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Development and Application of LC/HRPS for Quantification of Adenine Nucleotides, Creatine Phosphate, and Creatine in Sturgeon Spermatozoa

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ABSTRACT

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The objective of this study was to investigate the applicability of liquid chromatography coupled with high resolution product scan (LC/HRPS) mass spectrometry for simultaneous quantification of adenine nucleotides, creatine phosphate, and creatine levels after fixation of sturgeon sperm by perchloric acid. This approach has been used for the determination of adenine nucleotides, creatine, and creatine phosphate in biological tissues, but no applications to sperm cells have been reported to date. The results of method validation showed that specific correction factors for the calculation of macroergic phosphate content in sperm cells extracted by perchloric acid should be used to get accurate concentration values. The proposed LC/HRPS method is beneficial for the analysis of adenine nucleotides because it allows simultaneous quantification of all target analytes at low concentrations in a single run. This is an advantage compared to conventional methods based on colorimetric or chemiluminescent assays and even compared to sophisticated methods based on high resolution nuclear magnetic resonance.

Keywords: macroergic phosphates; high resolution mass spectrometry; extraction; method validation; sperm; chondrostean fishes

The spermatozoa of most fish species with external fertilization are maintained immotile in the seminal duct but initiate motility immediately after dispersion into water (Morisawa et al. 1983); this motility initiation is crucial for fertilization. Spermatozoa motility is primarily powered and regulated by creatine phosphate (CP) and adenylate phosphate metabolism. This metabolism is a very

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fast process, especially within the first 10 seconds post-activation, when the concentrations of CP and adenylate phosphates change up to several times (Dreanno et al. 1999). Adenosine triphosphate (ATP) production and CP synthesis occur in the middle part of the flagella, CP transport occurs along the flagellum, and ATP regeneration occurs, using CP and adenosine diphosphate (ADP), in the flagellum, which is why consideration of the contents of these components is important for the description of metabolic strategies (Cosson 2012). The content of each metabolite depends on the content of others: ATP could be regenerated from ADP in the axoneme by the adenylate kinase reaction (Cosson 2004), and CP also supports ATP levels during motility via the creatine–phosphokinase reaction (Lahnsteiner et al. 1992). Sperm motility highly depends on the energy of ATP hydrolysis catalyzed by dynein ATPase that is coupled to the sliding of adjacent microtubules, leading to flagellar movement (Morisawa and Okuno 1982; Christen et al. 1987; Saudrais et al. 1998).

Sturgeon spermatozoa are characterized by the elongated head commences with an acrosome with 8–12 posterolateral projections. The flagellum consists of an axoneme with a typical "9 + 2" structure of microtubules and presents a ribon-like structure due to two lateral membranous fins (Psenicka et al. 2008). Normal density of sturgeon spermatozoa (spz) is $0.46 \pm 0.25 \times 10^9$ spz/ml. Egg water, Ca^{2+}, and Mg^{2+} can trigger acrosome reaction. Released spermatozoa are immotile in seminal plasma and become motile only after dilution with water or specifically designed activating media (Ca^{2+} and K^+ ions are the main participants of cyclic adenosine monophosphate (cAMP) dependent pathways of signalling for sperm motility activation), suggesting the ionic mechanism of motility activation (Dzyuba et al. 2013). The duration of motility period is longer (up to several minutes) in comparison to some other species and supported by adenylate kinase and creatine phosphokinase reactions as the main sources of energy (Fedorov et al. 2015; Dzyuba et al. 2016).

