A metabolomics approach to elucidate effects of food deprivation in juvenile rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

The aim of this study was to evaluate if NMR-based metabolomics is a suitable method to generate an integrated view on metabolic processes during food deprivation in salmonid fish. Juvenile rainbow trout (*Oncorhynchus mykiss*) weighing 43-115 g were either fed *ad libitum* or deprived of food for 28 days at 10°C to investigate catabolic effects on energy reserves and metabolite patterns. The NMR analysis of plasma, liver and muscle extracts revealed significant fasting-induced changes in the metabolome. Altered plasma lipoprotein levels and tissue specific patterns of fatty acid mobilization were the most prominent responses, emphasizing the role of lipids as the primary energy source during fasting. In plasma, very low density lipoprotein levels increased in food deprived fish compared to fed, whereas levels of high density lipoprotein decreased. Signs of muscle protein catabolism were also observed as changes in the alanine dynamics. The results further suggest that mechanisms to preserve liver glycogen are present in the food deprived fish. This study thus demonstrates the utility of NMR-based metabolomics of tissue extracts and plasma to describe the integrated metabolic status of fish.

Keywords: fish, fasting, NMR, lipoprotein, fatty acid, glycogen, protein, amino acid
INTRODUCTION

Salmonid fish encounter periods of little or no food intake, e.g. due to low food availability during winter conditions. Thus, these species need to be able to handle large changes in energy stores and nutritional status, with major changes in metabolism and its regulation. Juvenile salmonids are highly competitive and exhibit rapid growth when food sources are sufficient. Low food intake and impaired growth at an early stage may result in lower performance during later life stages since condition and/or lipid stores are decisive for future survival and timing of sea migration (5, 11, 22). The catabolism of stored lipids appears to be central for salmonids to cope with severe restriction in food intake (1, 10, 24). Fasting leads to an increased release of fatty acids (FA) from lipid stores (mesenteric, muscle, belly flap and liver). The FAs are subsequently transported to the liver where they are included in triacylglycerols (TG), which are then incorporated in very low density lipoproteins (VLDL). These lipoproteins, which contain most triglycerides (3) are then released into the circulation (33). In line with this, the activities of key lipid metabolic enzymes, such as hepatic lipase (34) and lipoprotein lipase in both muscle and adipose tissue (1) increase during fasting. This leads to elevated plasma FA levels and enables maintained lipid uptake and metabolism by other tissues.

The role of carbohydrates and proteins as energy sources during short term fasting (days-weeks) or long term starvation (months) in different fish species are less clear (24, 29, 34). A rapid decrease in liver glycogen appears to be a common early response to food deprivation in vertebrates, but as fasting progresses, the liver glycogen levels may be replenished (12, 24). In coho salmon (Oncorhynchus kisutch), liver glycogen decreases one week into fasting, but is back to normal after three additional weeks of food deprivation (34). In line with this, plasma glucose levels during fasting tend to be rather stable, returning to “fed” levels after an initial decrease.
Protein breakdown has only been observed during prolonged food deprivation in salmonids, but not during the acute phase (24, 34). In brown trout (Salmo trutta), increased plasma amino acid levels, reflecting protein breakdown, is evident after 15 days of food deprivation, according to one study (23) whereas another study finds earliest signs of protein mobilization at day 50 (24).

In sockeye salmon (Oncorhynchus nerka), alanine can be used as a substrate for gluconeogenesis during the spawning migration (12), indicating protein breakdown in order to mobilize energy.

Previous studies on the metabolic response to food deprivation in salmonids have focused on selected parts of the metabolism using a number of in vivo or in vitro approaches. In some instances in vitro studies are preferred when responses of specific tissues are being investigated, e.g. by using primary cell cultures from liver (12) or adipose tissue (1). However, such “focused” approaches are insufficient to elucidate the integrated metabolism of the fish, taking into account the many pathways and the complex systems involved in metabolic regulation, including interplay between different tissues (26). For this, an integrated in vivo approach is needed.

Metabolomics is defined as the global profiling of small endogenous compounds (metabolites) in an organism, tissue or biofluid, and it has proven to be a reliable and reproducible method suitable for metabolite identification in fish and other organisms (16). Furthermore, the use of “exploratory” or “open” analytical methods, such as NMR-based metabolomics, provides the means for discovering important, unexpected responses that otherwise might pass unnoticed.

Studies on basic fish physiology applying metabolomics are still quite scarce, though progress in this area is being made (8, 16, 31, 38). The objective of this study was to use NMR-based metabolomics to obtain a whole animal perspective of which metabolites are up- or down-regulated during food deprivation in salmonids. Rainbow trout were therefore fasted for 28 days to reach a catabolic state. In order to better understand metabolic fluxes, blood plasma, liver and muscle samples were analyzed by $^1$H NMR spectroscopy and the resulting metabolite levels from
fed and fasted fish were compared using multivariate analytical tools. Growth and energy stores
were also monitored in order to estimate the energy status and general condition of the fish.

