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Microstructural and metabolic alterations in the zebrafish brain induced by toll-like receptor 2 deficiency: insights from ultra-high field magnetic resonance imaging and spectroscopy

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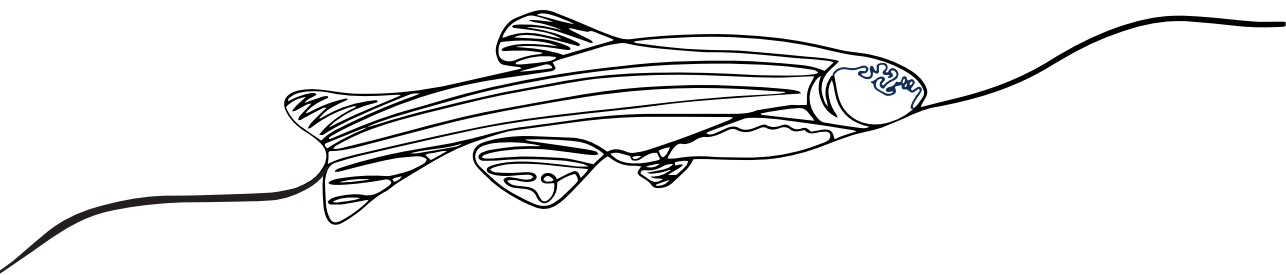
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**UNBEATABLE SUPER-
RESOLUTION OF VOXEL-
BASED MR SPECTROSCOPY
OF ZEBRAFISH BRAIN
ACHIEVED AT 28.2 T***

* Manuscript in preparation for publication

ABSTRACT

Localized ^1H magnetic resonance spectroscopy (MRS) is pivotal in clinical neurology, offering non-invasive insight into neurochemical composition in localized brain regions. Zebrafish (*Danio rerio*) serve as valuable models in neurological research. However, the size of the zebrafish brain and limited MRS sensitivity hinder comprehensive analysis. Here, we investigate the potential of ultra-high field (UHF) MR systems, particularly 28.2 T, for this purpose. This work pioneers application of localized ^1H spectroscopy in zebrafish brain at 28.2 T. We explore potentials and challenges associated with UHF localized ^1H MRS on the zebrafish brain, emphasizing the acquisition of highly resolved spectra to enable targeted molecular level information of distinct brain regions. Young adult zebrafish (three month old), were sacrificed and directly transferred to 5 mm glass NMR tubes. MR microscopy images were obtained at 28.2 T using a rapid acquisition with relaxation enhancement (RARE) sequence to select a volume of interest. Point resolved spectroscopy (PRESS) sequences was optimized for localized ^1H spectroscopy. PRESS sequence preceded by a variable pulse power and optimized relaxation delays (VAPOR) sequence for global water suppression interleaved with outer volume suppression. Chemical shift displacement effects were careful reduced by choosing appropriate bandwidths. Parameters including the gradient strength, excitation frequency, echo time and voxel volume were fine-tuned specifically targeting the 0 – 4.5 ppm chemical shift regions. Highly resolved cerebral metabolite spectra were acquired from localized regions in zebrafish brain, from volumes as small as 125 nL within approximately 30 minutes. This facilitated identification and quantification of several brain metabolites with super high resolution. Localized ^1H MRS at 28.2 T significantly enhance signal-to-noise ratio and improved baseline separation of metabolites signals from localized regions in the tiny zebrafish brain.