To understand the processes involved in fertilization of fish eggs, it is important to accurately measure macroergic compounds in the spermatozoa. The most frequently used approaches (colorimetric and chemiluminescent assays) allow quantification of only one substrate (e.g. ATP or CP), limiting the amount of obtainable data (Perchec et al. 1995; Lahnsteiner and Caberlotto 2012). To monitor the general energy status of spermatozoa, it is necessary to measure all the substrates. Therefore, simultaneous analysis of several interrelated metabolites (especially macroergic phosphates) is of great interest, as it allows us to obtain more information from a single measurement, which consequently can be used for the assessment of the role of macroergic phosphates in sperm motility (Ingermann 2008). The application of conventional methods is laborious and was carried out for only a few fish species (Saudrais et al. 1998). Multi-compound analysis based on high resolution nuclear magnetic resonance (NMR) has been applied to the sperm samples of several mammals (Smith et al. 1985; Lin et al. 2009); however, we have found only one article using this approach for the analysis of fish sperm (Dreanno et al. 2000). The high requirements for sample preparation, expensive instrumentation, and skilled personnel, together with relatively high limits of quantification (LOQ), result in a limited use of NMR methods for fish samples.

Novel liquid chromatography/mass spectrometry (LC/MS) analytical methods allow selective identification and quantification of low concentrations of biomolecules in complex biological matrices (Higashi et al. 2008; de Jong et al. 2010). Analytical methods using different LC/MS configurations have already been successfully applied to the quantification of nucleotides in different cells in various biological matrices (Witters et al. 1996; Buchholz et al. 2001; Qian et al. 2004). Up to date, no papers reporting the LC/MS analysis of adenine nucleotides in sperm have been published. To utilize this technique for the quantification of adenine nucleotides, CP, and creatine in sperm cells, proper extraction is required. The most common procedure for the fixation of sperm cells is based on the use of perchloric acid (PCA), which prevents alterations of target compounds after extraction (Lahnsteiner and Caberlotto 2012). This method is appropriate for LC/MS analysis (Klawitter et al. 2007); however, the influence of PCA on the extraction of CP and adenylate phosphates from fish sperm is still insufficiently investigated while it is widely used in studies of bacteria, plant, and mammalian cells (Chida et al. 2012).

The aim of the present work was to evaluate the applicability of the liquid chromatography/high resolution mass spectrometry in product scan mode (LC/HRPS) method for the simultaneous quanti-

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fication of ATP, ADP, adenosine monophosphate (AMP), cyclic adenosine monophosphate (cAMP), CP, and creatine after fixation of sturgeon sperm by PCA. Elaborating this method could open new possibilities for the study of sperm bioenergetics in comparison to existing methods.

MATERIAL AND METHODS

Sperm sampling and quality parameters evaluation. Sterlet, *Acipenser ruthenus*, was selected as a model sturgeon species. During the natural spawning season (April–May), 6 sterlet males (3–4 years, 0.6–1.0 kg body weight) were transferred from aquaculture ponds (water temperature 8–10°C) into a 0.8 m^3 closed water recirculation system. The system water temperature was increased to 15°C within 24 h, and, prior to experimentation, fish were held four days without feeding. Spermiation was stimulated by an intramuscular injection of carp pituitary powder (Rybníkářství Pohořelice a.s., Czech Republic, www.rybnikarstvi-pohorelice.cz) dissolved in 0.9% (w/v) NaCl solution (4 mg/kg body weight (BW)). 24 h post-stimulation, milt was collected from the urogenital (Wolffian) ducts by aspiration using a 4-mm plastic catheter connected to a 10 ml syringe.

To initiate the motility, sperm was diluted in activation medium (1 : 50) containing 5 mM Tris-HCl buffer, 5 mM NaCl, and 0.25% Pluronic (Sigma-Aldrich Chemie GmbH, Germany), pH 8.5. Spermatozoon motility was recorded from 10 s post-activation until cessation, using video microscopy combined with stroboscopic illumination (ExposureScope, Czech Republic). Sperm motility video records were analyzed using CASA-automated plugin (http://www.ucs.mun.ca/~cfpurchase/CASA_automated-files.zip) for ImageJ software (National Institutes of Health, USA) to assess motility percentage and curvilinear velocity (VCL) (Purchase and Earle 2012).

