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# EFFECT OF TIME AFTER HORMONAL STIMULATION ON SEMEN QUALITY INDICATORS OF COMMON CARP, *CYPRINUS CARPIO* (ACTINOPTERYGII: CYPRINIFORMES: CYPRINIDAE)

Beata I. CEJKO [1*], Radosław K. KOWALSKI [1], Dariusz KUCHARCZYK [2], Daniel ŻARSKI [2], Katarzyna TARGOŃSKA [2], and Jan GLOGOWSKI [1, 3]

[1] *Division of Gamete and Embryo Biology, Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences in Olsztyn, Poland*

[2] *Division of Lake and River Fisheries,* [3] *Division of Ichthyology, Faculty of Environmental Sciences and Fisheries, University of Warmia and Mazury in Olsztyn, Poland*

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**Background.** Obtaining the appropriate quantity of milt and spermatozoa of biologically good quality depends on a number of environmental factors. Additional factors may be involved while using a hormonal stimulation. The aim of this study was to analyse the effect of time, after stimulation with Ovopel [(D-Ala$^6$, Pro$^9$-NEt)--mGnRH+metoclopramide] (1 granule · kg$^{-1}$ body weight) on semen quality indicators of common carp, *Cyprinus carpio* L., over the period of 72 h post injection.

**Materials and methods.** The total volume of milt (TVM, mL), volume of milt per 1 kg of the male body weight (VOM, mL · kg$^{-1}$ b.w.), total sperm production (TSP, ×10$^9$), and their concentration (×10$^9$ mL$^{-1}$) in milt were determined. Additionally, the motility of spermatozoa (%) by means of the subjective method and the osmotic pressure of seminal plasma (mOsm · kg$^{-1}$) were determined. The milt was collected 24 h (group I, $n$ = 10), 48 h (group II, $n$ = 10), and 72 h (group III, $n$ = 10) after stimulation with Ovopel.

**Results.** No significant differences ($P > 0.05$) in the main parameters of milt i.e., the motility and concentration of spermatozoa in the milt, as well as in the osmotic pressure of seminal plasma were found between the experimental groups of the fish. Higher TSP and VOM values were recorded 24 h after Ovopel injection compared to samples obtained after 72 h ($P < 0.01$ and $P < 0.05$, respectively) and 48 h ($P > 0.05$). TVM values were also higher at 24 h after the injection than those noted at 48 and 72 h ($P < 0.01$).

**Conclusion.** The lack of significant differences in the motility and concentration of spermatozoa in milt at 24, 48, and 72 h after injection indicate that time after Ovopel administration does not have an influence on the main indicators of common carp milt quality. However, we noted significant differences in TSP, TVM, and VOM between samples obtaining 24 h and 72 h after hormonal stimulation. The highest quantity indicators i.e., the number of spermatozoa in milt and volume of obtained milt noted for samples obtained after 24 h suggest that this time is better for milt sampling than time after 48 and 72 h.

**Keywords:** *Cyprinus carpio*, milt, spermatozoa, seminal plasma osmotic pressure, Ovopel

## INTRODUCTION

Poland holds the second place, following Russia, in fish breeding and rearing in Central and Eastern Europe. The most important species in Polish aquaculture has been the common carp, *Cyprinus carpio* L. It is a consequence of its wide trophic spectrum and its high growth rate. It has also been an important object of angling. The long history of carp rearing in Poland goes back to the medieval times, and this fish is well established as a food item in traditional Polish cuisine (Brylińska 2000).