1. INTRODUCTION

Single voxel localized magnetic resonance spectroscopy (MRS) is a well-established, non-invasive, technique for monitoring metabolic changes in human clinical studies and pre-clinical studies using animal models¹. Unlike various other metabolomic methodologies, localized MRS bypasses the need for laborious extraction procedures. Moreover, it facilitates precise selection of tissue regions, enabling *in vivo* monitoring of metabolites within their native environments. While localized MRS is utilized for various tissue types²⁻⁵, clinical applications predominantly focus on brain tissue across a wide range of neurological disorders⁶⁻¹². In this respect, brain metabolites show a remarkable sensitivity to deviations from physiological homeostasis induced by pathological conditions. Specific metabolites offer insights into distinct cells and their corresponding cellular activities. For instance, localized MRS enables quantification of glutamate (Glu) and γ -aminobutyric acid (GABA), the principal excitatory and inhibitory neurotransmitters in the brain^{13,14}. Additionally, N-acetylaspartate (NAA), predominantly localized in neuronal cell bodies and axons, serves as a potential marker for monitoring neuronal integrity¹⁵ and *myo*-inositol (m-Ins), chiefly situated in glial cells, functions as a potential marker for osmotic stress or neuroinflammation¹⁶.

Zebrafish (*Danio rerio*) are exemplary model organisms. For scientific inquiry, zebrafish offer distinct advantages, including cost-effectiveness and easy maintenance, coupled with a rapid developmental pace. Moreover, zebrafish embryos are transparent, particularly beneficial in toxicity studies for precise observation of developmental stages¹⁷. Furthermore, toxicity studies utilizing zebrafish larvae using high-resolution magic angle spinning (HR-MAS) allows for metabolomic studies to reveal toxicity pathways at a cellular and molecular level¹⁸⁻²³. Additionally, the entire zebrafish genome is sequenced²⁴, revealing 84% of genes linked to human diseases possess a corresponding homologous gene in the zebrafish genome. These similarities with humans allow for genetically manipulation to create transgenic zebrafish models to study human diseases. For neurological disorders, extensive zebrafish models are available, including transgenic models of Alzheimer's disease, Parkinson's disease, brain tumours and epilepsy²⁵⁻²⁹. Despite their extensive use in research, studies utilizing localized MRS involving zebrafish are rare. Previously, we presented the first methodology of localized MRS for *in vivo* analysis of cerebral metabolites in zebrafish at 9.4 T (400 MHz)³⁰. High-resolution spectra were obtained, allowing for the identification and quantification of the most predominant metabolites, including creatine (Cr), Glu, NAA, m-Ins, glutamine (Gln), taurine (Tau), and GABA. However, the limited sensitivity of localized MRS, in combination with the small size of the zebrafish brain (few mm³), prevented the application from analysing individual brain structures.

Recent developments in MR systems operating at high fields and ultra-high fields (UHF), offer remarkable potential in elucidating intricate details within the zebrafish brain (Chapter 2). Elevated signal to noise ratios (SNR) in combination with extremely high gradients strengths, unveiled previously unseen details in the zebrafish brain through non-invasive magnetic resonance imaging (MRI), including the identification of tiny white matter structures through diffusion-based MRI tractography and shown in Chapter 2 and as previously by Ullman *et al.*³¹. However, despite these recent breakthroughs, the application of localized MRS in the zebrafish brain at UHF remains untapped. Integrating localized MRS into UHF MRI studies could provide valuable insights into neurochemical processes, paving the way for comprehensive investigations into zebrafish brain functions and pathology.

Localized MRS at UHFs holds tremendous promise due to anticipated enhancements in SNR and improved baseline separation of metabolites. These advancements enable monitoring of cerebral metabolites in smaller voxels than previously achievable, facilitating the precise examination of specific brain regions. However, utilization of UHFs for single voxel localized MRS also introduces specific challenges, notably increased chemical shift displacement (s_g). Chemical shift displacement effects, also called chemical shift misregistration or chemical shift artifacts, were first elucidated in 1984 through the registration shift of the fat signal³². These effects escalate with elevated magnetic field strengths and recede with stronger magnetic field gradients³³. Despite UHF MRI systems utilizing gradient strengths (G_{max}) up to 3 T/m³⁴, considerably higher than the typical strengths of 40 mT/m in medical imaging systems³⁵, they are insufficient to compensate for the increase in Larmor frequency. Consequently, chemical shift displacements are notably exacerbated in localized MRS utilizing UHF systems. Addressing this challenge requires sophisticated correction methods to accurately quantify metabolite concentrations and interpret spectroscopic data.