For the evaluation of sperm concentration, sperm was diluted in 0.9% NaCl solution at a ratio of 1 : 2000 (sperm/saline). Sperm concentration was calculated after counting the number of spermatozoa in 12 cells of a Burker hemocytometer (Meopta, Czech Republic) at ×200 magnification with an Olympus BX 50 phase-contrast microscope (Olympus, Japan).

Experimental design. Initially the LC/HRPS method for quantification of adenine nucleotides, creatine phosphate, and creatine in sturgeon spermatozoa was validated. Sperm sample from one male was used for the LC/HRPS method validation. Twenty replicates were used for this purpose (10 were fortified and 10 measured as is). After that samples from six males were used for the study of macroergic phosphates content during sperm motility. Samples were taken and fixed by PCA at 10, 60, 120, 240, and 480 s post-activation.

Preparation of samples for adenine nucleotides, CP, and creatine analysis. Three molar PCA was added at 1 : 1 v/v to sperm samples (Lahnsteiner and Caberlotto 2012). Samples were subsequently frozen in liquid nitrogen and held at –80°C for 14 days. The samples were thawed at +4°C and then stored on ice. The amount of standard mixture corresponding to 1 µg of the target compounds (ATP, ADP, AMP, cAMP, CP, and creatine) was added to each fortified sample prior to extraction. The sample preparation for LC/HRPS analysis was carried out as follows: (1) centrifugation at 17 000 *g* for 14 min to collect the protein-free supernatant to avoid possible interference; (2) adjusting the pH range to 4.0–8.0 by addition of 12M KOH to the supernatant; (3) centrifugation at 3000 *g* to separate the precipitate from the $KClO_4$ solution; (4) dilution of the supernatant with 1M Tris-Cl (pH = 7.0) at 1 : 1 v/v to adjust the pH to 7.0; and (5) filtering through 0.45 µm regenerated cellulose filters (LABICOM, Czech Republic) into autosampler vials.

LC/HRPS quantification of adenine nucleotides, CP, and creatine. Adenine nucleotides of interest, creatine, and CP were detected using a hybrid Q Exactive™ Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, USA) coupled to an Accela 1250 liquid chromatography (LC) pump (Thermo Fisher Scientific) and a HTS XT-CTC autosampler (CTC Analytics AG, Switzerland). The LC method previously published by Jiang et al. (2012) was adapted to the LC/HRPS method for the analysis of target compounds. In brief, a Hypercarb column (50 mm × 2.1 mm ID × 3 µm particles; Thermo Fisher Scientific) was used to separate the target analytes. The mobile phases used for the separation consisted of ultrapure water (aqua-MAX-Ultrasystem; Younglin Instrument Co., South Korea) and acetonitrile (LiChrosolv Hypergrade; Merck, Germany). Both were buffered with 2 mM ammonium acetate and the pH of water was adjusted to 10.0 using am-

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monia solution (measured off bottle with a pH meter). The same amount of ammonia used for water was used initially for acetonitrile, but some extra addition was necessary (see below in "Results and Discussion"). The LC gradient is reported in Supplementary Table S1.

Heated electrospray (HESI-II), in both positive/negative ion modes, was used for the ionization of target compounds. The analysis was performed using product scan acquisition with the mass inclusion list, optimized collision energies, and expected retention times of the target analytes. Precursor ions were isolated at the first quadrupole, operated with 0.7 mass units extraction window, and collision products were analyzed in the orbital trap, operated at resolution of 17 500 full width at half maximum (FWHM) (*m/z* 200). The automatic gain control (AGC) target was set to 1 000 000 with a maximum injection time of 50 ms. Collision energy was optimized for all analytes of interest. The *m/z* ratios for the precursor and product ions of target compounds, their collision energies, and their retention times are presented in Supplementary Table S2.

