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Swimming at different temperatures: The lipid composition of sperm from three freshwater fish species determined by mass spectrometry and nuclear magnetic resonance spectroscopy

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ABSTRACT

The spawning behavior of different fish species is as diverse as their habitats. A lot of factors influence the (phospho)lipid composition of fish sperm, including the water temperature at which spawning takes place. Therefore, this study aimed on the elucidation of the phospholipid composition of sperm from three fish species from different orders (common carp – *Cyprinus carpio*, northern pike – *Esox lucius* and burbot – *Lota lota*) with different spawning temperatures by nuclear magnetic resonance spectroscopy (NMR), matrix-assisted laser desorption/ionization mass spectrometry and thin-layer chromatography (TLC) coupled to electrospray ionization mass spectrometry as well as gas chromatography.

Next to the lipid composition that was different for carp, northern pike and burbot, regarding the moieties of the different (phospho)lipid classes (particularly sphingomyelin and acidic phospholipids) and the saturation degree of the fatty acyl residues, there were differences observed depending on the analytical method that was used. The results from TLC and NMR investigations differed regarding the amounts of the different phospholipids. Reasons for these discrepancies are discussed in detail.

1. Introduction

Being a non-monophyletic group of animals, fishes represent the most diverse group of vertebrates, which includes four classes comprising about 32,000 species (Nelson et al., 2016). Inside fishes, there are classes which diverged from each other about 500 million years ago (Betancur-R et al., 2017; Irisarri et al., 2017). The diversity of fishes is manifested by differences in their morphology, physiology, behavior and habitat-related adaptations. Fishes possess a large variety of reproduction modes and occupy a wide range of aquatic environments differing in salinity and temperature (Helfman, 2009; Jamieson, 2009), which results in taxon-specific physiological properties of gametes required for fertilization.

Temperature, as one fundamental physical regulatory factor, can influence qualitative and quantitative sperm parameters, such as the spermatozoon motility rate, motility duration, swimming velocities, tail beat frequency, ATP content, seminal fluid composition and morphological characteristics. Temperature effects are highly species specific and may differ depending on the characteristic spawning temperature (Dadras et al., 2017; Lahnsteiner and Mansour, 2012).

Regardless of the motility activation mode and the sensitivity to tonicity and temperature changes, the motility of sperm is initiated in response to external signals acting at the level of the spermatozoon plasma membrane (Dzyuba et al., 2014). Since membrane properties are mediated (among other factors) by the double bond content of the related lipids and some acidic phospholipids (PL) may be responsible for ion binding, the analysis of the lipid composition is very important. Unfortunately, the sperm lipid composition of different teleostean fish species was studied so far only using liquid and gas chromatography (GC) (Beirão et al., 2012; Drokin, 1993; Labbé and Maisse, 1996). The detailed analyses by nuclear magnetic resonance (NMR) spectroscopy and high performance thin-layer chromatography (HPTLC) coupled to

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electrospray ionization ion trap mass spectrometry (ESI-IT MS) have not yet been reported for the elucidation of differences in the sperm lipid compositions of fishes with different spawning temperatures and different modes of motility activation. Such an approach allows comparing the newly obtained data with already known data for fish with external fertilization and offers some advantages in comparison to LC/MS, for instance, a simple set-up and the possibility to analyze several samples at the same time (Fuchs et al., 2011).

We will show that the sperm of carp, northern pike and burbot differ characteristically in their lipid compositions. It will also be shown that (^{31}P) NMR provides some advantages compared to the other methods which allow the characterization of biological samples at the first glance.

2. Materials and methods

2.1. Ethics

All manipulations of animals were performed in accordance with the authorization for the use of experimental animals (Reference number: 2293/2015-MZE-17214 (16OZ22302/2014-17214), valid from January 22nd 2015 for five years) issued to the University of South Bohemia, Faculty of Fisheries and Protection of Waters (USB, FFPW) by the Ministry of Agriculture of the Czech Republic.

2.2. Fish rearing conditions and sperm collection

Experiments were conducted using mature males of northern pike, common carp and burbot. Fish were cultured in polyculture ponds (common carp and northern pike) and a water through system (burbot) with natural environment and feeding regime in USB, FFPW, Vodnany, Czech Republic. According to common fisheries practice, spermiation in carp was stimulated by intramuscular injection of carp pituitary powder dissolved in 0.9% NaCl solution at 1 mg kg^{-1} body weight, 24 h before sperm collection. Northern pike sperm was collected after intramuscular injection of long-acting hormonal preparation containing poly lactic-co-glycolic acid microspheres imbedded with mammalian gonadotropin-releasing hormone agonist (GnRHa, 20 µg kg^{-1}). In burbot, sperm was collected without hormonal treatment. Sperm was stripped by abdominal massage into dry collecting vials and stored on ice before experimentation. For each fish species, sperm samples from five male were collected during natural spawning season. Each stripped 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 (Horokhovatskyi et al., 2018).

2.3. Chemicals

All used solvents were obtained in the highest commercially available purity grade from Sigma-Aldrich (Taufkirchen, Germany). All chemicals were used as supplied.

2.4. Lipid extraction

Lipid extraction was performed according to the procedure by Bligh and Dyer (Bligh and Dyer, 1959). Briefly, fish sperm were mixed with methanol, chloroform and water (2 ml each) vigorously. Samples were centrifuged for 10 min at 2500 rpm, to achieve a separation into aqueous, protein-containing and organic layers. The lower (organic) layer was withdrawn by a Hamilton syringe and lipid extraction was repeated once more with an additional volume of 2 ml chloroform. Organic phases were combined. Aliquots of the organic phases were evaporated to dryness and dissolved either in deuterated chloroform ($CDCl_3$) or in 50 mM Tris buffer containing 200 mM sodium cholate and 5 mM EDTA (pH 7.65) for subsequent ^{1}H and ^{31}P NMR analyses, respectively. Aliquots for HPTLC and MS were dissolved in chloroform.

