Different glycolipids in sperm from different freshwater fishes – A high-performance thin-layer chromatography/electrospray ionization mass spectrometry study

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Rationale: Glycolipids play important roles in many physiological processes – despite their commonly low abundance. This study summarizes selected data on the (glyco) lipid composition of sperm from different fish species.

Methods: Lipid extraction of fish sperm was performed according to the procedure by Bligh and Dyer. The lipid composition of the organic extracts was analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) and electrospray ionization ion trap (ESI-IT)MS coupled to high-performance thin-layer chromatography (HPTLC).

Results: It was shown that sperm from carp, northern pike, rainbow trout and burbot contain high amounts of neutral and acidic glycosphingolipids as well as sulfoglycolipids. These particular lipids are presumably involved in reproduction requirements.

Conclusions: Phospholipids and glycolipids in crude lipid extracts can be analyzed in parallel by MS coupled to TLC. The direct application of tandem mass spectrometry (MS/MS) helps to elucidate the glycolipid structure. Thus, compositional analysis can be performed very rapidly.

1 | INTRODUCTION

Even though sperm cells have the same and only mission in every sexually reproducing species, namely to fertilize a female ooyte, sperm cells are the most heterogeneous cells regarding size, shape and molecular composition (reviewed in Ramón et al1). The molecular composition is particularly heterogeneous when it comes to lipids.2–6 In sperm most lipids are integrated parts of the different membrane systems, namely the sperm plasma membrane, the outer and the inner acrosomal membrane, the nuclear envelope and mitochondrial membranes.7 Due to distinct fertilization modalities among different species, the lipid composition of the sperm membrane has to be adjusted according to the respective requirements. The lipid composition changes continuously during the life-time of a spermatozoon8 and helps the sperm membrane to become more fluid and to overcome the different fertilization obstacles.9 In mammals, sperm membranes are mainly composed of phospholipids, particularly of phosphatidylcholines (PC) and phosphatidylethanolamines (PE), which can be easily differentiated by mass spectrometry. In addition to these lipids, there is also a special glycolipid called seminolipid which is exclusively present in the membranes of mature mammalian sperm.10 Seminolipid is a sulfoglycolipid with an ether-bound alkyl residue at the sn-2 position (also called an ether-glycerolipid) and, just like all other glycolipids, it is an important component of the outer cell membrane layer. Due to its negatively charged sulfate group, it provides the sperm cell membrane with an overall negative charge.
Seminolipid has been described as being important for the adhesion of mammalian sperm to the respective egg cell.\textsuperscript{11}

It should be noted that sperm–egg interaction during the fertilization process occurs in different taxa-specific environmental conditions (e.g., the environment of the female reproductive tract in species with internal fertilization and external water environment in species with external fertilization). These conditions are so diverse that it is really difficult to understand the biological meanings of the known differences in sperm lipid composition. In this respect, fishes with external fertilization are the group which is of interest for comparative studies oriented on understanding the basic principles of sperm physiology in relation to the fertilization process. While quite a lot of work in this field has already been done, the present study will focus on poorly described aspects of sperm lipidology in externally fertilizing fishes – the presence of glycolipids.

In the testes of salmon and trout, sulfogalactosylglycosylceramide has been detected as the most abundant glycolipid.\textsuperscript{12} However, there are so far no data available on whether this lipid is also present in the sperm of other fish species. In a recent study, we showed that a neutral glycosphingolipid, namely Galβ\textsuperscript{5000}-Cer(d18:1/16:0), represents one of the most abundant lipids in sterlet spermatozoa.\textsuperscript{13}

During earlier investigations of the phospholipid profiles of sperm from the common carp (\textit{Cyprinus carpio}), the northern pike (\textit{Esox lucius}) and the burbot (\textit{Lota lota}),\textsuperscript{14} dedicated lipid fractions, that could not be assigned to any common phospholipid class, were also detected. These spots presumably represent different glycolipids. Therefore, the present investigation aimed at elucidating the unknown lipid fractions from sperm of several freshwater fishes with external fertilization by coupling high-performance thin-layer chromatography (HPTLC) to electrospray ionization ion trap mass spectrometry (ESI-ITMS). This rapid method is very convenient in the field of lipid analysis, because (1) the different lipid classes of a separated lipid mixture are visible at first glance after staining of the TLC plate and (2) ion suppression effects which are the major problem with soft ionization mass spectrometric methods can be avoided.