Obtaining adequate volumes of milt and spermatozoa of biologically good quality depends, among other parameters, on the temperature, which is an important factor influencing the maturation of gametes in cyprinid fishes (Billard 1986, Billard et al. 1995). Inducing reproduction with hormonal substances can also affect the volume of milt and the motility of spermatozoa (Redondo-Müller et al. 1991). Injections of commercially available hormonal substances: natural gonadotropins i.e., human chorionic gonadotropin (hCG), homogenate (CPH) or extract of

* Correspondence: Dr Beata I. Cejko, Zakład Biologii Gamet i Zarodka, Instytut Rozrodu Zwierząt i Badań Żywności PAN, ul. Tuwima 10, 10-747 Olsztyn, Poland, phone: +48 89 539 31 33, e-mail: *b.cejko@pan.olsztyn.pl.*

(CPE) carp or bream pituitary (BPH), as well as natural gonadotropin-releasing hormone (sGnRH, mGnRH) and analogues (sGnRHa, mGnRHa), have proven to be effective tools when applied in aquaculture to induce ovulation and stimulate spermation in fish (Drori et al. 1994, Brzuska 1999, Kucharczyk et al. 1999, 2008, Szabó et al. 2002, Szabó 2003, Targońska et al. 2010). The literature, as well as the authors' own observations, indicate that in cyprinid fishes the volume of milt and the quantity of spermatozoa obtained are influenced by the length of time after hormonal stimulation (Saad and Billard 1987, Caille et al. 2006, Cejko et al. 2010). Such treatments aim at obtaining adequate quantities of gametes of biologically good quality that determine the survival of embryos, the viability of larvae, and their growth.

Among commercially available hormonal preparations, the synthetic analogue Ovopel [(D-Ala$^6$, Pro$^9$ NEt)--mGnRH+metoclopramide] is recommended for reproduction of cyprinid- (Horváth et al. 1997, Kucharczyk et al. 2005) and non-cyprinid fish (Brzuska 2001, Kucharczyk et al. 2001, Król et al. 2009). Recently conducted studies also indicate that Ovopel has an application in the reproduction of rheophilic cyprinid fishes i.e., asp, *Aspius aspius* (L.); ide, *Leuciscus idus* (L.); or chub, *Squalius cephalus* (L.) (see Cejko et al. 2008, 2010, Krejszeff et al. 2008, 2010, Żarski et al. 2009). In comparison to control group stimulation with Ovopel gives better results in ovulation, latency time, survival to eyed-egg stage, and motility of spermatozoa in milt and volume of obtained milt (Kucharczyk et al. 1999, Jamróz et al. 2008, Krejszeff et al. 2008, Żarski et al. 2009, Cejko et al. 2010). The effectiveness of the application of Ovopel to both males and females of numerous fish species as well as economic and practical considerations (Hakuć-Błażowska et al. 2009, 2010) have led to the application of this hormonal product as a means of stimulating spermation in many species of fish.

Milt from carp can be obtained after a short time i.e., 12, 18, or 24 h after hormonal stimulation (Saad and Billard 1987, Perchec et al. 1995, Christ et al. 1996), but our earlier observations indicate that in some rheophilic fish e.g., ide extending the time from 36 to 60 h and 84 h after hormonal stimulation had a significant influence on increasing the volume of the milt, the number of spermatozoa and their quality as expressed by the percentage of their motility (Cejko et al. 2010). The aim of this project was to verify whether extending the time from 24 to 48 h, and 72 h after administration of Ovopel would influence the qualitative and quantitative parameters of carp milt.

## MATERIAL AND METHODS

The male broodfish originated from the Halinów Fish Farm, near Warsaw where, in mid-May 2008, they were caught from earth ponds using pond trap tools. The fish were of spawners aged 2–5 years, weighing 1.40 ± 0.39 kg. The same day the males were transported in bags containing oxygen to the aquarium lab of the Division of Lake and River Fisheries of the University of Warmia and Mazury in Olsztyn. The time of transport did not exceed 5 h and, following their delivery to the hatchery the fish were placed in 1000-m$^3$ tanks equipped with a system for controlling thermal conditions (Kujawa et al. 1999). Water temperature was initially set at 17ºC and, after a 3-day acclimation period, increased (within one day) to 20ºC. Such a temperature was then maintained at a constant level until the end of the experiment.