2.5. ^{31}P nuclear magnetic resonance

The “mixed micelle” approach was used for ^{31}P NMR of phospholipids (Schiller et al., 2007). Dried organic extracts were dissolved in sodium cholate buffer. Spectra were recorded in 5 mm NMR tubes on a Bruker DRX 700 spectrometer operating at 283.3 MHz for ^{31}P at 310 K. All measurements were performed using a 5 mm TXI probe with composite pulse decoupling (Waltz-16) to eliminate ^{31}P-^{1}H coupling. Pulse intervals of the order of T_1 were used to allow quantitative analysis of PL integral intensities. Other NMR parameters were as follows: acquisition time: 2 s; data size: 16 k; 60° pulse; pulse delay 3 s; and a line-broadening of 1 Hz.

Spectra were processed using the software 1D WINNMR, version 6.2 (Bruker Analytische Messtechnik GmbH, Rheinstetten, Germany).

2.6. ^{1}H nuclear magnetic resonance

For ^{1}H NMR dried organic extracts were dissolved in 0.5 ml $CDCl_3$. Spectra were recorded in 5 mm NMR tubes on a Bruker DRX 700 spectrometer at 300 K due to the reduced boiling point of chloroform compared to water. Spectra (16k data points) were processed without any window functions, i.e. without Gauss or line broadening factor. Spectra were calibrated by setting the resonance of non-deuterated, residual solvent ($CHCl_3$) to 7.24 ppm.

2.7. MALDI-TOF MS

Chloroform solutions of organic extracts of fish sperm were mixed 1:1 (v/v) with 2,5-dihydroxybenzoic acid (DHB) in methanol as matrix (Schiller et al., 1999) for positive polarity and 9-aminoacridine (9-AA) in isopropanol/acetonitrile (60/40, v/v) for negative polarity (Sun et al., 2008) and vortexed for good homogeneity. One µl was transferred onto an aluminum-coated MALDI target (Bruker Daltonics GmbH, Bremen, Germany). MALDI-TOF spectra were recorded by using the predefined laser firing algorithm “random walk” on a Bruker Autoflex "Speed" mass spectrometer (Bruker Daltonics GmbH) which utilizes a pulsed 2000 Hz laser emitting at 355 nm. The extraction voltage was 20 kV. Gated matrix suppression was applied to prevent saturation of the detector by matrix ions. For each mass spectrum 500 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. In order to enhance the resolution all spectra were recorded in the reflector mode. Raw data were processed using the software "Flex Analysis" version 3.0 (Bruker Daltonics GmbH).

2.8. HPTLC coupled to ESI-IT MS

Dried organic 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 and lipids were visualized as described before (Schiller et al., 2003). The lipids in each spot were automatically eluted by a Plate Express™ TLC plate reader (Advion, Ithaca, NY, USA) with methanol as solvent and directly transferred into the ESI-IT mass spectrometer.

ESI-IT MS was performed on an Amazon SL mass spectrometer (Bruker Daltonics GmbH) by direct infusion. The following conditions were used: Spray voltage 4.5 kV, end plate offset 500 V, nebulizer gas 7 psi, drying gas (N_2) 3 l min^{-1}, capillary temperature 180 °C, flow rate 20 ml/min, sheath gas (He) flow rate 25 a.U. Spectra were recorded in the enhanced resolution mode by positive or negative ionization with a maximum ionization time of 50 ms.

For data acquisition and subsequent analysis, the software “Trap Control” and “Data Analysis” version 4.1 (Bruker Daltonics GmbH) were used, respectively.

2.9. Fatty acid analysis by gas chromatography

Dried lipid fractions from fish sperm were re-dissolved in chloroform/methanol (2:1, v/v) and a solution containing nonadecanoic acid (19:0) as the internal standard was added. A detailed sample preparation protocol for fatty acid derivatization was previously described (Dannenberger et al., 2017).

The fatty acid analysis was performed using capillary GC with a CP-Sil 88 for FAME column (100 m × 0.25 mm, Agilent, Santa Clara, CA, United States) and a PerkinElmer gas chromatograph CLARUS 680 with a flame ionization detector and split injection (PerkinElmer Instruments, Shelton, United States). The detailed GC conditions were recently described (Dannenberger et al., 2012).

The calibration was performed with the help of the reference standard mixture 'Sigma FAME' (Sigma-Aldrich, Deisenhofen, Germany) and the methyl esters of 18:1cis-11, 22:5n-3 and 18:2cis-9, trans-11 (Matreya LLC, State College, PA, USA), 20:3n-9, 22:3n-3 and 22:4n-6 (Sigma-Aldrich, Deisenhofen, Germany) and 18:4n-3 (Larodan, Limhamn, Sweden). The five-point calibration of single fatty acids ranged between 16 and 415 mg ml^{-1} and was checked after GC analysis of five samples.

2.10. Statistics

Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). Mean values and standard deviations were calculated. For the verification of significances nonparametric and two-tailed t tests were performed, and significance was indicated by $P < 0.05$.

3. Results and discussion

The water temperature needed for spawning differs among different fish species. Extremes of freshwater fish are the burbot spawning at very low water temperatures of 1–4 °C (Harrison et al., 2016) and the carp spawning above 18 °C (Edwards and Twomey, 1982). The required water temperature for the spawning of the northern pike is 8–12 °C (Bondarenko et al., 2015; Casselman and Lewis, 1996). These different requirements regarding the spawning temperature should result in changes of the PL composition of fish germ cells. Therefore, the PL composition of selected fish sperm was in the focus of this work. Furthermore, the results of different, independent analytical methods to elucidate the PL compositions were compared.