In the current study, we utilize localized MRS at 28.2 T. As these data represent the first localized MRS data obtained at 28.2 T, we present both the advantages, challenges, and limitations of this utilization. Besides optimization of sequence parameters involved in chemical shift displacement effects, we explore metabolic profiles at various echo times, voxel volumes, and number of averages. Subsequently, we employ optimized method parameters to generate a localized spectrum with prolonged measurement duration, facilitating the detection of highly resolved cerebral metabolites in the zebrafish brain. Following this, we perform a comparative quantitative assessment of spectra derived from various brain regions, aiming to elucidate cerebral metabolite profiles in specific brain regions.

2. METHODS

Zebrafish husbandry

The husbandry of the zebrafish was performed according to the regulations set forth by the local animal welfare committee of the university of Leiden (Licence numbers AVD1060020171767 and AVD10600202216175), in accordance with the international standards outlined by the EU Animal Protection Directive 2010/63/EU. All procedures followed standard protocols as previously described (www.zfin.org)³⁶. In this study, three-month-old male zebrafish were utilized. To minimize any metabolite degradation, zebrafish were euthanized just before the MRI examination, which was induced by immobilization through submersion in ice water at a temperature between 0 – 4 °C for 15 minutes until the halt of opercular movement. Following euthanization, specimens were shortly kept on ice until MRI and MRS measurements were conducted.

Magnetic resonance imaging and spectroscopy

MRI and MRS experiments were conducted using Bruker (Bruker Biospin GmbH, Germany) vertical bore systems operating at 17.6 T (750 MHz) or 28.2 T (1200 MHz), configured with a Micro5 or Micro5 iProbe gradient system, respectively. Both systems were water-cooled, provided $G_{\max} = 3$ T/m, used 5 mm inner diameter birdcage resonators, and GREAT60 gradient power supplies. Measurements were conducted at room-temperature. Data acquisition and processing on both systems was performed with Linux workstations, running Bruker imaging software ParaVision 360 v3.3 (Bruker Biospin GmbH, Germany). During analysis, zebrafish were embedded in perfluoropolyether (Fomblin Y, Solvay Solexis S.P.A.).

T_2 -weighted spin echo images were acquired by a rapid acquisition with relaxation enhancement (RARE) pulse sequence to select the position for single voxel localized MRS. RARE measurements were performed with an echo time $TE = 5.6$ ms, a repetition time $TR = 3000$ ms, using 4 refocussing pulses per excitation, and $ns = 16$ averages. The field of view (FOV) was 8×8 mm, at a matrix size of 256×256 voxels, obtaining a resolution of 31×31 μm , with a slice thickness of 200 μm .

For single voxel localized ^1H MRS, we utilized a point resolved spectroscopy (PRESS) pulse sequence³⁷. The PRESS sequence incorporates three transmitter slice-selective RF pulses (90° - 180° - 180°), applied simultaneous with x , y , and z gradients to target the specified voxel. Prior to each localized MRS measurement, magnetic field homogeneity within each voxel was achieved by local second-order shimming prior to data acquisition. After parameter optimization, standard PRESS measurements were performed with $TE = 15$ ms, $TR = 2000$ ms, $ns = 512$. The PRESS sequence used 2048 complex points and an acquisition bandwidth of 10 ppm. At 17.6 T and 28.2 T, the transmitter RF pulse bandwidth was $tBW = 10$ kHz or 15