2 | MATERIALS AND METHODS

2.1 | Fish rearing conditions and sperm collection

The study was performed on sperm from the common carp (\textit{Cyprinus carpio}), the northern pike (\textit{Esox lucius}), the burbot (\textit{Lota lota}) and the rainbow trout (\textit{Oncorhynchus mykiss}). Fish were reared and sperm samples were collected according to routine fisheries practice and permissions issued to the Faculty of Fisheries and Protection of Waters, University of South Bohemia in Ceske Budejovice by the Ministry of Agriculture of the Czech Republic (authorization for breeding and delivery of experimental animals, reference number: 44218/2015-MZE-1721417OZ14202/2015–17,214, and the authorization for the use of experimental animals, reference number: 2293/2015-MZE-1721416OZ22302/2014–17,214).

For each fish species, sperm samples from five males were collected by abdominal massage and stripped into dry collecting vials. One mL of each sperm sample was concentrated by centrifugation at 5000 g for 15 min at 4°C. The obtained cell pellet was frozen at −80°C until the time of lipid extraction.

2.2 | Chemicals

All solvents were obtained in the highest commercially available purity from Sigma-Aldrich (Taufkirchen, Germany). Lipids for the HPTLC lipid standard mix were purchased from Avanti Polar Lipids (Alabaster, AL, USA). All chemicals were used as supplied.

2.3 | Lipid extraction

Lipids were extracted according to the procedure by Bligh and Dyer\textsuperscript{15} with slight modifications. Briefly, 100 μL of concentrated sperm samples were mixed vigorously with 4 mL methanol/chloroform (1:1, v/v) in glass vials for cell lysis and good homogeneity. Then 2 mL of distilled water was added, and the batches were mixed vigorously and centrifuged for 10 min at 1400 g to achieve good phase separation. The lower organic (CHCl\textsubscript{3}) layer was withdrawn using a glass syringe and lipid extraction was repeated once more with an additional volume of 2 mL of chloroform. The organic phases were combined, and aliquots were evaporated to dryness.

2.4 | Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS)

Dried lipid extracts of fish sperm (see section 2.3) were mixed 1:1 (v/v) with 0.5 M 2,5-dihydroxybenzoic acid (DHB) in methanol as matrix\textsuperscript{16} for positive polarity and 10 mg/mL 9-aminoacridine (9-AA) in isopropanol/acetonitrile (60/40, v/v) for negative polarity\textsuperscript{17} and vortexed for good homogeneity. A sample of 0.75 μL was transferred onto an aluminum-coated MALDI target (Bruker Daltonics GmbH, Bremen, Germany). MALDI-TOF spectra were recorded on a Bruker Autoflex Speed time-of-flight mass spectrometer, which utilizes a 2 kHz solid-state laser emitting at 355 nm. Spectra were recorded in reflector mode by using the predefined laser firing algorithm “random walk.” The extraction voltage was 20 kV. Gated matrix suppression was applied to prevent saturation of the detector by matrix ions. For each mass spectrum 1000 single laser shots were averaged. Laser-induced sample alterations were kept to a minimum by setting the laser energy only slightly above the threshold level.
2.5 | HPTLC and ESI-MS

Lipid extracts were analyzed as described earlier. Briefly, dried lipid extracts were re-dissolved in chloroform and spotted onto a normal-phase HPTLC glass plate (Merck KGaA, Darmstadt, Germany) with the help of a Linomat device (CAMAG, Muttenz, Switzerland). Plates were developed with chloroform/ethanol/water/triethylamine (30:35:7:35, v/v/v/v) as the mobile phase and lipids were visualized by dipping the entire plate in primuline (Direct Yellow 59, Sigma-Aldrich; 50 mg/L in acetone/water (80:20, v/v)). Primuline is a non-destructive dye that binds non-covalently to the apolar fatty acyl residues of the lipids and can be released by changing the solvent and/or exposure to high vacuum. Thus, an additional step to remove primuline is not necessary and lipids are detected with their “correct” mass, not as primuline adducts or derivatives. The lipids in each spot were automatically eluted by a Plate Express™ TLC plate reader (Advia, Ithaca, NY, USA) with methanol as solvent and directly transferred into the ESI-IT mass spectrometer.