3.1. Nuclear magnetic resonance

^{31}P NMR is an excellent method to study the PL compositions of crude lipid extracts and can be performed without any previous separation into the individual phospholipid classes (Schröter et al., 2017). The addition of an excess of sodium cholate suppresses the aggregation of phospholipids to avoid severe broadening of the PL resonances (Fuchs et al., 2005). The ^{31}P NMR spectra of the different fish species are shown in Fig. 1**A** and the compositional data determined by integration of the individual resonances are summarized in Table 1.

The spectra show that the content of the acidic PL phosphatidylserine (PS) differs considerably. Sperm of the northern pike are characterized by the most pronounced PS moiety, compared to the carp and the burbot. Second, the sphingomyelin (SM) content is also most marked in the northern pike while particularly the burbot possesses only small amounts of SM. Third, the phosphatidylcholine (PC) content, which represents the main phospholipid in all three fishes, is highest in the burbot. In contrast, no significant changes are observed regarding the phosphatidylethanolamine (PE) content (approximately 32% in all three species). These results contradict a recent study about carp sperm, where a PC content of only 5% and a PE content of 55% was reported (Horokhovatskyi et al., 2016). This study used a different lipid extraction method (hexane/2-propanol) and separated the lipids by a TLC approach different from this study. However, such a low PC content in the presence of a very high PE content would be very surprising, because PE does not form proper PL bilayers but tends to form hexagonal phases (reviewed in (Verkleij, 1984). Additionally, our results are in agreement with another study that reported phosphatidylinositol (PI), PC and PE contents of 4.5%, 30.4% and 22.3%, respectively (Drokin, 1993). Despite the differences in the amounts of the individual PL classes, the sum of SM, PC and PS is approximately 60% in all three species.

Information about the cholesterol moieties can be achieved by ^{1}H NMR (Longo et al., 1995). Compared to ^{31}P NMR the frequency dispersion of ^{1}H NMR is much smaller and, thus, there are problems with overlapping resonances (Nicholson et al., 1995). Therefore, only the most indicative spectral range with a few selected resonances is shown in Fig. 1**B**. The sperm of northern pike exhibit a higher amount of cholesterol relative to the quaternary ammonium group of PC and SM than carp and burbot. The cholesterol content of carp sperm is in agreement with the study of Horokhovatskyi and colleagues, who reported a cholesterol content of approximately 25% using a TLC-based approach (Horokhovatskyi et al., 2016). In a nutshell, NMR is a convenient method to monitor quantitative changes of the overall PL composition but not suitable to investigate changes in the fatty acyl compositions. This can be more easily and accurately performed by mass spectrometric methods.

3.2. MALDI-TOF MS

MALDI MS is probably the most convenient method to study the lipid compositions of crude lipid extracts because the ion generation process is not significantly affected by potential impurities such as salts. The MALDI-TOF mass spectra of organic extracts of carp, northern pike and burbot sperm are shown in Fig. 2.

In the positive ion mode SM and PC are exclusively detectable. The contribution of SM 16:0 (*m/z* 703.6, 725.6 and 741.6 for the H^+, Na^+ and K^+ adducts, respectively) is obvious but the ratio relative to PC is different. The northern pike has the most significant SM moiety while SM is nearly lacking in the burbot. This result is in accordance with the ^{31}P NMR data. PC 16:0/18:1 (*m/z* 760.6 and 782.6) is abundant in the carp and the burbot while this PC species is nearly lacking in the northern pike sperm, which are dominated by PC 16:0/20:5 (*m/z* 780.6). Long-chained fatty acyl residues occur particularly in the carp sperm. The high amount of PC 16:0/18:1 in carp and burbot sperm is in accordance with previous studies that investigated the fatty acyl distributions in sperm lipids of different fish species (Blecha et al., 2018; Drokin, 1993).

Differences are even more obvious from the negative ion spectra: in the carp spectrum PI 18:0/20:4 (*m/z* 885.6) is exclusively detectable while in the burbot there are additional peaks which correspond to PE 38:5 (*m/z* 764.6). All the remaining major peaks do not represent PL but commonly used data bases such as LIPID MAPS (www.lipidmaps.org) suggest glycolipids for these *m/z* values. Glycolipids were not in the focus of this study which focusses exclusively on PL. Although there are obvious differences in the MALDI spectra, it has to be emphasized that suppression effects may play a significant role, i.e. PL with different head groups are detected with different sensitivities (Khoury et al., 2016). Additionally, the choice of the matrix (Leopold et al., 2018) and the different pK values (Moncelli et al., 1994) influence the shape of the obtained mass spectrum.

Another problem with the MALDI-TOF mass spectrometer used in this study is the limited resolution and its mass accuracy which is not high enough to discriminate differences in the fatty acyl composition and the adduct pattern of PL by the exact mass. Since MALDI tolerates high salt concentrations, this problem can be overcome by recording the mass spectra in the presence of an excess of cesium ions (Schiller et al., 2001). This makes the assignment of the observed peaks very