kHz, respectively. The excitation frequency was $\nu_1 = 3$ ppm, coinciding with the $\text{N}(\text{CH}_3)$ total creatine (tCr) signal. For the estimation of regional cerebral metabolite levels, three voxels were selected in the zebrafish brain; (1) in the forebrain, covering part of the pallium, (2) in the midbrain, covering part of the mesencephalon, cerebellum, and diencephalon, and (3) in the hindbrain, covering part of the cerebellum and rhombencephalon. In the midbrain, a PRESS voxel of $0.8 \times 0.8 \times 0.8 \text{ mm}^3$, with a voxel volume of $V_{\text{voxel}} = 512 \text{ nL}$ was selected. In the forebrain and hindbrain, a PRESS voxel of $0.6 \times 0.6 \times 0.6 \text{ mm}^3$, with $V_{\text{voxel}} = 216 \text{ nL}$ was selected. For suppression of the water signal, a variable pulse power and optimized relaxation delays (VAPOR) sequence³⁸ was applied prior to the PRESS pulses. Outer volume suppression (OVS) was integrated in an interleaved manner with the VAPOR sequence. The OVS consists of six suppression slices, each repeated three times and aligned parallel to the PRESS voxel, reducing the PRESS gradient spoilers.

Data processing

Identification and quantification of metabolites. Identification of cerebral metabolites was performed by comparison of obtained ^1H spectra to reference spectra of brain metabolites³⁹⁻⁴¹ and on our previously obtained NMR spectra of zebrafish brain extracts³⁰. Quantification of metabolites was executed using Chenomx NMR suite 8.2 (Chenomx Inc., Edmonton, Alberta, Canada). Available reference libraries provided by Chenomx were employed. Metabolite concentrations were estimated relative to tCr⁴². Statistical analysis for NMR quantification involved one-way analysis of variance (ANOVA) by OriginPro v.8 (Originlab, Northampton, MA, USA). Multiple comparisons were conducted using the t -test, and significance was established with $p < 0.05$.

Signal-to-noise ratio estimation. Unless specified otherwise, SNR was estimated based on the tCr signal at a chemical shift $\delta = 3.03$ ppm, utilizing build-in TopSpin functionalities with

$$SNR = \frac{I_{\text{tCr}}}{2 \cdot N} \quad (4.1)$$

Here, I_{tCr} is the maximum signal intensity of the tCr signal at $\delta = 3.03$ ppm, and N is the noise factor as fully described in the TopSpin manual (Processing commands and parameters user manual, version 007, Bruker Corporation).

Chemical shift displacement estimation. The estimation of s_δ was based on

$$s_\delta (\%) = \frac{|v_1 - v_x|}{tBW} \quad (4.2)$$

where v_x is the Larmor frequency of the metabolite of interest (Hz).

T_2 relaxation time estimation. T_2 relaxation times of individual metabolites were estimated using PRESS signal intensities acquired at various echo times. T_2 was estimated using a non-linear least squares algorithm to fit the mono-exponential function described by

$$I_{TE} = A + I_0 \cdot \exp\left(\frac{-TE}{T_2}\right) . \quad (4.3)$$

Here, I_{TE} represents the signal intensity at TE , A is the absolute bias, and I_0 is the signal intensity at TE_0 .

3. RESULTS AND DISCUSSION

It is well known that performing localized MRS at high magnetic fields significantly enhances both the SNR and spectral resolution. These improvements enable more precise identification and quantification of metabolites within a given region. However, MRI artifacts, particularly the chemical shift displacement effects, must be carefully considered and managed during method optimization. Here, we explore the challenge of conducting localized MRS at 28.2 T magnetic field strengths on the adult zebrafish brain, particularly addressing the chemical shift displacement effects. Next, we study the impact of TE and its consequential influence on acquired spectra. Moreover, we elucidate how the application of UHF localized MRS with ascending echo times can be leveraged to estimate T_2 relaxation times of distinct metabolites within the zebrafish brain. Hereafter, we explore the limits of localized MRS at UHF in terms of voxel volume and number of scans required for metabolite quantification. Finally, we apply optimized method parameters to produce a localized spectrum with extended measurement time, enabling the identification of cerebral metabolites within the zebrafish brain at UHF strengths. We then conduct a comparative quantitative analysis of spectra obtained from different brain regions to gain insights into the cerebral metabolite profiles.

