Spermatozoa motility and short-term sperm storage of colourful orfe (*Leuciscus idus* aberr orfus)

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Abstract

In this study the effect of six activating buffers on the movement parameters of sperm were determined and short-term storage of semen in TLP buffer was attempted (0.292 g NaCl; 0.012g KCl; 0.011g CaCl2; 0.004g MgCl2; 0.105g NaHCO3; 0.002g NaH2PO4; 50 mL; pH 8.6). Sperm was collected from five orfe individuals, and spermiation was stimulated by means of an intraperitoneal Ovopel injection. The basic parameters of spermatozoa motility were measured after the semen was diluted with six different activating solutions, previously used successfully in other fish species. The motility analysis was conducted on a Crismas apparatus. Additionally, short-term storage of semen in TLP buffer was attempted. Subsequently, motility parameters were verified after 0 (Control), 24 and 120 h of storage at 4°C. It has been found that Lainsteinher’s buffer (100 mM NaCl, 10 mM Tris, 0.5% albumin, 199 mMOSm/kg) was found to be the most effective in sperm activation. In this paper, the spermatozoa motility of colourful ide is indicated for the first time. Finally, there was a successful attempt at short-term sperm storage for five days. For artificial insemination, it is very important to select the most effective solution to stimulate sperm motility. Data regarding sperm manipulation of orfe are scarce, so the aim of the study was to determine the basic sperm quality parameters of the colour ide form, *i.e.* Leuciscus idus aberr orfus.

Introduction

The domestication of finfish species can cause considerable changes in body shape, scale type, colour forms and physiology (Balon, 2004). One such freshwater species, the ide, needs several generations to produce forms with different physiological reactions to artificial reproduction procedures that cause final oocyte maturation (Krejszeff et al., 2009). Colour forms have also been developed in the species. The orfe, Leuciscus idus aberr orfus, is a highly valued ornamental fish, which is suitable for small ornamental ponds (Korwin-Kossakowski, 1999). Besides, the fish reproduction process is conducted under control. Fish are caught in ponds in early spring, and males and females are subsequently placed separately in tanks with controlled photoperiod and temperature. Then, the fish are subjected to hormonal stimulation, usually with a commercial preparation named Ovopel, which has been highly successful in the species (Kucharczyk et al., 2008; Cejko et al. 2010b).

Generally, spermatozoa motility and concentration are determined first to show the biological quality of sperm. Targońska et al. (2008) proved the existence of the relationship between the percentage of motile ide sperm and the embryo survival to the eyed-egg stage. Available literature provides only scarce reports on ide semen. Some papers focus on the characterisation of basic determinants in sperm quality (Targońska et al., 2008), and others only give information on the cryopreservation of wild ide sperm, and the characterisation of their motility (Lainsteinher et al., 2000; Kowalski et al., 2006). On the other hand, there is no data on the colour form of ide that is more cultured.

Short-term sperm storage at low temperatures enables artificial fertilisation when the ovulation precedes spermiation; this is important for wild fish or valuable breeding lines (Głogowski et al., 2000). Fish sperm concentration is high, which restricts the possibility of long-term sperm storage in decreased metabolism condition (temperature from 0 to 4°C). In order to extend the sperm survival period, species-specific immobilising buffers are used. The sperm dilution leads to the elimination of the potentially negative effects of contamination (Cabrita et al., 2008). To date, there have been no reports on the possibility of short-term orfe sperm storage.

According to our experience, the addition of disaccharide-trehalose to immobilization buffers increases the survival rate of ide sperm. Some authors suggest that sugar such as trehalose might stabilize the sperm membrane at low temperatures (Park and Chapman, 2005), just as it keeps the permeability of cells membrane at low temperatures (Zhang et al., 2007). Therefore, we decided to add trehalose to buffer prepared for preservation of ornamental ide sperm.

The aim of this study was to determine the parameters of sperm motility with the use of the CASA system (Computer Assisted Sperm Analysis) after sperm activation with six different buffers. An attempt at short-term storage at 4°C of orfe sperm was also made.

Materials and methods

Sperm acquisition

Male fish (average weight 134±27 g; n=5) were obtained from a private fish farm near the town Pila (central Poland), where they were caught from earthen ponds (average daily temperature ~10°C) and transported within eight hours to the hatchery at the Department of Lake and River Fisheries of the University of Warmia and Mazury in Olsztyn. The fish were placed in 1000 L tanks with semi-closed recirculation of water, with controlled thermal (%0.1°C) and light (%15 min) conditions and extra aeration (Kujawa et al., 1999). The fish were acclimatised for five days up to the temperature of 12°C, and then the male fish were stimulated hormonally with an intraperitoneal injection of Ovopel [(D-Ala4, Pro2Net)-mGnRH + metoclopramide] (Unic-
trade, Budapest, Hungary). The first dose was 0.2 pellet kg\(^{-1}\) of body weight (1 pellet of Ovopel contains 18-20 µg mammalian analogue of GnRH and 8-10 mg of metoclopramide; Horvath et al., 1997); after 10 h, another injection was performed at 1 pellet kg\(^{-1}\) of body weight (as described by Kucharczyk et al., 2008). After 36 h following the stimulation, sperm was collected from the male fish with sterile syringes, while care was taken to prevent its contamination with urine, faeces or blood. Before each manipulation, the male orfes were anaesthetised in 2-fenoxyethanol solution (0.5 mL L\(^{-1}\)) (Merck, Darmstadt, Germany). The semen was transported in separate test tubes at 4°C to the Department of Gamete and Embryo Biology of the Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences in Olsztyn, where a sperm motility analysis was performed.