The performance of the method was assessed in terms of its linearity, repeatability, matrix effect, limit of quantification (LOQ), and trueness. For the quantification of target compounds, external calibration, ranging from 1 to 500 ng/ml, was used. The lowest standard concentration in the calibration curve was considered the LOQ (LOQ response should be identifiable and reproducible within a precision of 20%). Validation of the method was performed using 10 replicates. The matrix effect was evaluated by preparing a matrix-matching standard using sperm extract.

Ethical clearance. All experiments were performed according to the principles of the Ethical Committee for the Protection of Animals in Research of the University of South Bohemia (USB), Research Institute of Fish Culture and Hydrobiology (RIFCH), Vodňany, Czech Republic. The Faculty of Fisheries and Protection of Waters of the USB and the RIFCH ran all experiments under the authorization for the use of experimental animals, reference No. 53100/2013-MZE-17214, and the authorization for breeding of experimental animals and delivery of experimental animals, reference No. 53103/2013-MZE-17214, accredited by the Ministry of Agriculture of the Czech Republic. Scientists were trained to work with animals according to the Act No. 246/1992 on the protection of animals against cruelty. Technical workers were regularly trained according to the Public notice of the Ministry of Agriculture of the Czech Republic No. 419/2012.

Statistical analysis. Statistical analysis was conducted on ATP, ADP, AMP, CP, and creatine, expressed as nmol/10^9 spz, taking into account dilutions of the sperm sample during activation and extraction. Due to a low number of observations (n = 6), a nonparametric Kruskal–Wallis ANOVA, followed by multiple comparisons of mean ranks for all groups, was used to compare ATP, ADP, AMP, CP, and creatine content. Data were presented as medians with percentiles (25%). Motility percentage and VCL values for each individual sample were presented as mean ± SEM.

Statistical significance was accepted at $P < 0.05$. Analyses and graphing were conducted using STATISTICA software (Version 12, 2013).

RESULTS AND DISCUSSION

Chromatography. Although adapting a previously published method (Jiang et al. 2012), the preparation of the mobile phase was not straight forward. Particularly problems arose with the reproducibility (day-to-day repeatability) of the chromatography. It was difficult to find the exact ammonia amount to achieve acceptable chromatography, as illustrated in Figure 1. It is obvious from Figure 1 that pH is very important for successful chromatographic separation of adenine nucleotides. Another complication was observed when the acetonitrile phase stood for some time (about 1 day) and was then reused: the same phenomenon of peak shift and broadening was observed. We assumed that this problem could be caused by ammonia volatilization or by ammonia reacting with atmospheric CO_2. Filling the head space above the mobile phase with argon seemed to be a useful solution for this problem; however, the final adjustment of the ammonia content in newly prepared acetonitrile had to be performed empirically – as a sequence of ammonia additions and high performance liquid chromatography (HPLC) analysis of the standard solution.

Method performance. Method performance parameters are presented in Table 1. Good linearity ($r \geq 0.9975$) was obtained for all six analytes. LOQs were determined in the low ng/ml range, which

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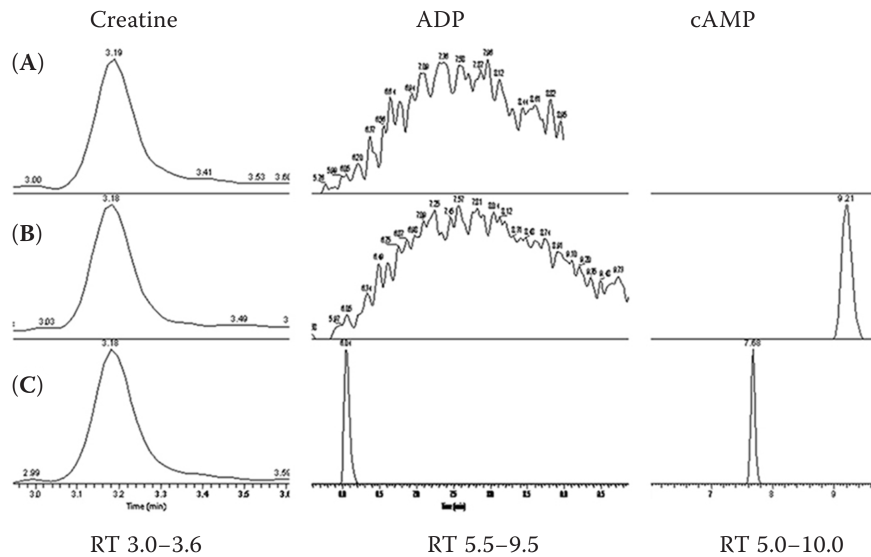