Hormonal stimulation was conducted by means of intraperitoneal injection of Ovopel (Unic-trade, Hungary) at 1 granule per kg$^{-1}$ body weight (1 granule contains 18–20 μg D-Ala$^6$, Pro$^9$ NEt-mGnRH and 8–10 mg metoclopramide). Before injection Ovopel granules were pulverized in a mortal and then dissolved in a saline of 0.7% NaCl. Dissolved material was taken up in a syringe and injected intraperitoneally at the dose 0.5 mm$^3$ fish body weight (Horváth et al. 1997). The milt was collected by means of delicate massage of the caudal region 24 h following the injection (group I, $n$ = 10), paying close attention to avoid the contamination of milt with urine, faeces or blood. After 48 h (group II, $n$ = 10) another batch of males was taken from the tanks from which milt was then collected under the same conditions as during the first collection. The last batch of milt (group III, $n$ = 10) was obtained 72 h after administration of Ovopel to the males. Group I was treated as the baseline group because milt was not obtained from fish representing control group. Prior to the collection of milt the fish were weighed and the milt from each individual was collected individually into sterile syringes calibrated at 0.01 mL, at each time measuring the volume of ejaculate obtained. All manipulations on spawners were carried out after anaesthetizing the fish by applying 2-phenoxyethanol (Sigma-Aldrich, St. Louis, MO) per 0.5 mL · L$^{-1}$ of water. The milt obtained was next transported on ice (+4ºC) to the Division of Gametes and Embryo Biology (DG&EB) of the Polish Academy of Sciences in Olsztyn, where further analyses of the material obtained were conducted.

Immediately after delivery of the milt to the DG&EB, the motility of spermatozoa was determined by means of the subjective method using light microscope and the magnification of 400×. Spermatozoa were activation by mixing 1 μL of milt with 30 μL of activation solution i.e., 68 mM NaCl + 50 mM urea (Woynarovich and Woynarovich 1980) with addition of 0.5% of BSA albumin (Sigma--Aldrich, St. Louis, MO). Motility of spermatozoa was determined immediately after activation by one observer and the value of spermatozoa motility was estimated in with the accuracy of 10%. This procedure was replicated twice to calculate the mean values (Cejko et al. 2010). To check whether the milt samples were contaminated with urine, the osmotic pressure of seminal plasma was also determined. Seminal plasma was obtained by centrifugation of milt samples for 10 min (133–166 s$^{-1}$), upon which the supernatant was collected and placed in tubes and its osmolality (mOsm · kg$^{-1}$) determined using a 5520 Vapor Pressure Osmometer (WESCOR, Logan, UT, USA).

The concentration of spermatozoa in milt (×10$^9$ per 1 mL) was determined on the base of the spectrophotometric method, as described by Ciereszko and Dabrowski (1993), first diluting the milt 1000× with 0.7% NaCl (Sigma-Aldrich, St. Louis, MO). The absorbance of test

samples was measured at α = 530 nm using a Beckman DU-640 spectrophotometer (Analytical Instruments, LLS, USA). The obtained absorbance values were then applied to the standard curve formula made for carp on base of the cytometric method (Ciereszko and Dabrowski 1993) and concentration values were read. The total volume of milt (TVM, mL) was measured during the collection of milt. To take the body weight of the males (kg) and TVM values the volume of milt obtained in mL per kg of body weight of spawners (VOM, mL · kg$^{-1}$ b.w.) was computed. Total spermatozoa production (TSP, ×10$^9$) in billions was computed based on data concerning TVM and concentration of spermatozoa in the milt.

The results were characterised for each group using the arithmetic mean ($\bar{x}$) and the standard deviation (±SD). The percentage data were subjected to normalization by arcsin transformation. In order to determine the significance of differences between groups for the percentage of motile spermatozoa, osmolality of seminal plasma, volume of milt obtained (TVM, VOM), and quantity of spermatozoa obtained (concentration, TSP), One-way ANOVA and Tukey's post-hoc test ($P < 0.05$) were applied. All statistical analyses were conducted using the GraphPad Prism 4 software package (GraphPad Software Inc., USA).

The presently reported study has been carried out in accordance with Polish regulations on experiments on animals (Decision No. 02/2010 dated 27 January 2010 of the Local Ethical Committee for Experiments on Animals; individual permit 27/IRZiBŻ PAN/2009).