ESI-ITMS was performed on an Amazon SL mass spectrometer (Bruker) by direct infusion. The following conditions were used: spray voltage 4.5 kV, end plate offset voltage 500 V, nebulizer gas (N2) pressure 7 psi, drying gas (N2) flow rate 3 L/min, capillary temperature 180°C, flow rate 3 μL/min, sheath gas (He) flow rate 25 auxiliary units (a.u.). Spectra were recorded in the enhanced resolution mode by positive or negative ionization with a maximum ionization time of 50 ms. MS/MS experiments were performed by collision-induced dissociation (CID) with the following parameters: isolation width 2 u, CID fragmentation cut-off 27%, “smartfrag”-enhanced amplitude, ramping 80–120%.

3 | RESULTS AND DISCUSSION

The investigation of the lipid composition of sperm from different freshwater fish species with external fertilization revealed some lipids that have not been described for fish sperm, so far. In addition to established phospholipids such as PC or PE, MALDI mass spectra, particularly in the negative ion mode, were dominated by unknown peaks (Figure 1A). Additional analyses of the organic fractions by 31P-nuclear magnetic resonance (NMR) spectroscopy did not reveal any unfamiliar resonances (data not shown). Thus, it was concluded that the unknown MALDI peaks (in addition to PI and PE) do not stem from any phosphorus-containing lipid species. However, HPTLC experiments also showed spots with unknown retention factors (Figure 1B). These spots were further investigated by combining HPTLC separation with ESI-MS detection to elucidate the structures of these lipids.

The HPTLC separation of lipids from carp sperm yielded an unknown spot characterized by an Rf value of approximately 0.24. The analysis of this spot by ESI-ITMS resulted in singly and doubly

**FIGURE 1** Depiction of unknown lipid peaks in the organic extracts of different freshwater fish sperm. A, Negative ion MALDI-TOF mass spectra of whole lipid extracts of northern pike, rainbow trout and burbot sperm were recorded with 9-AA (10 mg/mL in isopropanol/acetonitrile 60:40, v/v) as matrix. The most intense signals in the burbot sperm extracts could be assigned to well-known phospholipids, namely PE 38:5 (m/z 764.5) and PI 38:4 (m/z 885.6). The chemical structures of the other signals were determined by thin-layer chromatography coupled to ESI-ITMS and subsequent MS/MS experiments. B, For HPTLC separation of organic extracts of carp, northern pike, rainbow trout and burbot sperm, the extracts were automatically sprayed onto a silica-coated HPTLC plate and developed with chloroform/ethanol/water/triethylamine (30:35:7:35, v/v/v/v) as the mobile phase. After air-drying the plate was stained with 50 mg/L primuline (dissolved in acetone/water, 80:20, v/v). Red circles in (B) mark unknown spots. Their Rs values (given at the right end of the HPTLC image) were calculated by dividing the distance of the respective spot by the total distance traveled by the mobile phase [Color figure can be viewed at wileyonlinelibrary.com]
charged ions in the positive and negative ion mode, respectively (Figure 2A). The fragmentation (induced by CID) of the two singly charged ions at m/z 1151.7 and 1179.7 (in negative ion mode) generated product ions by characteristic mass losses, e.g. the loss of sialic acid (Δ = 291 u, including water loss), the loss of sialic acid and one hexose (Δ = 453 u), and the loss of sialic acid and two hexoses leaving the ceramide backbone (Δ = 615 u) (Figure 2B). These characteristic mass losses have already been suggested to be characteristic of acidic glycosphingolipids.18,19 Furthermore, the linkage of sialic acid could have been predicted by a specific loss of Δ = 221 u, as shown by Meisen and colleagues.18 Although these product ions are present in the MS² spectra of m/z 1151 and 1179, the signal intensities of these characteristic ions (m/z 930.6 and 958.6) are low. Therefore, a detailed characterization was not possible and both signals (as well as their doubly (negatively) charged ions at m/z 575.3 and 598.3) were assigned to the acidic glycosphingolipids NeuAc-Gal-Glc-Cer(d18:1/16:0) and NeuAc-Gal-Glc-Cer(d18:1/18:0), respectively (Figure 2C, D) without a deeper assignment of the linkages. Such glycolipids are also known as monosialodihexosylgangliosides, or abbreviated as GM3, comprising glucose, galactose, and sialic acid, one moiety each.20 GM3(d18:1/16:0) has already been described to be present in mullet sperm.21