Figure 1. Shift in retention time (RT) of selected compounds under different additions of ammonia to acetonitrile solution acetonitrile solution with the same amount of ammonia as was added to the water phase (**A**), the same acetonitrile solution + 2 ml of 10% ammonia solution (**B**), the same acetonitrile solution + 5 ml of 10% ammonia (**C**)
ADP = adenosine diphosphate, cAMP = cyclic adenosine monophosphate

allowed the quantification of target compounds in sperm at low concentrations.

The trueness of the method was evaluated by calculating the analyte recoveries from fortified sterlet spermatozoa samples. One fortification level (1 μg/ml) was tested in ten replicates. Average recoveries ranged from 52 to 86% in these samples. Relative standard deviations (RSDs) ranged from 2 to 20%.

The above-mentioned results show that even correction for the matrix effect (especially relevant for CP, where significant ion suppression was found) did not lead to acceptable recoveries for all the analytes in the range given by the Commission Decision 2002/657/EC on analytical methods performance (80–110%).

To compensate for the low recoveries of most of the target compounds, correction factors were established. Using these correction factors for measured concentrations, calculations improved the quantification accuracy and compensated for probable analyte loss during sampling and sample

Table 1. Method performance parameters

Analyte	Linearity (R^2)	LOQ (ng/ml)	Repeatability (% RSD)[1] (non-spiked samples)	Matrix effect (%)		Absolute recovery (%)[2]	Correction factor[3]
				ion suppression	ion enhancement		
CP	1	5	20	54	–	53 (13)	1.9
Creatine	0.9999	5	4	1	–	86 (2)	1.2
AMP	0.9999	1	12	3	–	54 (2)	1.9
ADP	0.9996	1	2	–	1	60 (4)	1.7
ATP	0.9975	1	2	–	4	73 (20)	1.4
cAMP	0.9997	5	nd	4	–	52 (3)	1.9

LOQ = limits of quantification, RSD = relative standard deviation, CP = creatine phosphate, AMP = adenosine monophosphate, ADP = adenosine diphosphate, ATP = adenosine triphosphate, cAMP = cyclic adenosine monophosphate, nd = not detected
[1]ten replicates were analyzed
[2]accounted for matrix effect; RSD (%) of ten replicates are given in brackets
[3]to calculate concentrations, multiply by the following factor

