Quality and quantity of smelt (Osmerus eperlanus L.) sperm in relation to time after hormonal stimulation

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SUMMARY

The effect of Ovaprim (salmon GnRH analogue and a dopamine antagonist) treatment on the quantity and quality of smelt (Osmerus eperlanus L.) sperm was studied in relation to time after hormonal stimulation. Sperm was obtained at 0, 24, 48 and 72 h after treatment (n=13/each time point). Computer Assisted Sperm Analysis (CASA) was used to evaluate sperm motility parameters and histological analysis was used to examine the testis morphology. Only a small volume of semen (1–5 µl) was collected at the beginning of the experiment (time 0) but it dramatically increased 24 h after hormonal treatment. A further increase in semen volume was recorded 48 h after hormonal stimulation. CASA parameters, such as percentage of motile cells, progressive motility, curvilinear velocity, straightlinear

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velocity, straightness and amplitude of lateral head displacement of stripped sperm increased 48 h after hormonal treatment, which indicates high quality of sperm. No further increase in sperm quality was recorded at 72 h. Compared to stripped semen, testicular semen was characterized by a slightly lower quality. In addition, histological analysis indicated that 24 h after hormonal treatment, a high number of spermatozoa was released from the testis. Ovaprim-stimulated smelt became clearly darker than the control fish. In conclusion, our results suggest that smelt semen should be collected 48 h after hormonal stimulation to ensure high quality of semen. This time may vary depending on maturation status of testis. Reproductive Biology 2012 12 2: 231–246.

Key words: smelt, hormonal stimulation, sperm motility, sperm quality, CASA

INTRODUCTION

In the past, European smelt (Osmerus eperlanus L.) was a fish species abundant in Polish lakes and the Baltic Sea coastal zone. However, due to anthropopression associated with eutrophication, the species is now endangered in Poland [23] and it is necessary to protect the naturally occurring populations of smelt1. Production of stocking material for hatcheries by means of artificial reproduction may help to protect the smelt population. Maturation of fish maintained in captivity may be affected by stress-related disorders of the hypothalamic-pituitary-gonadal axis [21]. Therefore, it is important to overcome the negative effects of stress and stimulate sexual maturation by using appropriate doses of hormones. In addition, hormone administration synchronizes reproductive processes, which is crucial for the efficiency of artificial spawning [13, 24]. Human chorionic gonadotropin (hCG) and synthetic analogues of GnRH (GnRHa), often in combination with dopamine antagonists, are the most popular agents used in artificial fish reproduction [24]. Problems with artificial

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reproduction of smelt often concern insufficient sperm production and poor maturity synchronization [17]. In contrast to GnRHa combined with dopamine antagonists [13], hCG stimulation in this species was found to be ineffective [12]. The time between hormonal treatment and production of good quality sperm can range from a few hours to a few days, depending on the species and water temperature [3, 15]. An increased amount of sperm was obtained in our previous study where the stripping time was fixed at 24 h after smelt treatment with GnRHa and a dopamine antagonist [13]. However, the obtained amounts of semen were not satisfactory. Therefore, the aim of the study was to examine the effect of time after hormonal treatment on sperm quantity and quality in smelt.

MATERIALS AND METHODS

Source of European smelt, acclimation, and hormonal stimulation

Smelt males were obtained in March 2009 from Lake Święcąjty (54° 10.8’N; 27° 46.4’E). The captured fish (n=52; weight: 6.9±2.1 g) were transported to a fish farm in Gawrych Ruda (NE Poland) to acclimate at 5°C. After two days, the fish were transported to the experimental tanks (0.35 m³/tank) in the Department of Ichthyology, University of Warmia and Mazury in Olsztyn. The physical and chemical parameters of water were monitored. Males and females were kept separately. After one-week acclimation period (water temperature 9.5°C, pH 7.7), the fish were stimulated with the Ovaprim (Syndel Laboratories Inc., Vancouver, Canada) at a dose of 0.5 ml/kg (an equivalent of 10 μg/kg of salmon GnRHa and 5 mg/kg of domperidone; [13]). Twenty four hours after injection the stimulated and non-stimulated smelt males were photographed to compare body coloration.