The separation of the lipids from northern pike sperm by HPTLC resulted in lipid fractions of unknown composition. The first band had an Rf value of approximately 0.28, which was thus
slightly higher than the GM3 spot of carp sperm under identical experimental conditions (vide supra). ESI-ITMS analysis of this spot revealed acidic glycosphingolipids that have also been found in the carp sperm, such as GM3(d18:1/16:0), represented by m/z 1151.7 (Figure 3). In addition, there were peaks with higher m/z values, namely m/z 1233.7 and 1261.7, which could be assigned to GM3(d18:1/22:1) and GM3(d18:1/24:1), respectively. The increased chain lengths, which could be verified by MS/MS of these GM3 molecules, are responsible for the shift in the Rf value compared with the carp sperm lipids. The ion with m/z 1165 has been assigned to a GM3 methyl ester by MS² experiments in the study dealing with mullet sperm21 and, thus, corresponds to GM3 methyl ester(d18:1/16:0). This also applies in our case, i.e. lipids with odd-chained fatty acids. This is not really surprising because the vast majority of fatty acids are made from acetyl-CoA which minimizes the contribution of odd-chained fatty acids.

The second unknown spot in northern pike had an Rf value of approximately 0.49. Because the amount of sample of the first TLC analysis was significant and analyses of the PE fraction indicated clear carry-over effects (data not shown), the experiment was performed a second time using a lower amount of sample to increase the chromatographic resolution. This spot yielded positively and negatively charged ions (Figure 4A). The most intense peaks at m/z 986.5 and 940.5 correspond to the same lipid with a neutral mass of 941.5 u, with m/z 986.5 being [M+2Na–H]⁻ and m/z 940.5 representing [M–H]⁻. The CID fragmentation of the positively charged ion at m/z 986.5 resulted in a product ion with m/z 722.6 after the loss of 264 u (Figure 4B). This loss has been described in an earlier study dealing with the characterization of sphingolipids by liquid chromatography (LC) coupled to ESI-MS/MS to be characteristic for the d18:1 sphingosine backbone.22 Additional fragmentation of m/z 722.6 resulted in the same mass losses as the neutral glycosphingolipid observed in sterlet sperm (also m/z 722.6).13 A negatively charged ion with m/z 940 has been described as II₃SO₃-LacCer (sulfate attached to C3 of the second hexose moiety, please see https://lipidmaps.org/resources/lipidweb/index.php?page=lipids/sphingo/sulfatid/index.htm for nomenclature) composed of 4-sphingenine and palmitic acid.23,24 The presence of a palmitic acid residue is substantiated by the neutral loss of 256 u while the presence of d18:1 sphingosine is obvious from the loss of 264 u.22 Losses of 162 and 180 u are representative of the presence of a hexose moiety. The losses of 120 u in the positive and 80 u in the negative ion mode (enlargement of the third spectrum in Figure 4B) represent −NaHSO₄ and −SO₃, respectively. However, there is no loss of one hexose modified with sulfate. This indicates that the sulfate is present at the first hexose unit (Figures 4C and 4D). The loss of 404 u might stem from the cleavage of two hexose moieties plus −SO₃.

