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Effect of Hormonal Stimulation on Milt Volume, Number of Sperm, and Sperm Motility in the Crucian Carp, *Carassius carassius* (L.)

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Abstract

The effects of three commercial hormonal preparations: carp pituitary extract (CPE), Ovopel, and Ovaprim, on quantitative and qualitative parameters of milt from the crucian carp, *Carassius carassius* (L.), during the spawning season were examined. Males in the control group were injected with 0.9% NaCl. The total volume of milt (ml), total sperm count (× 10^9), and concentration of sperm in milt (× 10^9/ml) were analyzed. Percent motile sperm, percent sperm with progressive movement, curvilinear velocity (μm/s), straight-linear velocity (μm/s), movement linearity (%), wobbling index (%), amplitude of lateral head displacement (μm), and beat cross frequency (Hz) were determined with a computer-assisted sperm analysis (CASA) system. The volume of milt, sperm count, and lateral head displacement were significantly greater in males receiving the Ovaprim treatment than in males stimulated by CPE, Ovopel, or the control but there were no significant differences in any other characteristic.

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Introduction

Recently, crucian carp *Carassius carassius* (L.) requires more active protection, although status of this species is listed as 'least concern' by Szczerbowski and Szczerbowski (2002). Populations of crucian carp have decreased in almost all its areas of distribution (Skrzypczak and Mamcarz, 2005; Tarkan et al., 2009) and the status of this species has become confused by the presence of congeners and hybrids (Wheeler, 2000). One reason is that endemic crucian carp populations have been driven out by the invasive prussian carp *Carassius gibelio* (Bloch). Owing to its high adaptability and tolerance to adverse environmental conditions, the prussian carp has conquered a considerable portion of inland and inshore waters that used to be the natural habitat of crucian carp (Vatemaa et al., 2005). Further, the crucian carp is able to form hybrids with other species, including the carp *Cyprinus carpio* L. and the prussian carp (Hänfling et al., 2005). This results in genetic pollution and the consequent vanishing of endemic populations of crucian carp in the natural environment (Wheeler, 2000).

Apart from applying legal measures, the protection of endangered species involves the production of fry under controlled conditions and the boosting of native populations by stocking (Philippart, 1995; Sarkar et al., 2006; Ross et al., 2008). Hormonal stimulation is used in reproduction biotechnology of nearly all species whose economic importance results in increasing aquaculture production (Targońska et al., 2010). The choice of hormonal preparation depends not only on the species but also on cost calculations (Hakuć-Błażowska et al., 2009).

Male cyprinids can be hormonally stimulated by preparations containing dopamine receptor antagonists, i.e., metoclopramide or domperidone (Cejko et al., 2011a, 2012a). Such treatment may not be necessary for domesticated species from which ova and milt can be obtained without hormonal stimulation (Krejszeff et al., 2009). However, a considerable problem with some fish is the small volume of milt (<0.1 ml) obtained under controlled conditions (Król et al., 2009). Our observations indicate that the total volume of milt obtained from crucian carp in controlled conditions without hormonal stimulation is relatively low (0.5 ml), insufficient to fertilize ova on a hatchery scale. Further, the effectiveness of insemination depends not only on the volume of milt but also on sperm motility parameters (Lahnsteinier et al., 1998; Gage et al., 2004).

Ovulation in crucian carp has been induced by gonadotropins, i.e., carp pituitary extract (CPE) and human chorionic gonadotropin (hCG) as well as the commercial preparations Ovopel analogue [(D-Ala^6, Pro^{9}NEt)-mGnRH+metoclopramide] (Targońska et al., 2009) and Dagin (Yaron, 2000). The aim of this study was to determine the effect of commercial hormonal preparations on the volume of milt, the number of sperm, and their motility as determined by a computer assisted sperm analysis (CASA) system.

Materials and Methods

Fish and hormones. Crucian carp males (n = 20) were caught during the spawning season (June, 2009) from a fishpond in the Knieja Fishery Farm near Częstochowa, southern Poland. The fish were 3-year-old breeding stock and segregated according to secondary sexual characteristics; spawning males were identified by a rash on their heads. The average daily water temperature in the pond did not exceed 17°C. The milters were transported on the same day to the aquarium hall at the Department of Lake and River Fisheries, University of Warmia and Mazury, Olsztyn, northeastern Poland, and stocked into 1-m^3 tanks with controllable temperature (±0.1°C) and photoperiod. The fish were kept together in the same tank and acclimated to 18°C for one week. Following adaptation the fish were floy-tagged, individually weighed, and five fish, each, were injected i.p. with one of three hormonal solutions mixed with 0.9% NaCl (Table 1). Five males were injected with 0.9% NaCl at 1 ml/l per kg body weight as the control group. The doses of hormonal preparations were 50% lower than the lowest effective doses used in the reproduction of cyprinid females because this amount is sufficient to induce spermiation (Kucharczyk et al., 2008). After hormonal stimulation, the water temperature in the tanks was raised to 20°C.