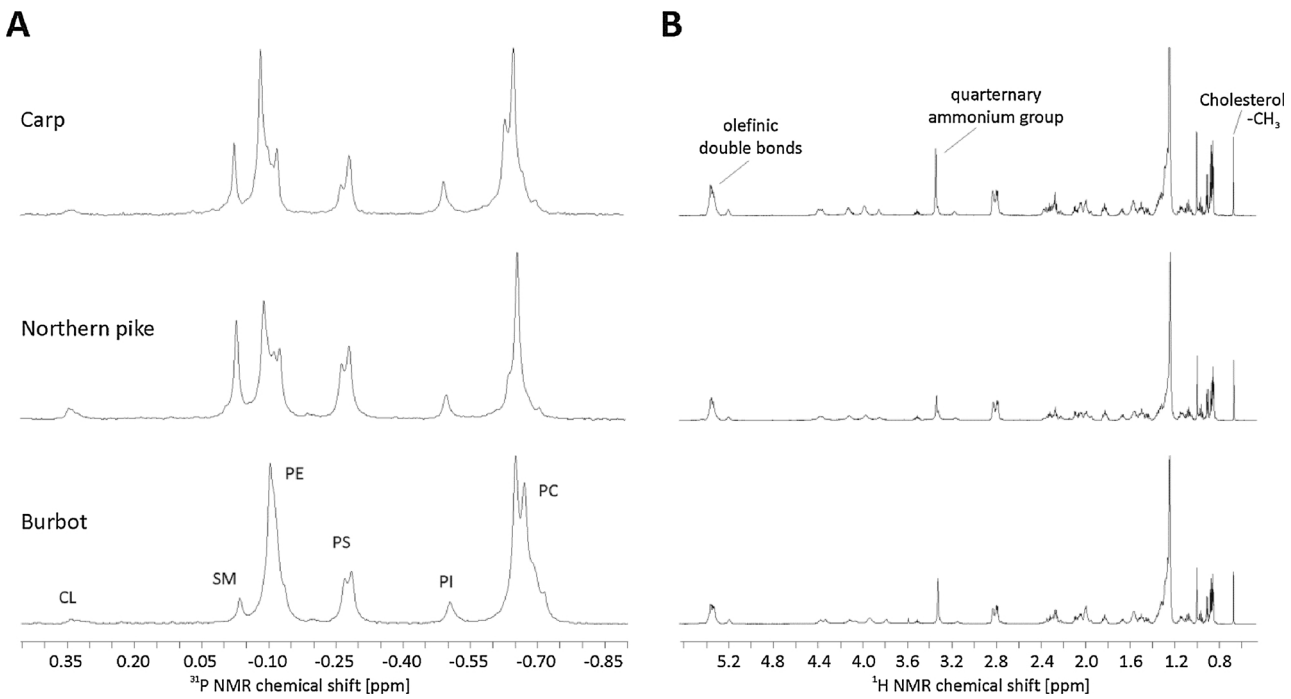

Fig. 1. NMR spectra of organic sperm extracts of the sperm of three different fish species. (**A**) For ^{31}P NMR lipid fractions were dissolved in 0.5 ml Na cholate buffer (pH 7.65) and spectra were recorded in 5 mm NMR tubes on a Bruker DRX 700 spectrometer operating at 283.3 MHz for ^{31}P at 310 K. The different phospholipid groups are abbreviated by their head groups: CL – cardiolipin, SM – sphingomyelin, PE – phosphatidylethanolamine, PS – phosphatidylserine, PI – phosphatidylinositol, PC – phosphatidylcholine. (**B**) ^{1}H NMR spectra were recorded at 300 K on lipid fractions dissolved in 0.5 ml $CDCl_3$.

Table 1
Total fatty acid composition of lipids in fish sperm. For a detailed protocol please see Section 2.9 of the text. Data represent the mean ± SD of four individuals, respectively, and are given in % of the total FA content. Significant differences are given as * ($P < 0.05$ regarding carp) and # ($P < 0.05$ regarding northern pike).

	common carp	northern pike	burbot
Saturated fatty acids (SFA)			
C12:0	0.23 ± 0.11	0.17 ± 0.09	0.08 ± 0.03
C14:0	0.07 ± 0.01	0.4 ± 0.07*	0.38 ± 0.02*
C16:0	18.12 ± 0.44	16.11 ± 0.68*	14.72 ± 0.33*/#
C18:0	7.78 ± 0.51	5.84 ± 0.64*	5.81 ± 0.26*
sum SFA[1]	27.22 ± 0.26	24.39 ± 0.89*	23.32 ± 0.63*
Monounsaturated fatty acids (MUFA)			
C16:1cis-9	1.36 ± 0.16	1.09 ± 0.11*	1.85 ± 0.13*/#
C18:1trans-9	0.08 ± 0.01	0.06 ± 0.02	0.29 ± 0.03*/#
C18:1trans-11	< 0.01	0.10 ± 0.01	0.22 ± 0.06#
C18:1cis-9	6.88 ± 0.56	2.26 ± 0.19*	11.38 ± 0.79*/#
C18:1cis-11	2.08 ± 0.12	1.06 ± 0.10*	2.83 ± 0.14*/#
sum MUFA[2]	10.61 ± 0.69	5.22 ± 0.28*	17.70 ± 1.07*/#
Polyunsaturated fatty acids (PUFA)			
C18:2trans-9,trans-12	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00
C18:2n-6 (LA)	3.75 ± 0.73	1.24 ± 0.42*	2.83 ± 0.74#
C18:3n-6	0.02 ± 0.00	0.13 ± 0.02*	0.17 ± 0.03*
C18:3n-3 (LNA)	2.23 ± 0.22	1.07 ± 0.20*	1.79 ± 0.21*/#
C18:4n-3	0.21 ± 0.14	0.24 ± 0.06	0.12 ± 0.02#
C20:3n-6	2.93 ± 0.32	0.60 ± 0.05*	2.66 ± 0.30#
C20:3n-3	0.14 ± 0.05	1.34 ± 0.06*	0.69 ± 0.03*/#
C20:4n-6	20.36 ± 2.61	22.35 ± 1.97	19.20 ± 0.87#
C20:5n-3 (EPA)	5.82 ± 1.14	24.11 ± 0.92*	13.86 ± 1.23*/#
C22:4n-6	1.84 ± 0.34	1.67 ± 0.25	1.54 ± 0.08
C22:5n-6	2.36 ± 0.32	1.30 ± 0.20*	2.15 ± 0.25#
C22:5n-3 (DPA)	2.63 ± 0.30	5.26 ± 0.48*	3.19 ± 0.13*/#
C22:6n-3 (DHA)	19.24 ± 1.66	9.87 ± 1.09*	9.16 ± 0.42*
sum PUFA[3]	62.18 ± 0.44	70.39 ± 0.89*	58.98 ± 1.24*/#
sum n-3 PUFA[4]	30.28 ± 2.22	41.89 ± 2.29*	28.80 ± 1.35#
sum n-6 PUFA[5]	31.88 ± 2.32	28.47 ± 2.12	30.17 ± 0.46