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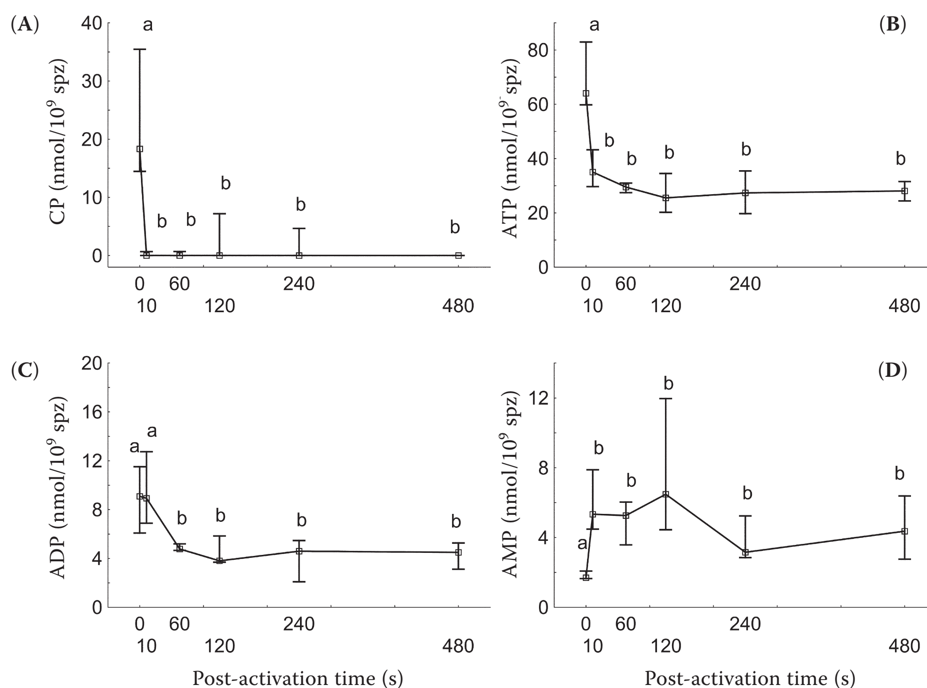

Figure 2. Creatine phosphate (CP) (**A**), adenosine triphosphate (ATP) (**B**), adenosine diphosphate (ADP) (**C**), and adenosine monophosphate (AMP) (**D**) content of sterlet spermatozoa (spz) after dilution with activation medium (n = 6)
[a,b]values with different letters differ significantly, as determined by the nonparametric Kruskal–Wallis ANOVA test (P < 0.05)
data are presented as medians with percentiles (25%)

handling. The study performed by Klawitter et al. (2007) with rat kidney tissue extracted in the same way reported acceptable recovery rates for ATP (76%) and ADP (80%), indicating that compound losses could be related to specific mechanisms in certain cells (Billard et al. 1999). However, the extraction efficiency of adenine nucleotides from sperm cells by PCA still requires to be estimated (even though it is the most frequently used approach). We believe that this is an important finding, as correct biological interpretation of the results must be based on the "true" proportions of energy metabolites. The correction of measured data with this factor is crucial; otherwise it can lead to misinterpretation of the real physiological state of the tested sperm.

Application of LC/HRPS for the quantification of adenine nucleotides, CP, and creatine in sterlet sperm. The method was successfully applied for the determination of CP, ATP, ADP, AMP, and creatine in sperm samples from six sterlet males under conditions of motility activation (Figure 2). The obtained dynamics of ATP content during the post-activation period (Figure 2b) were similar to those obtained for Siberian sturgeon (*Acipenser baerii*) using a bioluminescence method (Dreanno et al. 2000). The median creatine content was 419 nmol/10^9 spz (95% range, 254–520 nmol/10^9 spz) without a statistically significant difference during the entire post-activation period. cAMP was not detected, possibly due to dilution during the collection and preparation of samples. To avoid the influence of these factors, a 15-fold pre-concentration was used, but no cAMP was found. In any case, we obtained a complex picture of macroergic phosphates and their metabolites using this method. Motility percentage and velocity curvilinear (VCL) of spermatozoa were gradually decreased from 10 s (86.4 ± 3.9%, 139.7 ± 9.3 μm/s) to 120 s (50.2 ± 11.1%, 101.5 ± 14.8 μm/s) and finally to 480 s (5.3 ± 2.2%, 32.9 ± 7.6 μm/s).

There was an attempt to measure ATP, ADP, CP, and other substances simultaneously in the spermatozoa of turbot (*Psetta maxima*) with the help of NMR analysis, but data for the content of ATP, ADP, and CP were obtained only before motility activation, and not during the post-activation period. Possibility of simultaneous determination of ATP, ADP, AMP, CP, and creatine in fish spermatozoa during the motility period demon-

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strates the advantages of the proposed LC/HRPS method. Additionally, it is much more accessible to laboratories than NMR.