Chemical shift displacement at UHF localized MRS

Single voxel localized MRS at UHF strengths offers the distinct advantage of elevated SNR and enhanced metabolite separation compared to lower magnetic fields. These advancements facilitate advanced identification and quantification of metabolites. However, as the magnetic field strength increases, chemical shift displacement effects become more pronounced⁴³ (see Supplementary Figure S4.1). For example, in localized MRS spectra acquired with the PRESS sequence at 28.2 T, with $tBW = 5$ kHz and $v_1 = 4.7$ ppm, the $^3\text{CH}_3$ signal of lactate at $\delta = 1.32$ ppm exhibits $s_\delta = 81\%$ for each transmitter RF pulse. Consequently, only 0.67% of the lactate signal originating from within the selected PRESS voxel is effectively captured. In contrast, employing identical parameters with a clinical system operating at 1.5 T would result in $s_\delta = 4\%$ per PRESS transmitter RF pulse for the $^3\text{CH}_3$ signal of lactate at $\delta = 1.32$ ppm and a

significantly higher proportion, 88%, of the lactate signal from within the voxel is detected. This underscores the importance of understanding the effects of chemical shift displacement when transitioning to ultra-high magnetic field systems for single voxel localized MRS studies. To mitigate the impact of chemical shift displacement on targeted metabolites, ν_1 can be adjusted, to put it in closer proximity to the chemical shift of the metabolites of interest (see Equation 4.2). In Figure 4.1, we explored the impact of ν_1 on the metabolic profile obtained at 28.2 T. Apparently, a minor adjustment of ν_1 by ± 1 ppm, equivalent to ± 1200 Hz at 28.2 T, leads to notable changes in the obtained metabolic profile. The spectra obtained at $\nu_1 = 1$ ppm or $\nu_1 = 4.7$ ppm demonstrate metabolites with $s_g > 30\%$ results in an almost complete loss of the metabolite signal. Moreover, it becomes apparent that the chemical shift displacement at 28.2 T significantly alters the signal intensities of metabolites depending on ν_1 . For specific metabolites, e.g. lactate, which show significant differences in chemical shifts between their groups, the displacement of these chemical shifts plays a crucial role during quantification. This effect is clearly demonstrated in Table 4.1. Here the estimation of the lactate level relative to tCr for various ν_1 reveals significant discrepancies. At $\nu_1 = 2$ ppm, the ^2CH lactate signal at $\delta = 4.12$ ppm indicates a concentration of 0.41 relative to tCr, whereas the $^3\text{CH}_3$ signal at $\delta = 1.32$ ppm indicates a concentration of 2.89 relative to tCr. This underscores the importance of using consistent signals for metabolite quantification at specific excitation frequencies during metabolite comparison studies. For our specific objectives, the majority of brain metabolites exhibit signals within the range of $\delta = 4.1$ ppm to $\delta = 2$ ppm. These include Lac, Glu, Gln, m-Ins, Tau, tCr, GABA, and NAA.

Besides the impact of ν_1 , tBW of the excitation and refocussing pulses impact the magnitude of s_g . In Figure 4.2A, we probed the impact of tBW on the localized MRS spectra, acquired with $\nu_1 = 3$ ppm. At $tBW = 5$ kHz, a significant impact of lipid signals is visible especially around $\delta = 1.3$ ppm. The zebrafish brain is encapsulated by lipids, elucidated in Supplementary Figure S4.2, suppressing metabolite signals such as lactate and alanine. Although the PRESS voxel was fully placed inside the brain, chemical shift displacement effects caused the detection of the lipid signals at $tBW = 5$ kHz. At $tBW = 10$ kHz, the lipid signal is still observed, although significantly reduced. At $tBW = 15$ kHz or $tBW = 20$ kHz, the lipid signals are no longer observed, allowing for the identification and quantification of the lactate and alanine signals at $\delta = 1.32$ ppm and $\delta = 1.47$ ppm, respectively. However, as illustrated in Figure 4.2B, increasing tBW leads to diminished SNR, exhibiting a notable decrease of approximately 25% between $tBW = 15$ kHz to 20 kHz. In contrast, the decrease in SNR is less between 10 kHz and 15 kHz, at around 10%. Accordingly, further localized MRS acquisitions are performed utilizing $tBW = 15$ kHz, unless otherwise specified.