## RESULTS

After 24, 48, and 72 h following hormonal stimulation, no statistically significant differences were found between tested group in main parameters of milt i.e., spermatozoa motility ($P > 0.05$, Fig. 1), seminal plasma osmotic pressure ($P > 0.05$, Fig. 2), and concentration of spermatozoa in milt ($P > 0.05$, Fig. 3). The highest TSP values (129.6 ± 57.0 × 10$^9$) were characteristic of milt samples obtained 24 h after application of Ovopel. 48 h and 72 h after Ovopel application numbers of spermatozoa were lower, reaching values of: 72.3 ± 55.1 × 10$^9$ and 46.8 ± 56.8 × 10$^9$, however, significant differences in TSP values were found only between samples obtained 24 h and 72 h after injection ($P < 0.01$; Fig. 4).

The highest TVM values were obtained at 24 h after the application of Ovopel (6.9 ± 2.3 mL) with the lowest values recorded 48 h (3.4 ± 2.2 mL) and 72 h (2.6 ± 3.3 mL) after hormonal stimulation. No significant differences were found in TVM values between milt obtained after 48 h and 72 h. Significant differences in the values of this parameter were noted between 24 h and 48 h and 72 h ($P < 0.01$, Fig. 5). VOM values were at similar levels at 48 h and 72 h following injection (2.5 ± 1.4 mL · kg$^{-1}$ and 2.1 ± 2.7 mL · kg$^{-1}$ b.w., respectively). Almost twice higher VOM values (4.5 ± 0.9 mL · kg$^{-1}$ b.w.) were obtained from fish at 24 h after injection, but significant differences in VOM were found only between samples obtained after 24 h and 72 h ($P < 0.05$; Fig. 6).

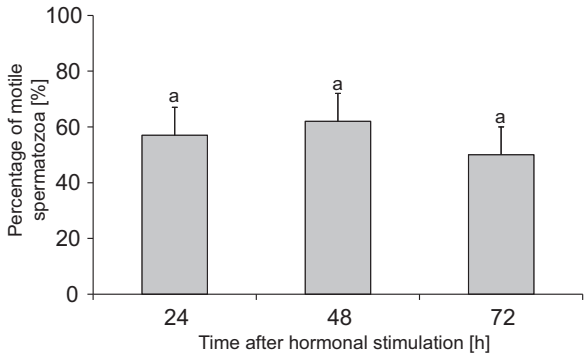


**Fig. 1.** Arithmetic mean and standard deviation (±SD) of spermatozoa motility (%) of common carp, *Cyprinus carpio*, obtained 24, 48, and 72 h after hormonal stimulation with Ovopel (1 granule · kg$^{-1}$ b.w.); Bars marked with the same letters are not significantly different

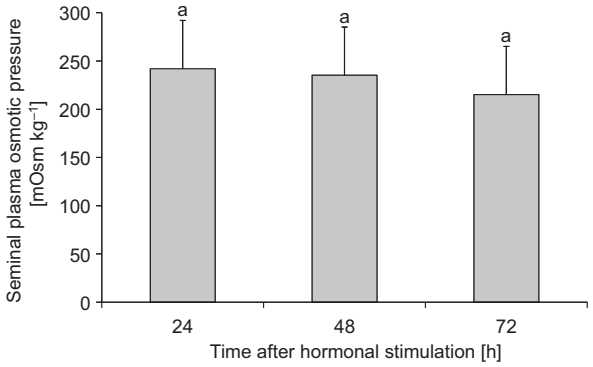


**Fig. 2.** Arithmetic mean and standard deviation (±SD) of seminal plasma osmotic pressure (mOsm · kg$^{-1}$) of common carp, *Cyprinus carpio*, obtained 24, 48, and 72 h after hormonal stimulation with Ovopel (1 granule · kg$^{-1}$ b.w.); Bars marked with the same letters are not significantly different