CONCLUSION

The developed method shows good performance for the target compounds in terms of linearity, LOQ, trueness, and repeatability. The obtained results revealed the necessity of using correction factors to account for the probable losses of target compounds during sample preparation. Currently, this is the first application of LC/MS for the quantification of all target analytes in fish sperm cells in a single run. Despite some problems with the liquid chromatography, the method promises a simple and fast multi-component determination of low levels of marcoergic phosphates and their metabolites.

While this method was successfully applied to simultaneous determination of ATP, ADP, AMP, CP, and creatine in spermatozoa from sterlet, it can be proposed for a study of sperm energy metabolism in other fish species.

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REFERENCES

Billard R., Cosson J., Fierville F., Brun R., Rouault T., Williot P. (1999): Motility analysis and energetics of the Siberian sturgeon Acipenser baerii spermatozoa. Journal of Applied Ichthyology, 15, 199–203.

Buchholz A., Takors R., Wandrey C. (2001): Quantification of intracellular metabolites in Escherichia coli K12 using liquid chromatographic-electrospray ionization tandem mass spectrometric techniques. Analytical Biochemistry, 295, 129–137.

Chida J., Yamane K., Takei T., Kido H. (2012): An efficient extraction method for quantitation of adenosine triphosphate in mammalian tissues and cells. Analytica Chimica Acta, 727, 8–12.

Christen R., Gatti J.L., Billard R. (1987): Trout sperm motility. The transient movement of trout sperm is related to changes in the concentration of ATP following the activation of the flagellar movement. European Journal of Biochemistry, 166, 667–671.

Cosson J. (2004): The ionic and osmotic factors controlling motility of fish spermatozoa. Aquaculture International, 12, 69–85.

Cosson J. (2012): ATP: the sperm movement energizer. In: Adenosine Triphosphate: Chemical Properties, Biosynthesis and Functions in Cells. Cell Biology Research Progress. Nova Publisher Inc., New York, USA, 1–46.

de Jong W.H.A., Wilkens M.H.L.I., de Vries E.G.E., Kema I.P. (2010): Automated mass spectrometric analysis of urinary and plasma serotonin. Analytical and Bioanalytical Chemistry, 396, 2609–2616.

Dreanno C., Cosson J., Suquet M., Seguin F., Dorange G., Billard R. (1999): Nucleotide content, oxydative phosphorylation, morphology, and fertilizing capacity of turbot (Psetta maxima) spermatozoa during the motility period. Molecular Reproduction and Development, 53, 230–243.

Dreanno C., Seguin F., Cosson J., Suquet M., Billard R. (2000): 1H-NMR and 31P-NMR analysis of energy metabolism of quiescent and motile turbot (Psetta maxima) spermatozoa. Journal of Experimental Zoology, 286, 513–522.

Dzyuba B., Cosson J., Boryshpolets S., Dzyuba V., Rodina M., Bondarenko O., Shaliutina A., Linhart O. (2013): Motility of sturgeon spermatozoa can sustain successive activations episodes. Animal Reproduction Science, 138, 305–313.

Dzyuba V., Dzyuba B., Cosson J., Rodina M. (2016): Enzyme activity in energy supply of spermatozoon motility in two taxonomically distant fish species (sterlet Acipenser ruthenus, Acipenseriformes and common carp Cyprinus carpio, Cypriniformes). Theriogenology, 85, 567–574.

Fedorov P., Dzyuba B., Fedorova G., Grabic R., Cosson J., Rodina M. (2015): Quantification of adenosine triphosphate, adenosine diphosphate, and creatine phosphate in sterlet spermatozoa during maturation. Journal of Animal Science, 93, 5214–5221.

Higashi T., Shibayama Y., Fuji M., Shimada K. (2008): Liquid chromatography-tandem mass spectrometric method for the determination of salivary 25-hydroxyvitamin D-3: a noninvasive tool for the assessment of vitamin D status. Analytical and Bioanalytical Chemistry, 391, 229–238.