### Identification of sperm quality

Before milt was collected, the fish were weighed (±0.1 g) and the total milt volume was measured during the collection process, using syringes calibrated every 0.01 mL. Sperm concentration was determined by the spectrophotometric method (Ciereszko and Dabrowski, 1993), using a previously prepared standard curve: \(y=62.283x+0.0178\); dilution x1000. Absorbance was measured at \(\lambda=530\) nm with a Beckman DU-640 spectrophotometer (Analytical Instruments, LLC, Golden Valley, MN, USA). To measure the osmotic pressure, a portion of milt was centrifuged twice (10,000 x g) in order to separate sperm from plasma. Osmotic pressure of seminal plasma was measured on a Löser apparatus, manufactured by Minitüb Abfüll-u. Labortechnik GmbH & Co.KG (Tiefenbach, Germany).

### Optimum buffer selection

Sperm motility was stimulated with the following buffers:
- **AS1**: 40 mM NaHCO\(_3\), 20 mM Tris, 0.5% albumin, pH 8.5; 101 mOsmkg\(^{-1}\) (Kowalski et al., 2010);
- **AS2**: 100 mM NaCl, 10 mM Tris, 0.5% albumin, pH 9.0; 199 mOsmkg\(^{-1}\) (Lahnsteiner et al., 1998);
- **AS3**: 0.4% NaCl, 0.3% urea, 0.5% albumin, pH 7.7; 181 mOsmkg\(^{-1}\) (Babiak et al., 1997a);
- **AS4**: 0.45 g NaCl, 0.1211 g Tris, 0.1126 g glycine, 0.0056 g CaCl\(_2\), 0.5% albumin, (50 mL), pH 9.0; 344 mOsmkg\(^{-1}\) (Billard et al., 1995);
- **AS5**: 120 mM NaCl, 0.5% albumin, pH 7.2; 225 mOsmkg\(^{-1}\) (Babiak et al., 1997b; Cejko et al., 2010b);

**Figure 1.** Motility parameters of orfe sperm at different activating solutions. Values identified by different letters were statistically different (P<0.01).
AS6: 119 mM NaHCO₃, 0.5% albumin, pH 9.0; 210 mOsm kg⁻¹ (Cejko et al., 2008).

The sperm movement was analysed with the CASA system (Computer Assisted Semen Analysis) on a Crismas apparatus (Image House Company Ltd., Skien, Norway). The image of spermatozoa motility was documented 1-2 s after activation, with a Basler 202K digital camera integrated with an Olympus BX51 microscope. The following sperm motility parameters were analysed:

- VCL (curvilinear velocity; μm s⁻¹),
- VSL (straight-linear velocity; μm s⁻¹),
- VAP (average path velocity; μm s⁻¹),
- LIN (linearity; %),
- STR (straightness of path; %),
- WOB (wobbling index; %),
- ALH (amplitude of lateral head displacement; μm),
- BCF (beat cross frequency; Hz),
- MOT (percentage of motile sperm; %),
- PRG (percentage of sperm with progressive movements; %).

**Short-term sperm storage**

For short-term storage, the semen was diluted at a 1:3 ratio in a TLP buffer (0.292 g NaCl; 0.012 g KCl; 0.011 g CaCl₂; 0.004 g MgCl₂; 0.105 g NaHCO₃; 0.002 g NaH₂PO₄; 50 ml; pH 8.6; Bavister, 1989) with an addition of 100 mM of trehalose. The prepared semen samples were stored at 4°C. Sperm motility was analysed with the CASA system (as described above), immediately after the semen was collected and diluted in TLP buffer (time: 0) and after 24 and 120 h (5 days) of storage. Sperm motility was stimulated with an AS2 buffer, which produced the best motility parameters of the ide sperm.

Statistical analysis was conducted with GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA) software by one-way ANOVA for paired samples. The differences between experiment treatments were determined with Tukey’s post-hoc test at a level of significance of $P=0.01$.

**Results and discussion**

**Sperm quality indicators**

The amount of sperm was 0.40±0.23 mL. The basic parameters of orfe sperm show that the sperm concentration of 11.14±3.83×10⁹ mL⁻¹ is close to the highest values reported so far for other rheophilic cyprinids. Kowalski et al. (2004) obtained ide sperm with a concentration of 5.7×10⁹ mL⁻¹. Chub (Leuciscus cephalus L.) sperm, described in the same

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Figure 2. Motility parameters of orfe sperm at the 0 time and after 24 and 120 h of storage at 4°C. Values identified by different letters were statistically different ($P<0.01$).
paper, was more concentrated (15.3 × 10^10 mL⁻¹).
Lahnsteiner et al. (2000) obtained sperm from chub with a mere 3-5 billion sperm per mL. Therefore, the values measured in this study lie within the upper and middle range of values for the Leuciscus species.