[1] Sum SFA: 10:0 + 11:0 + 12:0 + 13:0 + 14:0 + 15:0 + 16:0 + 17:0 + 18:0 + 20:0 + 21:0 + 22:0 + 23:0 + 24:0.
[2] Sum MUFA: 14:1 + 15:1 + 16:1 + 17:1 + 18:1t + 18:1c9 + C18:1c11 + C22:1 + C24:1.
[3] Sum PUFA: 18:2tr-9,tr-12 + 18:2n-6 + 18:3n-3 + 18:4n-3 + 20:3n-6 + 20:4n-6 + 20:5n-3 + 22:1 + 22:4n-6 + 22:5n-3 + 22:6n-3 + c9,tr11CLA + 18:3n-6 + 20:2n-6 + 20:3n-3 + 22:2n-6.
[4] Sum n-3 PUFA: 20:3n-3 + 22:6n-3 + 22:5n-3 + 20:5n-3 + 18:4n-3 + 18:3n-3.
[5] Sum n-6 PUFA: 22:2n-6 + 20:2n-6 + 18:3n-6 + 22:4n-6 + 20:3n-6 + 18:2n-6 + 20:4n-6.

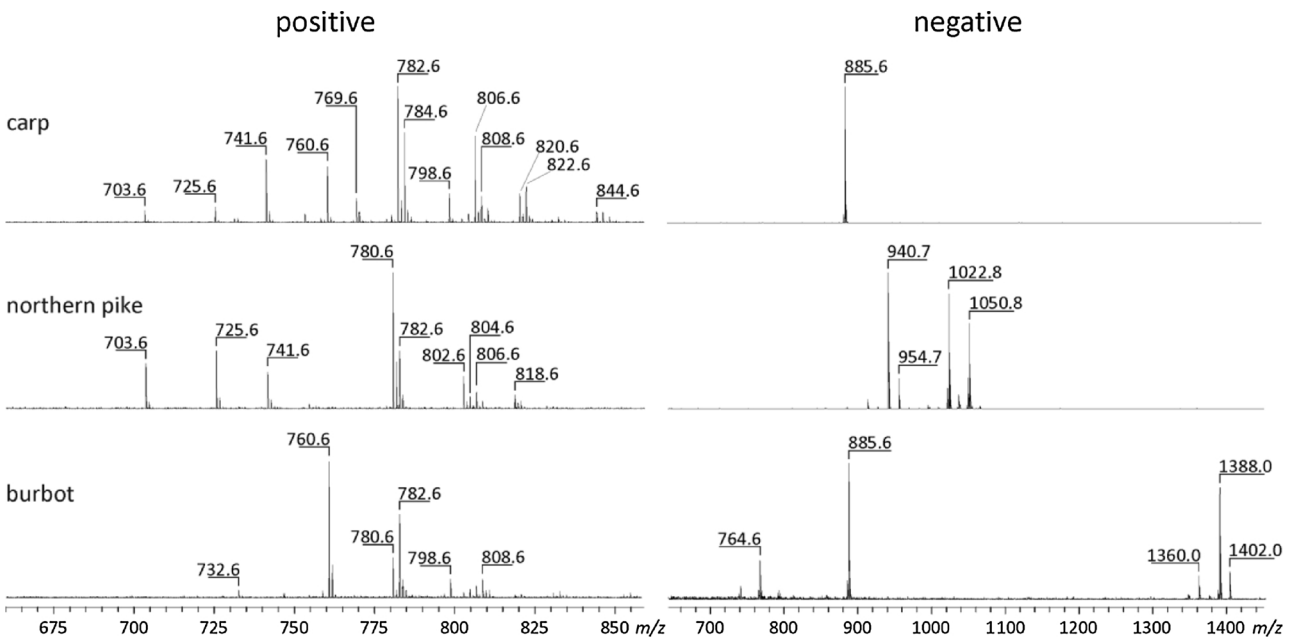

Fig. 2. MALDI-TOF mass spectra of organic extracts from three different fish species. Positive ion spectra (left) were recorded in the presence of DHB, while the negative ion spectra (right) were recorded with 9-AA as matrix. All peaks are labeled with the corresponding *m/z* ratio. For the assignment of the peaks see Suppl. Table S1.

simple because interferences between altered fatty acyl compositions and different alkali metal adducts can be excluded.

Since there is only a single Cs^+ adduct for each PL all assignments can be made unambiguously and confirm the data from the MALDI spectrum. For instance, SM 16:0 (Cs^+ adduct at *m/z* 835.6) is lacking in the burbot sperm while there are significant SM amounts in the northern pike and the carp sperm (**Suppl.** Fig. 1). This is in agreement with the results from NMR and MALDI without CsCl. Additionally, the fatty acyl composition of the northern pike sperm is very simple and consists particularly of PC 36:5 (*m/z* 912.6) and PC 16:0/20:4 (*m/z* 914.6). In the burbot sperm there are also less unsaturated (PC 16:0/18:1) and more unsaturated species such as PC 16:0/22:6. The carp is characterized by the most inhomogeneous fatty acyl distribution. These differences might also be caused by the different nutrition habits of the fish: both, the northern pike and the burbot are predators (Bonde and Maloney, 1960; Lawler, 1965) while the carp represents an omnivore (Hume et al., 1983). The impact of nutrition on the lipid composition of fish was investigated only recently (Fickler et al., 2019).