Ingermann R.L. (2008): Energy metabolism and respiration in fish spermatozoa. In: Alavi S.M.H., Cosson J., Coward K., Rafiee G. (eds): Fish Spermatology. Alfa Science International Ltd., Oxford, UK, 241–266.

Jiang Y., Sun C.J., Ding X.Q., Yuan D., Chen K.F., Gao B., Chen Y., Sun A.M. (2012): Simultaneous determination of adenine nucleotides, creatine phosphate and creatine in rat liver by high performance liquid chromatography-electrospray ionization-tandem mass spectrometry. Journal of Pharmaceutical and Biomedical Analysis, 66, 258–263.

doi: 10.17221/4/2016-CJAS

Klawitter J., Schmitz V., Leibfritz D., Christians U. (2007): Development and validation of an assay for the quantification of 11 nucleotides using LC/LC-electrospray ionization-MS. Analytical Biochemistry, 365, 230–239.

Lahnsteiner F., Caberlotto S. (2012): Motility of gilthead seabream Sparus aurata spermatozoa and its relation to temperature, energy metabolism and oxidative stress. Aquaculture, 370–371, 76–83.

Lahnsteiner F., Patzner R.A., Weismann T. (1992): Monosaccharides as energy resources during motility of spermatozoa in Leuciscus cephalus (Cyprinidae, Teleostei). Fish Physiology and Biochemistry, 10, 283–289.

Lin C.-Y., Hung P.-H., VandeVoort C.A., Miller M.G. (2009): 1H NMR to investigate metabolism and energy supply in rhesus macaque sperm. Reproductive Toxicology, 28, 75–80.

Morisawa M., Okuno M. (1982): Cyclic AMP induces maturation of trout sperm axoneme to initiate motility. Nature, 295, 703–704.

Morisawa M., Suzuki K., Shimizu H., Morisawa S., Yasuda K. (1983): Effects of osmolality and potassium on motility of spermatozoa from freshwater cyprinid fishes. Journal of Experimental Biology, 107, 95–103.

Perchec G., Jeulin C., Cosson J., Andre F., Billard R. (1995): Relationship between sperm ATP content and motility of carp spermatozoa. Journal of Cell Science, 108, 747–753.

Psenicka M., Hadi Alavi S.M., Rodina M., Cicova Z., Gela D., Cosson J., Nebesarova J., Linhart O. (2008): Morphology, chemical contents and physiology of chondrostean fish sperm: a comparative study between Siberian sturgeon (Acipenser baerii) and sterlet (Acipenser ruthenus). Journal of Applied Ichthyology, 24, 371–377.

Purchase C.F., Earle P.T. (2012): Modifications to the IMAGEJ computer assisted sperm analysis plugin greatly improve efficiency and fundamentally alter the scope of attainable data. Journal of Applied Ichthyology, 28, 1013–1016.

Qian T.X., Cai Z.W., Yang M.S. (2004): Determination of adenosine nucleotides in cultured cells by ion-pairing liquid chromatography-electrospray ionization mass spectrometry. Analytical Biochemistry, 325, 77–84.

Saudrais C., Fierville F., Loir M., Le Rumeur E., Cibert C., Cosson J. (1998): The use of phosphocreatine plus ADP as energy source for motility of membrane-deprived trout spermatozoa. Cell Motility and the Cytoskeleton, 41, 91–106.

Smith M.B., Babcock D.F., Lardy H.A. (1985): A 31P NMR study of the epididymis and epididymal sperm of the bull and hamster. Biology of Reproduction, 33, 1029–1040.

Witters E., Roef L., Newton R.P., VanDongen W., Esmans E.L., VanOnckelen H.A. (1996): Quantitation of cyclic nucleotides in biological samples by negative electrospray tandem mass spectrometry coupled to ion suppression liquid chromatography. Rapid Communications in Mass Spectrometry, 10, 225–231.

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