MATERIALS AND METHODS

Fish and experimental conditions

All animal experiments were approved by the local animal ethics committee in
Gothenburg, Sweden. Juvenile rainbow trout (Oncorhynchus mykiss) were brought from a local
fish farm (Vänneåns Fiskodling AB, in southern Sweden) to the Department of Zoology,
University of Gothenburg, and allowed to acclimate for 18 days. During this period, the fish were
kept in a 500 L tank with sand-filtered recirculating freshwater that was aerated and kept at
~10°C. Oxygen levels always exceeded 80% saturation. The photoperiod was set to 12L:12D.
The experiment was performed during October-November. At the start of the experiment, 40 fish
were quickly netted one at a time and anesthetized using 2-phenoxyethanol (0.4 mL × L⁻¹) and
subsequently weighed, measured (n=40; mean weight 74.7 ± 3.0 g; mean length 179.9 ± 2.3 mm;
mean condition factor 1.26 ± 0.01) and implanted with individual PIT-tags (ID100; Trovan Ltd).
The fish were randomly placed in four different 100 L glass aquaria with 10 fish in each
aquarium. In two tanks, the fish were deprived of food while in the other two, the fish were fed
commercial trout feed administered ad libitum by hand once a day until satiation, six days per
week, for 28 days. The aquaria were covered with black plastic to reduce potential stress on the
fish caused by people entering the room. The water and light conditions were the same as during
acclimation. Body weight (BW) and body (fork) length (FL) was recorded during anesthesia (as
above) at day 0, 18 and 28.
Sampling procedure

On day 28, all fish (one at a time, alternating among the four aquaria) were anesthetized as above and killed by a quick blow to the head, weighed and measured. Blood was withdrawn from the caudal vessels using heparinized syringes. The blood samples were centrifuged at 3000 \( \times g \) for 5 min at 4°C, and the obtained plasma transferred to new tubes and kept on ice for no more than 10 min, until frozen and stored at -80°C until analysis. Liver, muscle tissue [Norwegian quality cut (NQC)] (2) and mesenteric fat (adipose tissue in the abdominal cavity, including that surrounding the gut, pyloric ceca and intestine) were dissected. Liver and mesenteric fat weights were recorded. All samples were immediately frozen in liquid nitrogen and later stored at -80°C until analysis. For NMR analysis 15 plasma and 12 muscle/liver samples were randomly chosen from each treatment group.

Condition Factor (CF = 100 \( \times \) BW [g] \( \times \) FL [cm\(^{-3}\)]), Daily Growth Coefficient (DGC = 100 \( \times \) (BW at sampling\(^{0.333}\) – BW at start\(^{0.333}\)) \( \times \) time [days\(^{-1}\)] (7), Hepatosomatic Index (HSI = 100 \( \times \) liver weight \( \times \) BW\(^{-1}\)) and Mesenteric Fat Index (MFI = 100 \( \times \) mesenteric fat weight \( \times \) BW\(^{-1}\)) were calculated. Liver and muscle tissue lipids were extracted and total lipid content was quantified according to the method described by Silversand (37) adapted from Bligh and Dyer (4).

NMR-based metabolomics

Sample preparation. Muscle and liver samples (n=12) were prepared according to the two-step method described by Wu et al. (45) with minor adjustments. One-hundred mg tissue was put in a 2 mL test tube with 400 \( \mu \)L methanol and 85 \( \mu \)L deionized filtered water and was then homogenized with a stainless steel bead (5 mm, from Quiagen) in the TissueLyser.
(Quiagen) for 2 × 3 min at 25 Hz. The samples were kept cool by cooling mantles during the homogenization. The homogenate was transferred to a new tube and 400 μL chloroform plus 200 μL deionized filtered water were added. The solution was vortex-mixed and placed on ice for 10 min before centrifugation at 3000 × g for 10 min at 4°C. The polar and lipophilic phases were transferred to separate tubes and the solvents were evaporated under reduced pressure. Samples were stored at -80°C until further analysis.

$^1$H NMR spectroscopy. The NMR analyses were carried out at the Swedish NMR Centre at the University of Gothenburg, Sweden. The $^1$H NMR spectroscopy was conducted at 599.75 MHz on a Varian Inova 600 MHz spectrometer with a 600 MHz cold probe. Tuning and shimming was performed for each sample.