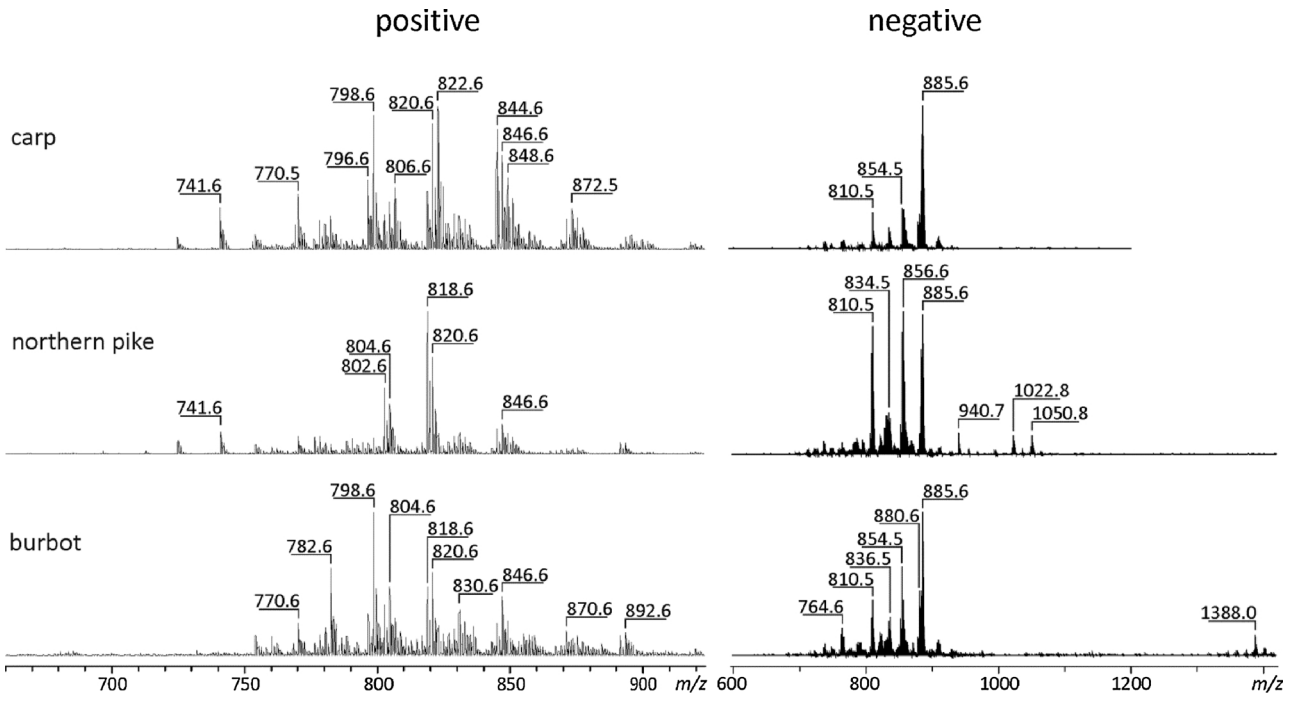

Fig. 3. ESI-IT mass spectra of organic extracts of sperm from three different fish species. Spectra were recorded in the positive (left) and the negative (right) ion mode. For the assignment of the observed peaks see Suppl. Table S2.

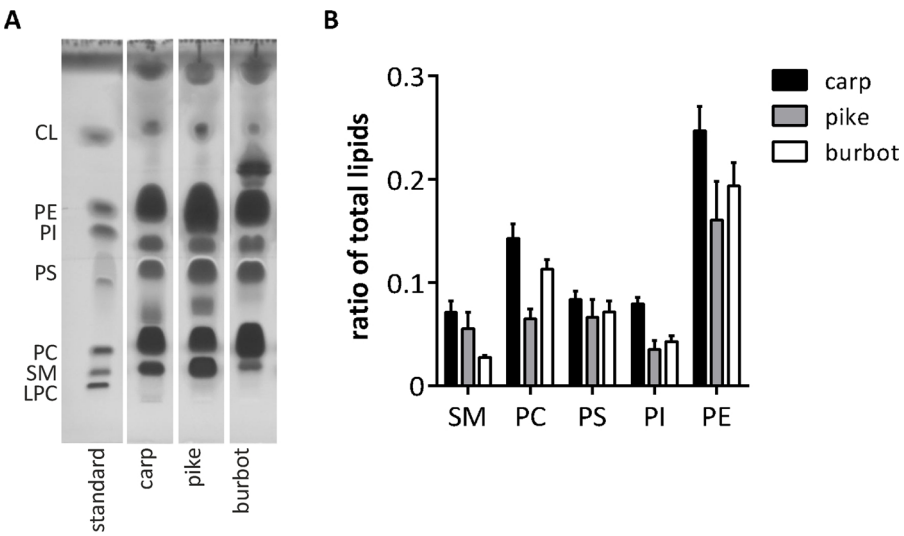

Fig. 4. HPTLC separation of organic extracts of sperm from three different fish species. Lipid fractions in chloroform were separated on a normal phase HPTLC plate by using chloroform/ethanol/water/triethylamine (30:35:7:35; by vol.) as the mobile phase. Lipids were visualized by primuline staining (**A**). The spot densities were quantified by ImageJ. The ratios represent the amount of one particular PL class compared to the total amount of all lipids. Data are presented by mean ± SD (n = 5) (**B**). Only the most abundant PL classes are given. Since subsequent MS analysis was planned, high amounts of lipids were applied onto the TLC plate which leads to considerable spot intensities.