Therefore, the ions at m/z 986.5 and 940.5 were assigned to II₃SO₃-LacCer(d18:1/16:0). Thus, the ions at m/z 1068.6 (+) and 1022.6 (−) could be assigned to II₃SO₃-LacCer(d18:1/22:1). The same sulfoglycolipid has also been found in rainbow trout sperm (data not shown). It is very surprising that a marked loss of the sulfate residue is only observed with low intensity. Former investigations using MALDI-TOFMS to characterize sulfated carbohydrates of the glycosaminoglycan type, for instance, showed that the spectra are dominated by a typical loss of 102 u (–SO₃Na + H) and that it is very difficult to suppress the generation of this production.25 The reasons that the sulfate loss only plays a minor role in the present ESI-ITMS investigation needs to be elucidated – although ionization by ESI is gentler than by MALDI.
indicates that the ions at m/z 1387.8 and 1401.8 contain at least three hexose moieties. It is remarkable that a loss of 291 u could not be detected in the case of burbot sperm glycolipids. Thus, these lipids do not contain a sialic acid residue. Regarding gametes, ions at m/z 1387.8 and 1401.8 have already been reported in the sperm of echinoidea (sea urchin) and assigned to NeuAc2Hex1/sulfate1(C18:1/t18:0) and NeuAc 2Hex1/sulfate1(C19:1/t18:0), respectively.27 However, according to the MS/MS spectra this assignment is not appropriate for burbot sperm.

It is obvious from the data given in this manuscript that there are major differences in the glycolipid composition of sperm from different fish species. Despite these differences, very limited attention has been paid to sperm glycolipids and their role remains largely unknown. The glycocalyx is involved in cell adhesion. In the context of sperm, it encodes information specific for its species of origin. This represents an example of reproductive incompatibility28 and might be important to prevent the generation of offspring from incompatible species. Glycolipids are modifications of the

![FIGURE 4](image)

ESI-IT mass spectra of an unknown lipid fraction from northern pike sperm after HPTLC separation. A, Singly charged ions were detected in the positive as well as in the negative ion mode with a mass difference of 46, representing the exchange of two protons by Na+. Additional information is available in Table S1 (supporting information). B, Ions at m/z 986 and 940 were fragmented by tandem mass spectrometry (CID) in the respective ion mode. Collecting all the available data, the observed ions could be assigned to a sulfoglycosphingolipid (C and D). The Y1 and Y0 product ions represent the loss of a hexose (B1) and the loss of a hexose plus a sulfated hexose moiety (B2), respectively. The linkage of the hexose moieties is only exemplarily given and remains to be elucidated.
molecular architecture of the cell surface. The hexoses bound to those lipids may be either glucose or galactose residues of different numbers which result in alterations in the hydrophobicity. Gangliosides have already been found in the sperm heads of rainbow trout together with related epitope structures near the egg micropyle, of the position where the sperm enter the female oocyte.29 In sea urchin sperm gangliosides and sulfatides were found to be highly expressed in low-density detergent-insoluble moieties of the sperm membrane30 which may bind to molecules on the surface of the egg cell. Unfortunately, there are no data available on the outer membrane lipid composition of the eggs of the respective fish species. However, one could assume that the different glycolipid patterns in carp, pike, trout and burbot sperm could be crucial for the recognition of the egg cell via the adhesion between spermatozoon and egg. From the available information it seems likely that all sperm cells (at least those from vertebrates) contain glyco (sphingo)lipids. However, sperm glyco (sphingo)lipids comprise a wide variety. The seminolipid of mammals as well as the acidic glycosphingolipids found in stingray,13 which also relies on internal fertilization, are representatives of this lipid class with a relatively low molecular weight compared with those found in sperm from fish with an external fertilization mode. Therefore, the fertilization mode (internal vs external) might require different sizes of glyco (sphingo)lipids. There could be a connection between gametes’ glyco (sphingo)lipid composition and the spawning behavior as well as the habitat of the respective fishes. This aspect requires future study but the short duration of the fish sperm motility period (in the range of 1 min) could be considered as an additional argument for the existence of sperm-egg binding mechanisms in fish with external fertilization which is realized via glycolipid presence in spermatozoa accompanied with their epitopes on the egg surface.

From an analytical point of view, this study shows that a simultaneous detection of phospho- and glyco (sphingo)lipids from one crude lipid mixture of a biological sample is possible by just one TLC-based approach. The solvents and solvent mixtures as well as the methods are capable of a fast and easy separation of these structurally very different lipid molecules.

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