Plasma. The plasma samples (n=15) were allowed to thaw at room temperature one at a time, in random order, before preparation for $^1$H NMR. After thawing, 150 μL plasma and 200 μL D$_2$O was mixed and transferred to a 5 mm Shigemi NMR tube (bottom length 15 mm, matched with D$_2$O; Shigemi Inc. USA). Plasma one-dimensional $^1$H NMR spectra were acquired at 25°C using a 8.0 μs pulse (90°) with an acquisition time of 1.63 s, spectral width of 10 kHz, 60 dB transmitter power, 0.5 s relaxation delay with 128 scans collected into 32 k data points. To suppress the water signal a presaturation (presat) pulse was applied for 1.5 s with a power of 10 dB (‘presat spectra’). To attenuate resonances from macromolecules the Carr-Purcell-Meiboom-Gill (CPMG) sequence (20) [relaxation delay – 90° - (τ – 180° -τ)$_n$ – acquire] was also used for recording spectra of the plasma samples (‘CPMG spectra’) using $n$=100 and a total CPMG time of 80 ms.

Liver and muscle hydrophilic extracts. Tissue hydrophilic extracts were dissolved in 80 μL 0.2 M Na$_2$HPO$_4$/NaH$_2$PO$_4$ buffer (pH 7.4) and 270 μL D$_2$O (containing TSP) and transferred
to a Shigemi NMR tube. The settings for 1D $^1$H NMR spectroscopy were the same as for plasma, except the pulse width that was set to 8.4 µs.

Liver and muscle lipophilic extracts. Lipid extracts from each fish were resuspended in 700 µL CD$_3$OD:CDCl$_3$ (1:2 mix) and transferred to a 5 mm NMR tube. 1D $^1$H NMR spectra were collected at 4°C using a standard sequence (90° pulse, 7.2 µs pulse width, 2.4 s acquisition time, 10 kHz spectral width, 60 dB transmitter power, 5.5 s relaxation delay, 64 scans and 65 k data points).

Data handling and statistics

All free induction decays were Fourier transformed and the resulting spectra were phased and baseline-corrected using NMR Processor (v11.0, ACD labs, Toronto, Canada). Chemical shifts were referenced to the valine methyl doublet at 1.042 ppm for plasma, TSP (0 ppm) for hydrophilic extracts and TMS (0 ppm) for lipid extracts, respectively. The regions 0.05-8.0 and 0.5-8.5 ppm for plasma presat and CPMG spectra, respectively, 0.05-9.5 for tissue hydrophilic extract spectra and 0.5-7.0 for tissue lipid extract spectra were divided into 0.01 ppm wide buckets and (the signal intensities were) integrated. Solvent peak regions 4.67-4.95 (presat), 4.66-5.10 (CPMG), 4.7-5.0 (hydrophilic extracts), 3.33-3.39 (lipid extracts) and 4.57-4.97 (lipid extracts) were excluded before integration and bucketing. The sum of all integrals was set to 100 for each spectrum to normalize the signal intensity and allowing the identification of relative changes in metabolite levels in the different spectra.

The data sets of bucket integrals were imported to SIMCA-P (version 11, Umetrics, Umeå, Sweden). For each data set, all variables (buckets) were Pareto scaled and Principal Component Analysis (PCA) (44) was performed to get a general overview of the data and search for outliers (data not shown). No outliers were found and all observations (n=15 for plasma samples for each of the treatment groups fed and fasted and n=12 for tissue extracts for each of
the treatment groups fed and fasted) were included in the data analyses. Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) (41) was conducted and the output was used to search for differences between fasted and fed fish. OPLS-DA is a supervised method, where information on class affiliation is included in the model which maximizes the separation according to class (e.g. treatment group) in the first component. With OPLS-DA unwanted variation is removed by focusing all variation from treatment effects into the first component. This facilitates the identification of variables separating the classes. Buckets were sorted according to their contribution to the model (the term ‘p’ in SIMCA-P). The buckets which contributed considerably to the separation (‘variable magnitude’ absolute value >0.04) and were significantly different (‘variable confidence’ absolute value >0.5) between the treatment groups were selected for identification. The OPLS-DA models were evaluated by assessing the model fit (R²) and prediction quality (Q²) using the inbuilt feature of the software. As a measure of the model quality, the likelihood of acquiring higher R² and Q² values by chance was evaluated using the automated validation function in SIMCA-P. For each model 9990 permutations were performed, where class (i.e. treatment group) was randomly assigned to each individual fish and a new model was created. The R² and Q² values from these randomized models were then compared to the values from the original, empirically derived models.

NMR peak assignments were initially based on published spectral data and tables (9, 13, 18, 25, 30, 36, 39) as well as databases (43). To confirm these assignments, ¹H-¹H COSY and ¹H-¹³C HSQC analyses were run on pooled samples from both treatment groups. To further examine the chemical shift positions of different lipoproteins within the ‘lipoprotein peaks’ a diffusion-edited NMR experiment based on Liu et al. (19) was conducted.