Experimental design and semen collection

Available volume of sperm was collected just before the hormonal stimulation (time 0) and then 24, 48 and 72 h after stimulation from different
males at each time point. After the collection of the stripped sperm, the fish were sacrificed, and then the testicular sperm was collected from the same fish. The stripped sperm (n=13/each time point) was collected with the use of a micropipette and placed in plastic tubes (1.5 ml) which were kept at 4°C for two hours until the analysis. Because of strong asymmetry in testis size [12], the sperm was collected only from larger cranial testes (caudal testes were often 5×smaller). Testicular sperm was collected by dissecting the testes (n=13/each time point) and cutting them into small pieces. Liberated sperm was placed into vials by micropipette. Such an approach allowed us to distinguish between stripped (mature) and testicular (not fully matured) semen.

Semen characteristics and histological analysis

The volume of each sample was measured and sperm concentration was determined [4]. Total sperm production (TSP) was calculated based on sperm concentration and sperm volume of each individual. In addition, the gonadosomatic index (GSI) was calculated [12].

Three fish from each group (time point) were sacrificed for histological analysis. The cranial lobe of the testes was removed and fixed in Bouin’s solution for histological study. Gonads were dehydrated in increasing ethyl alcohol solutions, kept in xylene and embedded in paraffin. Paraffin sections (5 µm) were stained with hematoxylin/eosin [18], and examined by light microscopy LEICA DM 3000 (Leica Microsystem, Germany). The serial cross sections were analyzed for the shape and type of germ cells present in testes using LEICA QWinPro software (Leica Microsystem, Switzerland).

Sperm motility analysis

Sperm motility parameters were determined using the Computer Assisted Sperm Analysis (CASA) system. For sperm motility activation, a solution composed of 20 mM Tris, 50 mM NaHCO₃ and 0.5 g/ml bovine serum albumin (Sigma-Aldrich, MO, USA; pH 8.5; [13]) was used with a dilution ratio of 1:400 for stripped sperm and 1:800 for testicular sperm. Activated
sperm was placed on Teflon-coated slide glasses (Tekdon, Inc., Myakka City, FL) and covered with standard cover slips. Recording of sperm movement started six seconds after activation. Recordings were made with a Basler a202K (Basler, Germany) digital camera integrated with an Olympus BX51 (Olympus, Japan) microscope. The recording velocity was 47 frames per...
Figure 2. Images of histological sections of the cranial lobe of the smelt testes: before (A), 24 h (B), 48 h (C) and 72 h (D) after hormonal stimulation; sz: spermatozoa; sg: spermatogonia; sc: Sertoli cells.

Figure 3. Images of histological sections of the European smelt testis; sc: Sertoli cells, sg: spermatogonia.
Figure 4. Gonado-somatic index (GSI; A) and sperm concentration in stripped (B) and testicular (C) samples of smelt semen in relation to time of hormonal stimulation (n=13). Data are shown as box-and-whisker plots (lower whisker: minimum; lower box line: 25\textsuperscript{th} percentile; middle box line: median; upper box line: 75\textsuperscript{th} percentile; upper whisker: maximum). Different letters depict statistically significant differences (p≤0.001).
second. The first 200 frames from each recording were analyzed using Image House software from CRISMAS Company Image House Ltd. (Denmark).

The recorded parameters included: straightlinear velocity (VSL, μm/s), curvilinear velocity (VCL, μm/s), beat cross frequency (BCF, Hz), amplitude of lateral head displacement (ALH, μm), linearity of movements (LIN=VSL/VCL×100%), straightness (STR=VSL/VAP×100%; VAP: average path velocity), percentage of motile cells (MOT, %) and progressive motility (percentage of spermatozoa characterized by linear movements of ≥50%).

Figure 5. The volume of stripped sperm (A) and total sperm production (B) of European smelt in relation to time of hormonal stimulation (n=13). Data are shown as box-and-whisker plots (lower whisker: minimum; lower box line: 25th percentile; middle box line: median; upper box line: 75th percentile; upper whisker: maximum). Different letters depict statistically significant differences (p≤0.001).
Statistical analysis

All data are presented as mean±SD. The results were statistically analyzed using GraphPad Prism software. One-way ANOVA followed by Tukey post-hoc tests were applied to compare the weight of testes, GSI, volume of semen, TSP and sperm concentration. A two-way ANOVA was applied to compare CASA parameters of the stripped and testicular sperm at different time points. Differences between groups were identified using the Bonferroni post-hoc test.