Table 1. Hormonal preparations and doses used to stimulate spermiation of crucian carp, *Carassius carassius* (L.).

Hormone		*Dose (per kg)*	*Source*
CPE	Carp pituitary extract	2 mg	Argent, USA
Ovopel[1]	[(D-Ala^6, Pro^{9}NEt)-mGnRH+metoclopramide]	0.5 pellet	Unic-trade, Hungary
Ovaprim[2]	[(D-Arg^6, Pro^{9}NEt)-sGnRH+domperidone]	0.25 ml	Syndel, Canada

[1] one pellet contains 18-20 µg mGnRHa (mammalian analogue) and 8-10 mg metoclopramide
[2] 1 ml contains 20 µg sGnRHa (salmon analogue) and 10 mg domperidone

Obtaining milt. Twenty-four hours after hormonal stimulation, the males were individually weighed; average body weight was 222±59 g for the control group, 110±43 g for the CPE group, 375±285 g for Ovopel group, and 194±120 g for the Ovaprim group. The males were anesthetized with 0.5 ml/l 2-phenoxyethanol (Sigma-Aldrich, St. Louis, MO, USA) and milt was collected by abdominal massage into sterile calibrated 1.0-ml syringes, being careful not to contaminate the samples with urine, feces, or blood. The amount of milt collected was recorded to 0.1 ml.

Volume of milt and the number of sperm. Sperm concentration in the milt ($\times 10^9$/ml) was determined by the spectrophotometric method (Ciereszko and Dabrowski, 1993). Sperm samples were diluted 1000 times in two replicates with 0.7% NaCl. Absorption of the samples was measured with a Beckman DU-640 spectrophotometer (Analytical Instruments, LLC, Golden Valley, MN, USA) at λ= 530 nm. Absorption measurement results (average of two replicates) were applied to the standard curve formula: y = 54,337x + 0,0256, where x equals earlier absorption measurement results for crucian carp using the Bürker chamber (cytometric method), and the concentration value was determined. Milt volume and sperm concentration were used to determine the total sperm count ($\times 10^9$).

Sperm motility. A computer-assisted sperm analysis (CASA) system was used to determine percent motile sperm, percent sperm with progressive movement, i.e., the percent sperm moving with straightness according to the formula: 100(VSL/VAP) where VSL = straight-linear velocity in µm/s and VAP = average path velocity in µm/s, movement linearity (%), wobbling index (%), amplitude of lateral head displacement (µm), and beat cross frequency (Hz). Sperm movement was activated in 25 µl Woynarovich's liquid (68 mM NaCl, 50 mM urea) and 0.5% albumin (Cejko et al., 2011b). Subsequently, 1 µl milt mixture and the buffer (Woynarovich's liquid) were transferred to a teflon-coated glass microscope slide with twelve 30-µm deep wells (Tekdon, Inc., Myakka City, FL, USA). Recordings were made approximately six seconds after movement activation. Sperm movements were recorded with a Basler a202K digital camera (Basler, Germany) integrated with an Olympus BX51 microscope (lens Plan FL N 20×/0.5 NH ph1; Olympus, Toyko, Japan). The frame rate was 46.6/s. The first 200 frames in each recording were analyzed with CRISMAS (Image House Ltd., Denmark) software, using the following acquire and track settings: AcquireTime delay 0, ImageFields max 40, ImagesPerRecord 200, SpermTracks min 10,000, ClassMethod VAP, CombineLevel 10, NoMoveLng 5, TrackImmotileLevel 5, TrackMotileLevel 25, TrackProgressiveSTRLevel 80, TrackTailsUse True, and VelocityMax 400. Two replicates were made for each male for each parameter and the averages were calculated.

Statistical analysis. Results are reported as treatment averages±SD (n = 5). The significance of differences was verified using one-way analysis of variance and Tukey's post hoc test ($p<0.05$). The normality of distribution was tested before analysis and percentage values were arcsine transformed. The analysis was determined with the GraphPad Prism program (GraphPad Software Inc., San Diego, CA, USA).

The present study was carried out in accordance with Polish regulations on experiments on animals (Decision No. 30N/2011 of the Local Ethical Committee for Experiments on Animals; individual permit 60/In/IRZiBŻ PAN/2012).

Results

Total milt volume, number of sperm, and amplitude of lateral head displacement were significantly greater following stimulation with Ovaprim than with CPE, Ovopel, or the control (Table 2). There were no significant differences of sperm concentration, %motile sperm, %progressive movement of sperm, curvilinear velocity, straight-linear velocity, %movement linearity, wobbling index, or beat cross frequency.

Table 2. Sperm parameters in crucian carp *Carassius carassius* (L.), determined by a computer-assisted sperm analysis system in untreated control fish and in fish treated with carp pituitary extract (CPE), Ovopel [(D-Ala^6, Pro^{9}NEt)-mGnRH+metoclopramide], or Ovaprim [(D-Arg^6, Pro^{9}NEt)-sGnRH+domperidone].