The morphological data were analyzed using a General linear model (GLM) with ‘treatment’ as fixed factor and tank as covariate, using 0.05 as significance limit. Variables that
did not fulfill the criteria for homogenous variance (Bartlett test >0.05) were log transformed during the statistical analysis. Values are presented as mean ± standard error of the mean (se), if not stated otherwise.

RESULTS

Morphometric data, liver and mesenteric lipid extraction analyses

BW, FL or CF did not differ between the groups of fish in the four tanks at the start of the experiment (GLM, p>0.05). After 28 days, the BW of fed fish had increased from 80 to 113 g, as well as FL from 184 to 208 mm. The BW of the fasted fish had decreased from 69 to 62 g, but FL had increased slightly from 176 to 179 mm. Thus, fasted fish had significantly lower BW and FL than the fed fish at day 28, as well as a negative Daily Growth Coefficient (Table 1).

Liver and mesenteric fat weights were lower in fasted fish than in fed fish after 28 days (Table 1). Also, HSI was lower in fasted fish than in fed fish at day 28 (Table 1). However, no difference in MFI between fed and fasted fish was observed (Table 1). The muscle lipid content was lower in fasted fish compared to in fed fish (Table 1). Liver lipid content did not differ between the treatments groups (Table 1).

NMR data

Examples of 1H NMR spectra of plasma and tissue extracts are presented in Figure 1. In the presat spectra, resonances from several types of metabolites, e.g. lipoproteins, lipids and glycoprotein acetylts, are shown. In the CPMG spectra, signals from larger molecules are suppressed and smaller compounds, e.g. lactate, choline and glucose, are more lucid. Spectra from liver and muscle hydrophilic extracts contain various peaks representing water-soluble metabolites, such as alanine, lactate and glycogen. Tissue lipid extract spectra present
information on different lipid classes, mostly various fatty acid groups. By separating the water-
and lipid-soluble compounds, spectra with fewer metabolites are obtained, thus reducing peak
overlap.

A distinguishable separation in the first component of OPLS-DA scores plots is apparent
for all datasets (Figure 2). The separation along the x-axis (OPLS-DA component 1) represents
differences related to the treatment. All other variation in the NMR data is visualized as
separation in the y direction (second component). The cross-validation of plasma metabolomics
OPLS-DA models (1+1 component) gave $R^2 > 0.7$ and $Q^2 > 0.6$. For the hydrophilic tissue
extracts (1+1 component) the same values were $R^2 > 0.8$ and $Q^2 > 0.7$ and the lipid extracts (1+3
components) resulted in $R^2 > 0.8$ and $Q^2 > 0.3$. The $R^2$ and $Q^2$ values in all our models were
significantly higher than in the random models. The p-values were <0.0001 for the $R^2$ for all
models and for the $Q^2$ of plasma presat and CPMG as well as liver and muscle hydrophilic
extracts. The p-value for the $Q^2$ of liver lipophilic extracts was 0.007 and for muscle lipophilic
extracts the p-value was 0.0001 for the $Q^2$.

**Plasma.** Plasma NMR-results are presented in Table 2. The top-ranking components from
the OPLS-DA analysis of plasma NMR data belong to lipid metabolism, where increased
unsaturated lipids in fasted fish are mainly responsible for the separation of the metabolomes of
the two treatment groups (Table 2). Treatment group differences in lipoprotein levels were also
significant, the most evident difference being higher VLDL and lower high density lipoprotein
(HDL) levels in fasted fish compared with fed fish. Apart from the same changes as in the presat,
results from CPMG spectra showed that there were a number of differences between treatment
groups with regards to metabolites with smaller MW. The most pronounced result was down-
regulated choline in fasted fish in comparison with fed fish. Other differences included lower
levels of β-glucose and lactate in fasted fish compared with fed fish.
Liver. Among the water-soluble liver metabolites, lower levels of alanine and lactate were observed in fasted compared with fed fish (Table 2). In addition, fasted fish had increased liver glycogen compared with fed fish (Table 2). Liver lipid profiles further showed that total liver FA levels were lower in fasted fish than in fed fish (Table 2). Fasted fish had higher levels of unsaturated \((n-3)\) FA, and of the more specific FA, such as 18:2 and non-DHA FA in the liver than fed fish (Table 2).

Muscle. Muscle phosphocreatine and alanine levels were higher in fasted fish compared with fed fish (Table 2). At the same time, lactate and betaine levels were lower in fasted fish than in fed fish. Muscle lipids showed similar responses to fasting as in the liver. Total FA levels were elevated along with increased \(n\)-3 and 18:2 FA in fasted fish compared with fed fish. Additionally, levels of 18:1 \((n-9)\) FA were lower in muscle of fasted fish compared with fed fish.