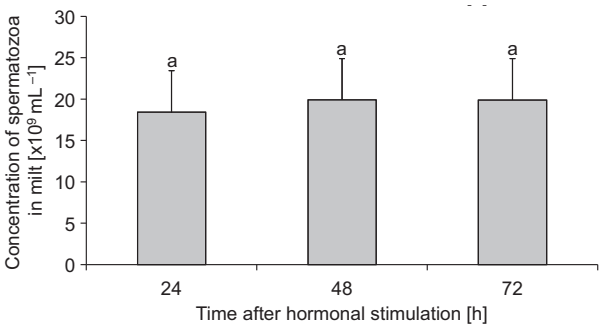


**Fig. 3.** Arithmetic mean and standard deviation (±SD) of concentration of spermatozoa in milt (×10$^9$ mL$^{-1}$) of common carp, *Cyprinus carpio*, obtained 24, 48, and 72 h after hormonal stimulation with Ovopel (1 granule · kg$^{-1}$ b.w.); Bars marked with the same letters are not significantly different

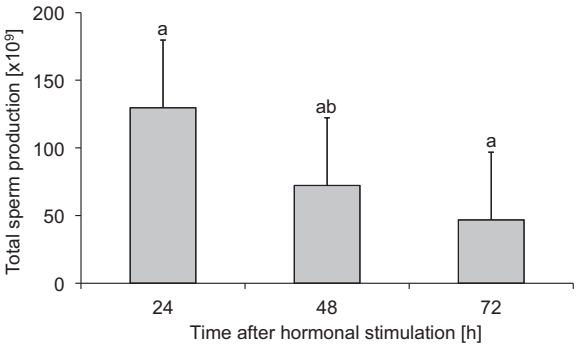


**Fig. 4.** Arithmetic mean and standard deviation (±SD) of total sperm production ($\times 10^9$) of common carp, *Cyprinus carpio*, obtained 24, 48, and 72 h after hormonal stimulation with Ovopel (1 granule · $kg^{-1}$ b.w.); Bars marked with different letters are significantly different

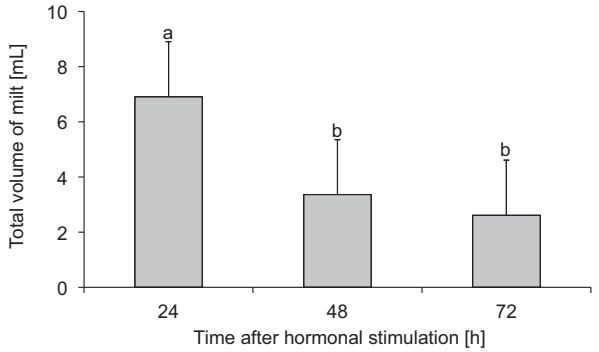


**Fig. 5.** Arithmetic mean and standard deviation (±SD) of total volume of milt of common carp, *Cyprinus carpio*, obtained 24, 48, and 72 h after hormonal stimulation with Ovopel (1 granule · $kg^{-1}$ b.w.); Bars marked with different letters are significantly different

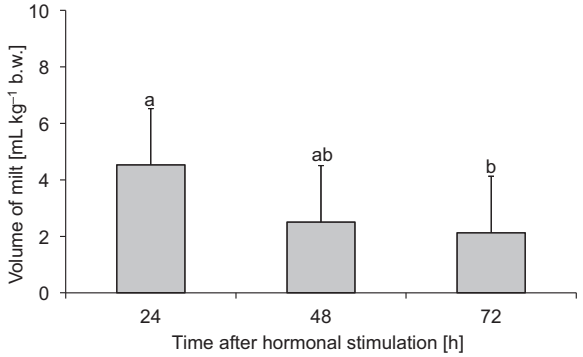


**Fig. 6.** Arithmetic mean and standard deviation (±SD) of volume of milt per kg of body weight (b.w.) of common carp, *Cyprinus carpio*, obtained 24, 48, and 72 h after hormonal stimulation with Ovopel (1 granule · $kg^{-1}$ b.w.); Bars marked with different letters are significantly different

## DISCUSSION

The maturation of spermatozoa in fish takes place in the spermatic ducts and is hormonally controlled (11-ketotestosterone and 17α, 20β-dihydroxy-4-pregnen-3-one), while the entire process of spermatogenesis is determined by environmental factors and linked to the species reproduction strategy (Billard 1986, Yaron 1995). Hormonal stimulation—plays a key role in the biotechnology of controlled cyprinid fish reproduction and, as a consequence of the dynamics of the spermatozoa maturation process of carp—and it is effective several hours after its application (Christ et al. 1996). In some rheophilic fish, where spawning takes place in colder waters, the time it takes males to reach full sexual maturity is longer e.g., 60–84 h for ide (Cejko et al. 2010).