	Control	*CPE*	*Ovopel*	*Ovaprim*
Volume of milt (ml)	0.14±0.06^a	0.29±0.31^a	0.26±0.25^a	1.88±0.72^b
Sperm count (x 10^9)	2.60±1.02^a	5.60±6.53^a	4.39±5.58^a	24.10±8.99^b
Sperm concentration (10^9/ml)	15.06±3.11	17.23±2.54	13.61±4.56	12.85±1.38
Motile sperm (%)	61.83±10.60	62.39±11.70	51.89±18.75	67.50±4.69
Progressive motility (%)	39.67±8.04	31.52±4.72	30.04±9.06	31.43±23.57
Curvilinear velocity (μm/s)	223.6±25.63	202.7±21.44	230.0±39.77	234.1±40.97
Straight-linear velocity (μm/s)	170.4±35.59	131.4±18.29	175.3±51.54	144.9±75.55
Movement linearity (%)	56.75±6.37	51.04±3.12	57.35±9.76	49.28±13.03
Wobbling index (%)	69.43±5.02	65.29±2.06	70.63±5.09	67.42±7.91
Lateral head displacement (μm)	1.67±0.25^a	1.76±0.32^a	1.51±0.42^a	2.50±0.33^b
Beat cross frequency (Hz)	10.99±1.38	10.66±0.84	12.10±3.61	11.84±2.18

Values in a row with different superscripts significantly differ (p<0.05).

Discussion

There were significant increases in milt volume and sperm number using Ovaprim, as opposed to CPE or Ovopel, where the total milt volume and total sperm count were similar to those in the control group. The high value for lateral head displacement using Ovaprim might indicate the good condition of the sperm. Ovaprim was effective in inducing spermiation in rheophilic cyprinids such as the chub *Leuciscus cephalus* (Cejko et al., 2011a) and the dace *Leuciscus leuciscus* (Cejko et al., 2012a). The positive effects of Ovaprim may be due to higher susceptibility of cyprinid males to salmon analogue sGnRHa (Peter et al., 1993) than to the mammalian mGnRHa found in Ovopel (Horváth et al., 1997). Most probably, the salmon form of GnRH is a native form for cyprinids (Podhorec and Kouřil, 2009).

Hypophysation (stimulation with CPE) is highly effective in inducing spermiation in male cyprinids (Cejko et al., 2011a, 2012a). However, stimulation with Ovaprim produced significantly higher values of total volume of milt and total sperm count. CPE contains gonadotropins that are the source of native hormones regulating the processes of reproduction. However, a very strong mechanism inhibits the release of gonadotropins from the pituitary gland in cyprinids (Mikołajczyk et al., 2004), silurids (Silverstein et al., 1999), and mullets (Glubokov et al., 1994). This strong dopaminergic effect can be inhibited by the addition of dopamine receptor antagonists such as metoclopramide, a component of Ovopel, or domperidone, a component of Ovaprim and Dagin. The effectiveness of this method (known as Linpe) was successfully applied in reproduction of Cyprinidae in artificial conditions (Yaron et al., 2009). Therefore, as shown in this study, using CPE in reproduction might be ineffective as hypophysation in the crucian carp did not bring satisfactory results in either the volume of milt or number of sperm. In contrast, stimulation with CPE in the chub and the dace resulted in a significant increase in the total milt volume compared to the control (Cejko et al., 2011a, 2012a). Thus, despite the close phylogenetic relation between crucian carp and common carp, CPE does not effectively stimulate maturation in male crucian carp.

Sperm velocity directly affects the ability of sperm to fertilize eggs (Gage et al., 2004). In Teleostei, curvilinear and straight-linear velocity have broad ranges, probably due to the reproductive strategy of the species. Sperm velocity can change but

curvilinear and straight-linear velocity did not increase or decrease following hormonal stimulation of the crucian carp in the present study. Of all the CASA-tested parameters, only lateral head displacement was significantly higher in the Ovaprim group. Lateral head displacement is higher in smelt (*Osmerus eperlanus* L.) sperm obtained from the spermatic duct than in sperm obtained from the testes (Kowalski et al., 2006), perhaps indicating that lateral head displacement is associated with maturity of the fish sperm. Lateral head displacement was connected with the highest sperm speed and milt volume after hormonal treatment of barbel (Cejko et al., 2012b). Higher lateral head displacement is likely to benefit the fertilization potential of crucian carp sperm because the penetration of ova, even by the micropyle, requires physical force, similar to the case in mammals when the sperm moves towards the *zona pellucida* (Lewis, 2007). This force might be greater when the head displacement is wider during sperm movement.

Hormonal stimulation with Ovaprim in the crucian carp produced significantly higher production of milt and sperm with greater lateral head displacement than stimulation with Ovopel or hypophysation, indicating the higher effectiveness of a salmon analogue (sGnRH+domperidone) than a mammalian analogue (mGnRH+metoclopramide) or gonadotropic hormones (CPE) in inducing spermiation in crucian carp. Thus, Ovaprim can be recommended for the stimulation of spermiation in the crucian carp under controlled conditions.

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