DISCUSSION

The present study demonstrates significant fasting-induced changes in the metabolome of rainbow trout identified by NMR-based metabolomics. Analysis of plasma, liver and muscle extracts clearly shows that an altered lipid metabolism is a central process during a catabolic state in rainbow trout. However, the condition of the fish, together with the size of the hepatic glycogen stores and the mesenteric fat deposits indicate that there are still significant energy reserves available after 28 days of food deprivation. This maintenance of energy stores even after four weeks of fasting at 10°C is similar to observations in fasted brown trout (24), indicating that salmonids have the capacity to tolerate several months with little or no food.

Changes in lipoprotein levels is the major contributing factor for differences in plasma constituents/metabolites between fed and fasted fish, emphasizing the importance of lipids as the
primary energy source during fasting. Fasting leads to the release of FA from various stores, after which the FA are further metabolized into TG in the liver and secreted back to the blood as VLDL (33). According to the present results, lipids from muscle and adipose stores are utilized first, whilst liver lipid stores are not used to the same extent during the first four weeks of fasting. This is in accordance with the view that mesenteric adipose tissue is the most important lipid source in salmonids, followed by lipids in muscle and liver (35). However, although total liver lipid concentration appears similar between fed and fasted fish, using lipid extraction, the NMR results show decreased total FA in both liver and muscle in food deprived fish. This indicates that hepatic lipids have also been used during the period of fasting. The relatively small size of the liver (compared with mesenteric adipose and muscle tissue) limits the amount of lipids available for mobilization from this source. One advantage with NMR is that it is more sensitive than the gravimetric method, and can detect such subtle changes as appear to have occurred in the liver. At the same time, NMR separates different lipid classes/fatty acids and thus gives a more detailed qualitative description of lipid metabolic patterns. In agreement with previous studies, the fatty acid 18:1 \((n-9)\), which is one of the most readily used energy sources (via \(\beta\)-oxidation) in teleosts (40), is lower in muscle of the fasted fish than in their fed counterparts (14). Both 18:1 \((n-9)\) and 20:1 \((n-9)\) are mobilized from liver and muscle stores in fasted rainbow trout, saving the \((n-3)\) FA (14). In the present study, higher levels of 18:2 FA in muscle and liver of fasted fish are observed. Previously, 18:2 FA has been shown to decrease somewhat in muscle, but to increase in mesenteric adipose tissue and liver of fasted rainbow trout (14). Taken together, these results demonstrate clear tissue-specific patterns of FA mobilization.

The elevated alanine levels in the muscle of the fasted fish indicate ongoing protein catabolism in this tissue. Alanine levels also increased in white muscle tissue in spawning migrating sockeye salmon (21). Although juveniles potentially are more sensitive to fasting than
are adult fish, our observation indicate that there are commonalities between life stages in protein metabolism during fasting in salmonids. Alanine is likely used as substrate for glycogen and/or glucose production in the liver (6, 28, 34), but it may also be oxidized in the liver and used as a direct energy source (28). This scenario is further supported by the decreased levels of alanine in the liver of the fasted fish. In fish, increased hepatic uptake of alanine (6) and the preferred use of alanine as a substrate in gluconeogenesis (12, 28) enable hepatic glycogen levels to increase during fasting. The increase of liver glycogen concentration may, to some extent, arise as a compensation for the decreased liver weight. In addition to alanine, lactate is a preferred substrate for gluconeogenesis in fish, which may lead to maintained or even increased liver glycogen stores during fasting (12). The decreased hepatic levels of alanine and lactate, along with the increased glycogen of fasted fish suggest that maintained glycogen levels may be important as a part of the metabolic adaptation during a catabolic situation in rainbow trout. Rapid (within days) depletion of liver glycogen stores during fasting is reported to occur in fish (24, 34). Apart from this, choline levels are lower in the fasted than in the fed fish (Table 2). Choline is a precursor to phosphatidylcholine and is important in the formation of lipoproteins as well as several other steps in the general lipid metabolism (46).

Together, these data suggest that the initial phase of fasting in juvenile rainbow trout involves the use of easily accessible energy, both lipids and glycogen. Sustained fasting may eventually cause the need for protein catabolism to replenish glycogen stores. However, the relative increase in alanine levels in muscle of the fasted fish in this study also indicate that proteins are mobilized as energy in these fish even during a period of fasting where there is still a fair amount of energy reserves left in mesenteric lipid stores. This is in line with results from brown trout where muscle protein began to decrease while there were still adipose reserves remaining (24).
The higher levels of phosphocreatine in muscle of fasted fish implies that readily available energy for burst swimming is maintained, and that foraging activities and predator avoidance behavior are not necessarily compromised during periods of fasting. Sustained phosphocreatine levels during fasting are in accordance with a previous study, where white muscle of rainbow trout had increased phosphocreatine levels after one-week fasting at 15°C (17). Thus, this may be a general mechanism that allows fish to maintain important behavior responses to a certain extent during fasting. It is, however, clear that fasting does affect behavioral responses to some degree, as rainbow trout fasted for 12 days have less fighting ability than their fed counterparts (15).