3.3. ESI-IT MS

In addition to the biological problem, another topic of this study was to clarify which information can be most accurately achieved by which method. For this purpose, the ESI-IT mass spectra of the same samples as already characterized by MALDI MS are shown in Fig. 3. There are some remarkable differences in comparison to the MALDI spectra.

The achieved signal-to-noise ratio is obviously poorer compared to the MALDI mass spectra. This is presumably not caused by the different mass spectrometers and their different sensitivities but by the residual salt content of the investigated samples. The salt content of the samples is also obvious from the detection of different alkali metal adducts of the respective PL. This sheds light on the considerable sensitivity of (ESI) MS to salts.

Although ESI-IT MS detects the same PC species as MALDI-TOF MS, the yields of the H^+ adducts are lower in the ESI-IT spectra and it is obvious that there are also PL species with an increased chain length such as PC 16:0/22:6 (*m/z* 844.6 for the K^+ adduct) and a higher double content (K^+ adduct of PC 40:7 at *m/z* 870.6).

In contrast to MALDI-TOF MS, SM is detected with poor sensitivity by ESI-IT MS. Nevertheless, it is clear that the typical SM peaks are lacking in the case of the burbot. This agrees with the MALDI and NMR data which both indicated a relatively low SM content of the burbot sperm.

Particularly remarkable are the negative ion spectra: while in the negative ion MALDI spectra PI was primarily detectable, the ESI spectra exhibit a number of additional peaks which can be mainly assigned to PS. PS species were not detectable at all by MALDI MS. This is remarkable because PS represents an acidic PL and should be detectable with a comparable sensitivity as PI. Again, there is no PE detectable. In contrast to PS or PI that already exhibit a negative charge, PE is a zwitterion and needs to be ionized by the addition or the subtraction of a proton (for the characteristics of the PL head groups see (Pulfer and Murphy, 2003)). Although the comparison of peak intensities should be regarded with great caution, the (relative) PS content is most marked in the case of the northern pike. This agrees with the NMR spectra. The two most abundant PS species correspond to PS 18:0/20:4 (*m/z* 810.5) and PS 18:0/22:6 (*m/z* 834.5). This high content of double bonds is expected for spermatozoa and has been described earlier (Engel et al., 2017; Fuchs et al., 2009; Schiller et al., 2003).

3.4. HPTLC-ESI-IT MS

In order to overcome ion suppression effects, which prevent the detection of poorly detectable PL with small concentrations, all samples were separated by HPTLC into the individual lipid classes prior to MS.

The visualization of lipids on the HPTLC plate revealed obvious differences regarding the lipid compositions of sperm samples from the three different fish species. SM, PC, PS, PI and PE were present in all three fish species but in different amounts (Fig. 4A).

Fig. 4**B** shows the moieties of SM, PC, PS, PI and PE related to the total amount of lipids. According to these data PE is the most abundant lipid species in the investigated fish sperm (carp 24.7% ± 2.3%, northern pike 18.7 ± 4.4%, burbot 19.4 ± 2.3%), and differences are particularly significant between carp and burbot ($P = 0.016$). The PC moieties vary between 6.5 ± 1.0% in the northern pike and 14.3 ± 1.4% in carp. Whereas in carp SM accounts for 7.1 ± 1.1% of all lipids, the SM content is lower in the northern pike (5.5 ± 1.6%, $P = 0.155$) and even significantly lower in the burbot (2.8 ± 0.2%, $P = 0.003$). The same is true for the PI content which is significantly lower in the northern pike (3.5 ± 0.9%, $P < 0.001$) and the burbot (4.3 ± 0.6%, $P < 0.001$) compared to the carp sperm (8.0 ± 0.6%).

Even though, the differences in the SM contents in the investigated fish species are obvious from both, the NMR and the TLC experiments, the TLC data differ considerably from the data obtained by ^{31}P NMR. While PE is the most abundant lipid species according to TLC, PC is the most abundant lipid species in the NMR spectra. One reason for this discrepancy might be the binding efficiency of primuline which varies for the different PL classes and, thus, the PE moiety might be overestimated (White et al., 1998). Therefore, quantitative data determined by primuline staining should be discussed with great caution - although relative differences (i.e. relative differences between the individual lipid classes) can be reliably obtained. Furthermore, it needs to be emphasized that only lipids containing at least one phosphate group, namely PL, are detectable by ^{31}P NMR while HPTLC separation indicates that there are also other non-phospholipids present in the samples.

To elucidate the composition of the individual PL fractions, spots were directly analyzed by ESI-IT MS. Representative mass spectra of the relevant spots are shown in **Suppl. Figs. 2-5**. The assignments of all observed signals are additionally given in **Suppl. Tabs. S3-S6**.

The SM composition of the investigated fish species differs with respect to the chain length of the fatty acyl residue. In carp SM there are only middle chained residues, such as C16 (*m/z* 703.6, 725.6) and C18 (*m/z* 731.6, 753.6). In northern pike there are also minor amounts of C22 (*m/z* 785.6, 807.6) and C24 residues (*m/z* 835.6). In burbot these long chain residues even dominate the SM spectra with additional signals at *m/z* 821.6 and 849.6 that could not be assigned to any SM species with even acyl residues. Therefore, it is likely that these peaks

represent odd-chained SM species - although the mechanisms leading to these species in burbot are unknown. Also the PC spectra differ between the three fish species. While there are only a few PC species in the northern pike sperm, the composition of the carp is more complex. Whereas the carp PC fractions are dominated by mono-, di- and polyunsaturated PC with three to six double bonds, there are nearly exclusively PC molecules with four to six double bonds and only very few monounsaturated PC in the northern pike. This agrees well with the ^{31}P NMR data where different line-widths of the PC resonances were detectable which indicates differences in the fatty acyl compositions (Komoroski et al., 2008).