The osmolality of orfe seminal plasma was equal to 252 ± 19 mOsm kg⁻¹ and it was higher compared to that of wild ide, in which it ranged from 118.31 to 203.19 mOsm kg⁻¹ (Cejko et al., 2010b). Decreased osmotic pressure may be a sign of samples being contaminated with urine and is observed frequently in different species when semen is collected in controlled conditions (Glogowski et al., 2000). Hence, the results of this study indicate that semen samples used in previous studies may have been contaminated with urine to a lesser extent. Physiological changes caused by domestication cannot be ruled out either. In order to exclude any possible variables, including urine contamination, it is necessary to standardise the procedure of sperm sample taken from cyprinids, as it has been done for salmonids, in which a catheter is used (Glogowski et al., 2000).

**Optimum buffer selecting**

The composition of buffers to activate spermatozoa motility is selected individually for each species. This study has determined the effect of six different activating liquids, earlier successfully used in other species, on orfe spermatozoa motility parameters. The highest values of the parameters were measured when AS1, AS2 and AS3 buffers were used (Figure 1). The lowest (P < 0.01) sperm motility was observed after Billard’s buffer (AS4) was added to semen. The highest curvilinear and straight-line velocity was achieved by the activation of ide sperm in AS2 buffer, 90.02 and 72.22 m s⁻¹, respectively. In addition, the percentage of sperm with progressive motility was the highest after activation with AS2 buffer (16%). Decreasing osmotic pressure affects the motility of fish sperm (Alavi et al., 2007). In cyprinids, excessively high osmotic pressure, above the values typical of seminal plasma, 170 to 204 mOsm kg⁻¹ for ide (Cejko et al., 2010a); 252 for orfe (this paper), has an inhibiting effect on sperm motility (Billard et al., 1995). This probably explains why a considerable decrease in all the sperm motility parameters was observed in the AS4 buffer with the highest osmotic pressure (344 mOsm kg⁻¹). It seems that differences in the osmolality of activating liquids had a considerable effect on the parameters of orfe spermatozoa motility, with those lower than the osmolality of seminal plasma having a beneficial effect on the motility. In theory, an AS1 buffer should activate most effectively orfe spermatozoa motility because its osmotic pressure was the lowest (101 mOsm kg⁻¹), but the best results were achieved with AS2 liquid, whose osmolality was higher (199 mOsm kg⁻¹). Therefore, it can be suggested based on the results of this study that pH, osmotic pressure and ionic composition are important in the activation of spermatozoa motility. This is indicated by a comparison of results of sperm activation with AS2 and AS6 buffers. The pH values of both were the same and the osmotic pressure was also similar, although sperm motility parameters, such as VCL, VSL, VAP, LIN and PRG, were statistically significantly different. Similar relationships have also been observed in other species. For example, the LIN parameter of sperm in pikeperch (Sander lucioperca L.) was affected by salt solutions used as an activating solution (Sarosiek et al., 2004). The results helped to select the optimum activating liquid for orfe sperm (AS2) (Lahnsteiner et al., 1998).

Moreover, further detailed research is needed into the role of ionic composition of activating liquids in the mechanism of the activation of orfe spermatozoa motility.

**Short-term sperm storage**

The optimal artificial reproduction of fish is the possibility of short-time cooled (4°C) sperm storage, without being frozen. The method can be successfully employed to store sperm when male fish mature earlier than females. Developing an effective method of short-term semen storage provides the possibility of collecting it even in field conditions. Unlike in cryopreservation, this method does not result in a dramatic loss in sperm quality (Cabrila et al., 1998) and only requires the use of immobilising solutions because without dilution, semen quickly loses its quality. For example, in carp, Cyprinus carpio (L.), it happens as early as after 24 h of storage (Jezińska and Witeśka, 1999; Linhart et al., 2003), whereas in tench, Tinca tinca (L.), after only a few hours (Linhart et al., 2006). It has been shown in this study that sperm dilution in a TLP buffer results in an increase in the values of such parameters of spermatozoa motility as: VCL, VSL and VAP after 24 h of storage of orfe sperm (Figure 2). The value of the key parameter (MOT) decreased from 46% to 33% after 24-hour storage. After another four days of storage, most sperm motility parameters did not differ from the values measured after 24 h of storage. The greatest differences were found in two parameters: MOT and PRG, for example the percentage of motile sperm (MOT) decreased to 13% after five days of storage.

**Conclusions**

In the study, six activating liquids were used to determined sperm motility of the orfe. The best results were obtained from the application of Lahnsteiner buffer. Furthermore, all analysis on semen storage concluded that a method could be developed which allows for short-term storage of colourful ide forms.

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