The nutritional state and general condition of the fish at the start of the fasting period is important and will influence how well the fish can tolerate fasting (10). In the present study, the fish had a high condition factor of 1.26 when fasting was initiated, and this is certainly a contributing factor to why mesenteric fat stores were not depleted after four weeks of fasting. Although the present study was carried out on hatchery-reared rainbow trout under laboratory condition, the metabolomics approach may be a valuable research tool in the future in assessing nutritional status of wild fish in nature. As wild fish are generally leaner than hatchery-raised fish, their metabolic responses to fasting might differ, both quantitatively and qualitatively. Further studies on wild fish are needed to elucidate such differences, as it is possible that domesticated fish such as the rainbow trout in the present study have different responses and regulation of appetite and growth as well as storage and mobilization patterns.

NMR metabolomics is, in general, a robust and reproducible method to analyze multiple metabolites in biofluids or tissue extracts in parallel (27, 42), however its utility to assess metabolic fluxes in fish has, to our best knowledge, received little attention (32). In contrast to many other methods, including various mass-spectroscopy-based analyses, the method is non-
destructive, i.e. the sample can be reused. A potential disadvantage is the relatively large sample volume required, partly circumvented here by using Shigemi tubes with a narrow diameter, rendering the method applicable also to small fish. Despite the lower sensitivity of NMR compared with mass-spectrometry, limiting the number of measurable metabolites, an interpretable picture of the metabolic status was generated. Another common challenge with NMR metabolomics is the discrimination of peaks from different metabolites, both due to overlapping peaks arising from the complex mix of molecules and by the presence of macromolecules, such as lipids, generating particularly broad resonances. Here two approaches were successfully applied to increase the ability to discriminate peaks; we physically separated molecules in the complex samples, e.g. by generating hydrophilic and hydrophobic extracts, prior to the analyses. We also applied a pulse programme attenuating resonances from macromolecules (CPMG).

**PERSPECTIVES AND SIGNIFICANCE**

The NMR metabolomics analysis shows that a large number of plasma, liver and muscle metabolites are affected during fasting in juvenile rainbow trout. The fish primarily mobilizes lipid stores as energy during fasting to cope with a negative energy balance. Protein and carbohydrate metabolism was also affected by fasting, as muscle protein was catabolized and mechanisms to preserve liver glycogen were initiated. This study demonstrates the applicability of NMR-based metabolomics and it is concluded that this method is suitable for further studies within integrative fish physiology.

**GRANTS**
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Table 1. Morphological characteristics of Rainbow trout fed (Control) or food deprived for 28 days (Fasted).

Table 1 legend:
HSI: Hepatosomatic index; MFI: Mesenteric fat index; DGC: Daily growth coefficient. Values represent Mean ± SE. ANOVA, * p<0.05; ** p<0.01; *** p<0.001

Table 2. Metabolite changes in Rainbow trout after 28 days of fasting. Plasma, liver and muscle metabolites are listed in order of their contribution to the class separation in the OPLS-DA model.

Table 2 legend:
VLDL: Very low density lipoprotein; LDL: Low density lipoprotein; HDL: High density lipoprotein; FA.: Fatty acid;

Figure 1. Representative 600 MHz $^1$H NMR spectra of rainbow trout plasma and tissue extracts. Plasma presat (A), Plasma CPMG (B), Liver hydrophilic extracts (C), Liver lipid extracts (D), Muscle hydrophilic extracts (E) and Muscle lipid extracts (F). 1, Very Low Density Lipoprotein (VLDL) –CH$_3$; 2, High Density Lipoprotein (HDL) –(CH$_2$)$_n$; 3, Glycoprotein acetyls; 4, Unsaturated lipids; 5, Lactate; 6, Lipid (CH$_2$ CO); 7, Choline; 8, Alanine; 9, Glycogen; 10, fatty acids (CH$_2$)$_n$; 11, (n-3) fatty acids; 12, non-DHA fatty acids (CH$_2$ - COOH); 13, 18:2 (n-2) fatty acids; 14, Phosphocreatine; 15, 18:1 (n-9) fatty acids.

Figure 2. OPLS-DA scores plots of metabolomics data of plasma and tissue extracts from fed (×) and fasted (•) rainbow trout. Plasma presat (A), Plasma CPMG (B), Liver hydrophilic extracts (C), Liver lipid extracts (D), Muscle hydrophilic extracts (E) and Muscle lipid
experiments (F). The separation along OPLS-DA component 1 represents differences related to the treatment. All other variation in the NMR data is visualized as separation in the second component.

