperatury wody po stymulacji hormonalnej na ilość i jakość mlecza jazia *Leuciscus idus* (L.)

Przeanalizowano całkowitą objętość pozyskanego mlecza (TVM, ml), objętość przypadającą na kg^{-1} masy ciała samców (VOM, ml kg^{-1} m.c.), koncentrację plemników w mleczu ($\times 10^9$ ml^{-1}), całkowitą ilość wyprodukowanych przez samce plemników (TSP, $\times 10^9$), ich ilość w przeliczeniu na kg masy ciała (TNS, $\times 10^9$ kg^{-1} m.c.), oraz jakość określoną odsetkiem plemników ruchliwych (%), w zależności od temperatury wody w jakiej przetrzymywano samce jazia po stymulacji Ovopelem (1 granulka kg^{-1} m.c.). Po iniekcji hormonalnej ryby przetrzymywano w temp. 10°C (N = 11), natomiast w dwóch pozostałych grupach, temperaturę wody w basenach podniesiono odpowiednio do 12°C (N = 11) oraz 14°C (N = 11). Mlecz pozyskiwano po 36 h od stymulacji do sterylnych strzykawek, zwracając uwagę aby jego prób nie zanieczyścić moczem, fekaliami lub krwią. We wszystkich badanych grupach, średnie wartości ruchliwości plemników w mleczu, nie przekraczały 40% i nie różniły się istotnie między sobą ($P > 0,05$). Osmolalność plazmy nasienia dla samców, którym po stymulacji Ovopelem podniesiono temp. wody do 14°C kształtowała się na najwyższym poziomie, tj. 154 mOsm kg^{-1}, natomiast w niższych temp. wartości osmolalności były niższe, aczkolwiek statystycznie nieistotnie ($P > 0,05$). Pomimo obserwowanego przez nas wzrostu objętości pozyskanego mlecza, tj. TVM, jak również wzrostu ilości wyprodukowanych plemników, tj. TSP, TNS oraz ich koncentracji w mleczu, wraz ze wzrostem termiki wody z 10 do 12°C wartości te nie różniły się istotnie między badanymi grupami ryb ($P > 0,05$). Przesłanką do rekomendowania temperatury niższej niż 14°C dla samców jazia przetrzymywanych przed tarłem, są koszty ekonomiczne związane z podgrzaniem znacznych ilości wody w wylęgarniach. Wydaje się również, że w przeciwieństwie do samic, u samców, nie ma konieczności zmiany termiki wody po stymulacji hormonalnej, dlatego prezentowane wyniki badań i ich interpretacja mogą znaleźć zastosowanie w optymalizacji biotechniki rozrodu jazia.