RESULTS

Effect of hormonal stimulation on smelt body coloration

The hormonally stimulated fish were characterized by darker body coloration compared to control fish, and the coloration persisted until 72 hours after the stimulation (fig. 1A). In contrast to control fish, a high clustering of dark spots was observed in the dorsal part of the peritoneum of the stimulated fish (fig. 1B).

Effects of hormonal stimulation on testis maturation

Before hormonal stimulation, the ampullae of the cranial lobe of the smelt testes were filled with spermatozoa and were separated by a layer of connective tissue with spermatogonia (fig. 2A). 24 and 48 h after stimulation, the structure of the testes was clearly loosened and spermatozoa did not uniformly fill the ampullae, leaving free space near the walls (fig. 2B, C). Seventy two hours after treatment, the number of spermatozoa in the lumen of the ampullae visibly decreased. In addition, the walls of connective tissue with proliferating spermatogonia became thinner and spermatocytes embedded in Sertoli cells appeared (fig. 2D). Sertoli cells and germ cells are shown in Figure 3. A significant increase in GSI was observed 24 h after hormonal treatment, and no further increase was observed at 48 and 72 h (fig. 4A).
Effects of hormonal stimulation on sperm quality parameters

The concentration of the stripped sperm increased 24 h after hormonal treatment but returned to its initial value afterwards (fig. 4B). Testicular semen was initially characterized by a very high sperm concentration (approximately 52×10⁹/ml) and decreased after 24 and 48 h after hormonal stimulation (fig. 4C).

Only a small volume of semen (1–5 µl) was collected at the beginning of the experiment (time 0) but it dramatically increased 24 h after hormonal treatment. A further increase in semen volume was recorded 48 h after hormonal stimulation. No change in milt volume was observed 72 h after the treatment (fig. 5A). In control fish, an increase in milt volume
statistically significant differences between testicular and stripped semen values at the one-time point; \( p \leq 0.05 \); MOT: percentage of motile cells; VCL: curvilinear velocity; VSL: straightline velocity; LIN: linearity of movements; STR: straightness; ALH: amplitude of lateral head displacement; BCF: beat cross frequency.

was not observed during the experiment (data not shown). In addition, an increased total sperm production was observed after hormonal stimulation (fig. 5B).

For the stripped sperm, the percentage of motile cells, progressive motility, VCL, VSL, STR and ALH increased 48 h after hormonal treatment (fig. 6). The increase in VCL was significant already 24 h after treatment. No further increases were recorded after 48 h. Hormonal stimulation did not affect the LIN and BCF. For testicular sperm, all sperm motility parameters increased 24 h after hormonal treatment. Afterwards the percentage of motile cells, progressive motility, ALH and BCF remained unchanged, while LIN and STR decreased 48 h after treatment, and VCL and VSL decreased 72 h after treatment.
At time 0 all CASA motility parameters, except for the percentage of motile cells, were higher for stripped sperm than for testicular sperm. Twenty four hours after hormonal stimulation, no differences were found between the two sperm types. Progressive motility and VCL were higher for stripped sperm than for testicular sperm 48 h and 72 h after hormonal treatment. At 72 h VSL was also higher for stripped sperm.

DISCUSSION

The results of the current paper expand our previous data [13] and provide evidence that in smelt, hormonal stimulation with GnRHa and a dopamine antagonist is the most effective when semen is collected 48 h rather than 24 h after treatment. We found that the highest volume of semen and the best sperm quality, as indicated by CASA parameters, were obtained 48 h after treatment. Histological analysis of the testis demonstrated that high number of spermatozoa was released from the testis after hormonal treatment.

In our earlier study [13], semen was collected 24 h after Ovaprim treatment which was in agreement with the common practice of Polish hatcheries. Our present results suggest that GnRHa and dopamine antagonist in male smelt may induce LH surge lasting longer than 24 h, however, this hypothesis has yet to be tested. The LH surge induced by a single GnRH treatment can range from a few hours to a few days, depending on the species and water temperature [5, 9, 15]. The semen collected from smelt 72 h after hormonal stimulation had similar quality parameters as samples collected at 48 h time point. However, a slight decrease in some sperm motility parameters was observed which suggests the initiation of sperm aging [2]. Thus, our results suggest that period between 24 and 48 h after hormonal treatment is optimal for sperm collection in smelt.