Figure 2 Legend:
OPLS-DA, Orthogonal Partial Least-Squares Discriminant Analysis.

Appendix 1. Unidentified metabolite changes in Rainbow trout after 28 days of food deprivation. Peaks from plasma, liver and muscle samples are listed in order of their contribution to the class separation in the OPLS-DA model.

Appendix 1 Legend:
OPLS-DA: Orthogonal Partial Least Squares Discriminant Analysis.
Table 1. Morphological characteristics of Rainbow trout fed (Control) or food deprived for 28 days (Fasted).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Fasted</th>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>113.0 ± 8.7</td>
<td>62.4 ± 3.7</td>
<td>***</td>
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<tr>
<td>Length (mm)</td>
<td>208.1 ± 5.0</td>
<td>179 ± 3.7</td>
<td>***</td>
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<tr>
<td>Condition Factor</td>
<td>1.22 ± 0.01</td>
<td>1.07 ± 0.01</td>
<td>***</td>
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<tr>
<td>Growth Rate (DGC)</td>
<td>1.88 ± 0.21</td>
<td>-0.46 ± 0.06</td>
<td>***</td>
</tr>
<tr>
<td>Liver (g)</td>
<td>1.22 ± 0.13</td>
<td>0.47 ± 0.04</td>
<td>***</td>
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<tr>
<td>Mesenteric fat (g)</td>
<td>2.43 ± 0.24</td>
<td>1.43 ± 0.10</td>
<td>**</td>
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<tr>
<td>HSI (%)</td>
<td>1.06 ± 0.09</td>
<td>0.75 ± 0.03</td>
<td>***</td>
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<tr>
<td>MFI (%)</td>
<td>2.17 ± 0.17</td>
<td>2.34 ± 0.18</td>
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<tr>
<td>Liver lipid cont. (%)</td>
<td>2.54 ± 0.49</td>
<td>2.46 ± 0.36</td>
<td></td>
</tr>
<tr>
<td>Muscle lipid cont. (%)</td>
<td>4.38 ± 0.47</td>
<td>2.38 ± 0.32</td>
<td>**</td>
</tr>
</tbody>
</table>

HSI: Hepatosomatic index; MFI: Mesenteric fat index; DGC: Daily growth coefficient.

Values represent Mean ± SE. ANOVA, * p<0.05; ** p<0.01; *** p<0.001
Figure 1. Representative 600 MHz $^1$H NMR spectra of rainbow trout plasma and tissue extracts. Plasma presat (A), Plasma CPMG (B), Liver hydrophilic extracts (C), Liver lipid extracts (D), Muscle hydrophilic extracts (E) and Muscle lipid extracts (F). 1, Very Low Density Lipoprotein (VLDL) -CH$_3$; 2, High Density Lipoprotein (HDL) –(CH$_2$)$_n$; 3, Glycoprotein acetyl; 4, Unsaturated lipids; 5, Lactate; 6, Lipid (CH$_3$CO); 7, Choline; 8, Alanine; 9, Glycogen; 10, fatty acids (CH$_2$)$_n$; 11, n-3 fatty acids; 12, non-DHA fatty acids (CH$_2$-COOH); 13, 18:2 (n-2) fatty acids; 14, Phosphocreatine; 15, 18:1 (n-9) fatty acids.
Figure 2. OPLS-DA scores plots of metabolomics data of plasma and tissue extracts from fed (x) and fasted (•) rainbow trout. Plasma presat (A), Plasma CPMG (B), Liver hydrophilic extracts (C), Liver lipid extracts (D), Muscle hydrophilic extracts (E) and Muscle lipid extracts (F). The separation along OPLS-DA component 1 represents differences related to the treatment. All other variation in the NMR data is visualized as separation in the second component.