The estimated values of spermatozoa motility in carp milt do not indicate that extending the time after hormonal stimulation can significantly influence an increase in the parameters of semen quality. Our observations indicate that during this time a significant increase or decrease in the values of spermatozoa concentration in milt or seminal plasma osmolality does not take place. The values of these parameters at each of experimental time point were at a similar and relatively high level. In the case of rheophilic fish like ide, the highest percentage of motility spermatozoa (59%) was characteristic only of milt samples obtained 84 h after hormonal stimulation. Concentration also proved to be the higher after 60–84 h, reaching the value of $11 \times 10^9$ $mL^{-1}$ when compared to the value recorded 36 h ($9 \times 10^9$ $mL^{-1}$) after the administration of Ovopel (Cejko et al. 2010). These differences most likely resulted from the different temperatures, lower for ide, at which males of different species were kept during controlled reproduction.

In the case of carp extending the time after hormonal stimulation from 24 to 72 h noticeably decreased the number of spermatozoa obtained, which was clearly reflected by significant differences in TSP values of milt samples collected at these two experimental time points. The significant decrease observed in the values of this parameter indicates that the time of 24 h after hormonal stimulation was sufficient to obtain the highest number of spermatozoa. The maturation of sperm occurs in spermatogenous cells where, during spermatogenesis, they are released from the cells into the light of the lobule. In the case of carp this occurs several hours before spawning (Billard 1986) and, on the basis of the results presented by this study, it can be concluded that the process of spermatogenesis in testes was completed, resulting in the positive response of the carp to hormonal stimulation. It also seems that extending the length of time after administration of Ovopel to these fish does not lead to an increase in the number of spermatozoa produced by the males (TSP). In the case of tench, *Tinca tinca* (L.), extending the time from 24 to 48 h, and 72 h also did not result in such an increase, regardless of the hormonal substance applied (CPE or LHRHa), (Caille et al. 2006).

The total volume of milt obtained from carp increases from 2.9 mL in March, to 6.5 mL at the peak of the breeding season i.e., in May (Christ et al. 1996). In the case of tench, TVM and VOM values also proved to be dependent on the time after hormonal stimulation as well as the dose and type of hormonal substance applied (Caille et al. 2006). The highest TVM and VOM values were found 24 h after application of CPH at 2 mg · $kg^{-1}$ b.w. After 48 and 72 h

following stimulation the volume of milt obtained significantly decreased as compared to that reported after 24 h (Caille et al. 2006). With administration of LHRHa at 40 μg · $kg^{-1}$ b.w. higher TVM and VOM values were also noted in the case of fish the milt of which was collected during the first experimental period i.e., after 24 h, when compared to those of milt collected at later periods i.e., after 48 and 72 h (Caille et al. 2006). Our observations indicate that TVM values were at their highest level 24 h after hormonal stimulation, which is consistent with results obtained by Christ et al. (1996). In our study, a significant decrease in the volume of milt as expressed by TVM and VOM values was noted 72 h fter hormonal stimulation. This suggests that the quality of milt 24 h after stimulation with Ovopel is characteristic of carp males ready for spawning (Billard et al. 1995).

Extending the time after stimulation from 24 h to 48 h and 72 h did not result in a significant increase in the values of motility and other sperm characteristics. Moreover, following stimulation of carp with Ovopel a decrease in the volume of milt obtained (TVM, VOM) and number of spermatozoa (TSP) occurred. Higher average TVM, VOM and TSP values reported during the first of the experimental time points (24 h) compared to those observed at the later times (48 and 72 h), as well as a lack of significant differences in the values of main milt quality parameters between the experimental groups of fish indicate that the best results are obtained 24 h after treating carp males with Ovopel.

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