It is assumed that fish sperm motility parameters measured by CASA are important for the evaluation of the effectiveness of fertilization [8, 14, 20]. In the current study, a clear upward trend was found in motility percentage and velocity parameters of stripped sperm (VSL, VCL) for samples collected at 24 h and 48 h after treatment, which indicates a gradual increase in sperm
quality. Our previous study demonstrated that ALH could also be a good indicator of smelt spermatozoa maturity [13]. In this study, we found that for stripped sperm ALH was higher 48 h after treatment as compared to 24 h. Summing up, high values of VSL, VCL and ALH recorded in samples collected 48 h after hormonal treatment strongly suggests that semen collected at this time is fully mature and of high quality.

The dynamic changes in sperm quality and quantity after hormonal stimulation suggest a two-step mechanism of smelt sperm maturation. In the first step, most spermatozoa are released into the spermatic duct. This assumption is supported by the detection of the highest sperm concentration 24 h after hormonal treatment. However, CASA analysis showed low motility parameters indicating that at this time spermatozoa were not fully mature. In the second step, between 24–48 h, final sperm maturation occurs as indicated by an increase in the CASA parameters. This suggests that, similar to other salmonid fish [16], the final maturation of smelt sperm occurs in the spermatic duct. This step was likely accompanied by further semen hydration and, consequently, by an increase in semen volume. It is well known that hydration is related to final sperm maturation [1, 22]. Further experiments are necessary to verify the proposed two-step model of sperm maturation in smelt.

Smelt testicular sperm is an additional source of sperm, especially when the volume of semen obtained via stripping is not sufficient [17]. Our results demonstrated that the quality of testicular sperm, expressed by CASA parameters, increased dramatically 24 h after hormonal treatment. The fish sperm quality indicators such as VSL, VCL and ALH remained high until 48 h after hormonal treatment, but were slightly lower than those of the stripped sperm. This suggests that testicular sperm has lower quality compared to the stripped sperm. Therefore, it may be necessary to use larger number of sperm for fertilization. Perhaps the use of testicular semen should be preceded by in vitro sperm maturation as described for sex-reversed rainbow trout (Oncorhynchus mykiss Walbaum, 1792; [10]).

Histological analysis is a useful tool for description of the testis after hormonal treatment. The thin layer of lobule walls observed 24 h after treatment together with the free space at the ampullae walls indicated the full
maturation of testis as it was described for salmonids [6]. This is consistent with our observation that most spermatozoa were released to the spermatic duct 24 h after treatment. Moreover, 72 h after treatment the testis structure changed drastically, and large lobule walls as well as Sertoli cells with vacuoles were observed – such changes are recognized as a sign of the end of the testis maturation in salmonids [6]. Indeed, testicular sperm motility parameters such as VCL, VSL, LIN and STR were lower at this time when compared to samples collected 24 h after treatment and this may be due to sperm aging. These results are in agreement with the well-established effect of sperm aging on sperm motility [2]. Summing up, the combination of CASA and histological analysis provided an excellent insight into gonad maturation in male smelt.

Smelt stimulated with Ovaprim became clearly darker after hormonal injection. This effect was probably due to an increased melanin production by melanocytes in response to high levels of gonadotrophins [11, 19]. Color changes were described in some cichlid species [7] and Australian eels (*Anguilla australis* Richardson 1841); [19]). The clear response of body coloration to hormonal stimulation demonstrated in this study can potentially be used for the maturity determination of male smelt.

Semen volume and sperm concentration at time 0 and 24 h after hormonal treatment demonstrated in our previous report [13] were different from those reported in the current study. In contrast to previous results, where the initial semen volume was quite high (16 µl), only small amount of semen (2 µl) was initially obtained in the current study. This may be due to differences in the maturation status of the examined fish i.e. fish from the current experiment could be less mature. It should be emphasized that the initial assessment of maturity status may be important for hormonal stimulation of smelt.

In conclusion, our results suggest that smelt semen should be collected 48 h after hormonal stimulation to ensure high quality of semen. This time may vary depending on maturation status of testis. Change in coloration can be used as an indicator of gonadal maturity of male smelt.
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Hormonal stimulation of smelt