Potassium ions which aggravated the assignments of the peaks in the MALDI mass spectra are nearly completely removed subsequent to TLC separation - maybe due to the poor solubility of potassium salts in methanol which was used to extract the PL from the silica gel.

The most pronounced difference in the PE species detected in the three fish species is stemming from the chain length. Species such as PE 16:0/20:5 (*m/z* 736.5) and PE 16:0/20:4 (*m/z* 738.5) are nearly completely missing in the burbot sperm while they are abundant in the carp and the northern pike. Small amounts of ether PE (*m/z* 750.5) were detected in the northern pike and in the burbot. In carp sperm PE species with 42 (*m/z* 810.5 and 812.5) and 44 C atoms (*m/z* 834.5, 836.5 and 838.5) were also detected. It is also important to note that in contrast to PC there were no saturated and monounsaturated PE molecules detectable in any of the investigated fish species. Overall, the content of unsaturated acyl residues was higher in the PE than in the PC fraction.

Analysis of the PS fractions revealed only slight differences in the fatty acyl compositions between the three fish species. For instance, a comparison of the peak intensities of PS 38:5 (*m/z* 808.5) and PS 38:4 (*m/z* 810.5) reveals that the content of eicosapentaenoic acid (20:5) is very low in the carp.

3.5. Total fatty acid composition analyzed by gas chromatography

The GC investigation of the fatty acyl residues incorporated in the different lipids revealed that the moiety of saturated fatty acids decreases slightly from carp to burbot (Table 1). In contrast, the proportion of monounsaturated fatty acids (MUFA, primarily oleic acid) is highest in burbot. Sperm of the northern pike are characterized by a very high degree of polyunsaturated fatty acids (PUFA, particularly docosahexaenoic acid). This is in agreement with the results from the HPTLC-ESI MS experiments. Furthermore, the GC results show that the sum n-6 PUFA proportions are nearly the same in all three fish species, but the n-3 PUFA proportions are approximately 10% higher in the sperm of the northern pike compared to carp and burbot. Different PUFA, such as C22:5, C22:6 and C20:4 are involved in processes regulating spermatogenesis, and sperm motility as well as the fertilization capacity of sperm in different fish (Baeza et al., 2015; Mansour et al., 2011). Besides structural aspects due to its double bond content, C20:4n-6 plays a tremendous role in signal transduction and is the source of messenger molecules such as leukotrienes and prostaglandins. However, the role of these signaling molecules in fish fertility has to be investigated. Even though this study focusses on sperm, the high amount of unsaturated fatty acids (MUFA + PUFA ≈ 76%) in the sperm of the northern pike is in accordance with (Kluytmans and Zandee, 1973) who reported a high degree of unsaturated FA in northern pike testis. The amounts of the different fatty acids in burbot sperm are in accordance with the results of a recent study comparing cultured and wild burbot (Blecha et al., 2018). In contrast to the lipid metabolite identification by TLC, MALDI MS and ESI MS, GC of lipid mixtures only gives information on the total fatty acid composition of all lipid classes (PL and non-PL) without information about their origin.

3.6. Conclusion

The hypothesis of this study was that the lipid composition of sperm from fish with an external fertilization mode differs depending on the spawning temperature. According to the current knowledge about the relation between membrane fluidity and temperature, characteristic changes in the double bonds, the cholesterol and the SM content of sperm from fish species spawning at different water temperatures were expected. By NMR and MS analyses it could be shown that there are characteristic differences in (a) the cholesterol, the SM, the PC, the PI and the PS content, (b) the length of the fatty acids and (c) the number of double bonds. The different contents of acidic PL are presumably important because they mediate the ion binding capacity of sperm which is an important event during the fertilization process. The fish with the lowest spawning temperature, the burbot, was characterized by the lowest PUFA but the highest MUFA proportion, while the northern pike had an elevated amount of PUFA, particularly based on the n-3 fatty acids. Eicosapentaenoic acid was about four times higher in the northern pike than in the sperm from carp and about twice as high as observed in the burbot. The fish with the highest spawning temperature, the common carp had the most diverse PL composition and was characterized by an elevated SM content. Considering the theory behind the liquid-crystalline ordered phase of biological membranes (McMullen et al., 2004), the high cholesterol content in the sperm of northern pike is in agreement with the high degree of unsaturation in the PL moieties. Furthermore, the affinity of cholesterol is different for PL with different head groups and highest for SM and PS. This is in accordance with a high SM and PS content in northern pike, too. However, next to the spawning temperature there are other differences in the spawning behavior and differences in the nutrition in the three fish species which might explain the different lipid compositions. Therefore, we conclude that different spawning temperatures are not necessarily reflected by the PL compositions of sperm.

Additionally, this study comprises some important methodological aspects. In particular, it was shown that the evaluation of the spot intensities of primuline-stained TLC plates yields different results than NMR and should be, thus, regarded with caution.

To our best knowledge this was the first investigation of PL of fish sperm with different, modern analytical methods.

Declaration of contributions

Conception and design: KME, BD, JS. Analysis and interpretation of the data: KME, BD, DD, JS. Drafting of the article: KME, BD, SS, JS. Critical revision of the article for important intellectual content: KME, BD, SS, JS. Final approval of the article: KME, BD, SS, PP, TP, DD, JS. Provision of study materials or patients: BD, PP, TP. Statistical expertise: KME. Obtaining of funding: BD, JS. Administrative, technical, or logistic support: DD, PP, TP. Collection and assembly of data: KME, DD, JS.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.chemphyslip.2019.03.014.

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