OPLS-DA, Orthogonal Partial Least-Squares Discriminant Analysis.
Table 2. Metabolite changes in Rainbow trout after 28 days of fasting. Plasma, liver and muscle metabolites are listed in order of their contribution to the class separation in the OPLS-DA model.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Change relative to control (%)</th>
<th>Pathway</th>
<th>¹H Chemical shift (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma, presat</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unsaturated lipids (C=C)</td>
<td>Up 133</td>
<td>Lipid metabolism</td>
<td>5.3</td>
</tr>
<tr>
<td>HDL</td>
<td>Up 151; 123</td>
<td>Lipid metabolism</td>
<td>2.79; 0.92</td>
</tr>
<tr>
<td>VLDL</td>
<td>Down 90; 79</td>
<td>Lipid metabolism</td>
<td>1.22; 0.82</td>
</tr>
<tr>
<td>LDL or VLDL</td>
<td>Up 118</td>
<td>Lipid metabolism</td>
<td>0.85</td>
</tr>
<tr>
<td>Glycoprotein acetyllys</td>
<td>Up 131</td>
<td>Lipid metabolism</td>
<td>2.02</td>
</tr>
<tr>
<td>Lipid (CH₃=C)</td>
<td>Down 75</td>
<td>Lipid metabolism</td>
<td>1.95</td>
</tr>
<tr>
<td>Plasma, CPMG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Choline</td>
<td>Down 77</td>
<td>Glycine metabolism; Glyceroethanolipid metabolism</td>
<td>3.22</td>
</tr>
<tr>
<td>HDL</td>
<td>Down 81; 73</td>
<td>Lipid metabolism</td>
<td>1.22; 0.82</td>
</tr>
<tr>
<td>Unsaturated lipids (C=C)</td>
<td>Up 138</td>
<td>Lipid metabolism</td>
<td>5.32</td>
</tr>
<tr>
<td>VLDL</td>
<td>Up 150; 148; 212</td>
<td>Lipid metabolism</td>
<td>0.92; 2.79; 1.62</td>
</tr>
<tr>
<td>LDL</td>
<td>Up 82</td>
<td>Lipid metabolism</td>
<td>0.86</td>
</tr>
<tr>
<td>β-glucose</td>
<td>Down 79; 71; 69; 72; 56; 67</td>
<td>Glycogen / Glucoegenesis</td>
<td>3.21; 3.42; 3.84; 3.89; 4.64</td>
</tr>
<tr>
<td>Lactate</td>
<td>Down 76; 63</td>
<td>Glycogen / Glucoegenesis</td>
<td>1.33; 4.11</td>
</tr>
<tr>
<td>Glycoprotein acetyllys</td>
<td>Up 132</td>
<td>Lipid metabolism</td>
<td>2.04</td>
</tr>
<tr>
<td>Lipid (CH₃,CO)</td>
<td>Up 147</td>
<td>Lipid metabolism</td>
<td>2.22</td>
</tr>
<tr>
<td>Lipid (CH₃=C)</td>
<td>Down 66</td>
<td>Lipid metabolism</td>
<td>1.96</td>
</tr>
<tr>
<td>Liver, hydrophilic extracts</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>Down 33; 83</td>
<td>Alanine and aspartate metabolism; Cysteine metabolism</td>
<td>1.48; 3.79</td>
</tr>
<tr>
<td>Lactate</td>
<td>Down 52; 50</td>
<td>Glycogen / Glucoegenesis</td>
<td>1.33; 4.12</td>
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<tr>
<td>Glycogen</td>
<td>Up 184</td>
<td>Lipid metabolism</td>
<td>5.41</td>
</tr>
<tr>
<td>Liver, lipid extracts</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(CH₃)n FA</td>
<td>Down 94</td>
<td>Lipid metabolism</td>
<td>1.29</td>
</tr>
<tr>
<td>n-3 FA</td>
<td>Up 117</td>
<td>Lipid metabolism</td>
<td>2.07</td>
</tr>
<tr>
<td>18:2 (n-6) FA</td>
<td>Up 162</td>
<td>Lipid metabolism</td>
<td>2.78</td>
</tr>
<tr>
<td>Non-DHA C2 (methylene) FA</td>
<td>Up 115</td>
<td>Lipid metabolism</td>
<td>2.33</td>
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<td>Muscle, hydrophilic extracts</td>
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<tr>
<td>Phosphocreatine</td>
<td>Up 126; 127</td>
<td>Arginine and proline metabolism</td>
<td>3.03; 3.93</td>
</tr>
<tr>
<td>Lactate</td>
<td>Down 76; 73</td>
<td>Glycogen / Glucoegenesis</td>
<td>1.33; 4.11</td>
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<tr>
<td>Betaine</td>
<td>Down 53; 28</td>
<td>Glycine, serine and threonine metabolism</td>
<td>3.27, 3.90</td>
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<tr>
<td>Alanine</td>
<td>Up 146</td>
<td>Alanine and aspartate metabolism; Cysteine metabolism</td>
<td>1.48</td>
</tr>
<tr>
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<td>(CH₃)n FA</td>
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<td>2.78</td>
</tr>
<tr>
<td>18:1 (n-9) FA</td>
<td>Down 94</td>
<td>Lipid metabolism</td>
<td>2.03</td>
</tr>
</tbody>
</table>

VLDDL: Very low density lipoprotein, LDL: Low density lipoprotein, HDL: High density lipoprotein, FA: Fatty acid.
OPLS-DA: Orthogonal Partial Least Squares Discriminant Analysis. Pathway category from KEGG (Kyoto Encyclopedia of Genes and Genomes) PATI