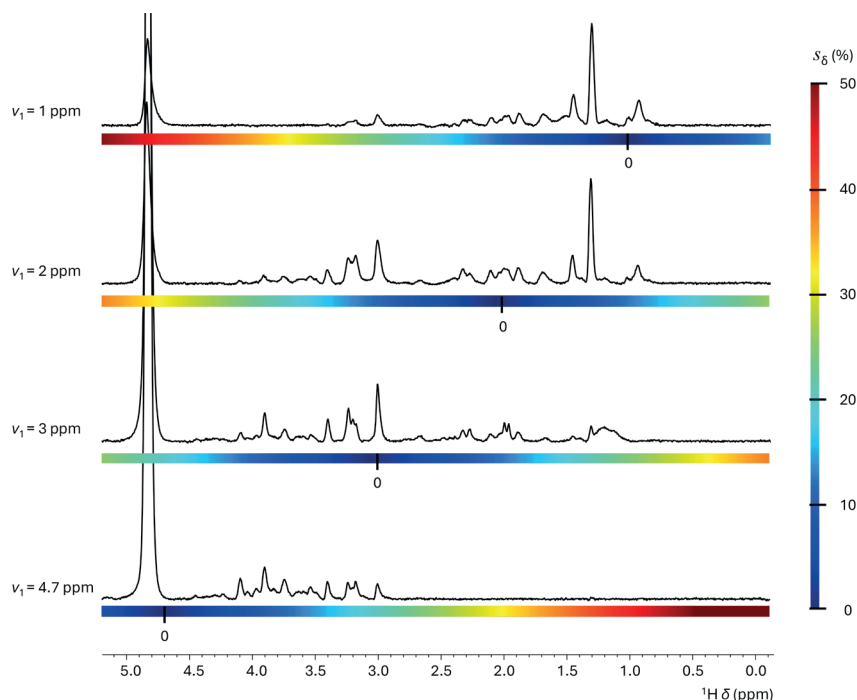


Figure 4.1. Impact of the excitation frequency v_1 on the acquired single voxel localized ^1H MRS spectra from adult zebrafish mid-brain at 28.2 T. All PRESS spectra were acquired from the same precise location within the zebrafish brain, employing uniform parameters as specified, while adjusting v_1 as specified for each spectrum, delineated by $s_\delta = 0$. To facilitate direct comparison, spectral intensities were normalized based on the noise level of the base line. The s_δ colour bars show the magnitude of chemical shift displacement per transmitter pulse, further elucidated in Supplementary Figure S4.1B. Acquisition details: $TR = 2000$ ms, $TE = 15$ ms, $ns = 1024$, a voxel size of $0.8 \times 0.8 \times 0.8$ mm³ with $V_{\text{voxel}} = 512$ nL, 2048 acquisition points, acquisition bandwidth 11904 Hz / 9.91 ppm, 4 dummy scans, $tBW = 10$ kHz. Water suppression and outer volume suppression were applied as specified in the method section.

Table 4.1. Impact of v_1 on the lactate level estimation for both the ^2CH group at $\delta = 4.12$ ppm and the $^3\text{CH}_3$ group at $\delta = 1.32$ ppm. (-) indicates the concentration could not be estimated. All concentrations are given relative to the $t\text{Cr}$ signal at $\delta = 3.03$ ppm.

Excitation frequency	^2CH Lac	$^3\text{CH}_3$ Lac
	$\delta = 4.12$ ppm	$\delta = 1.32$ ppm
4.7	6.04	-
3	0.64	0.35
2	0.41	2.89
1	-	7.17

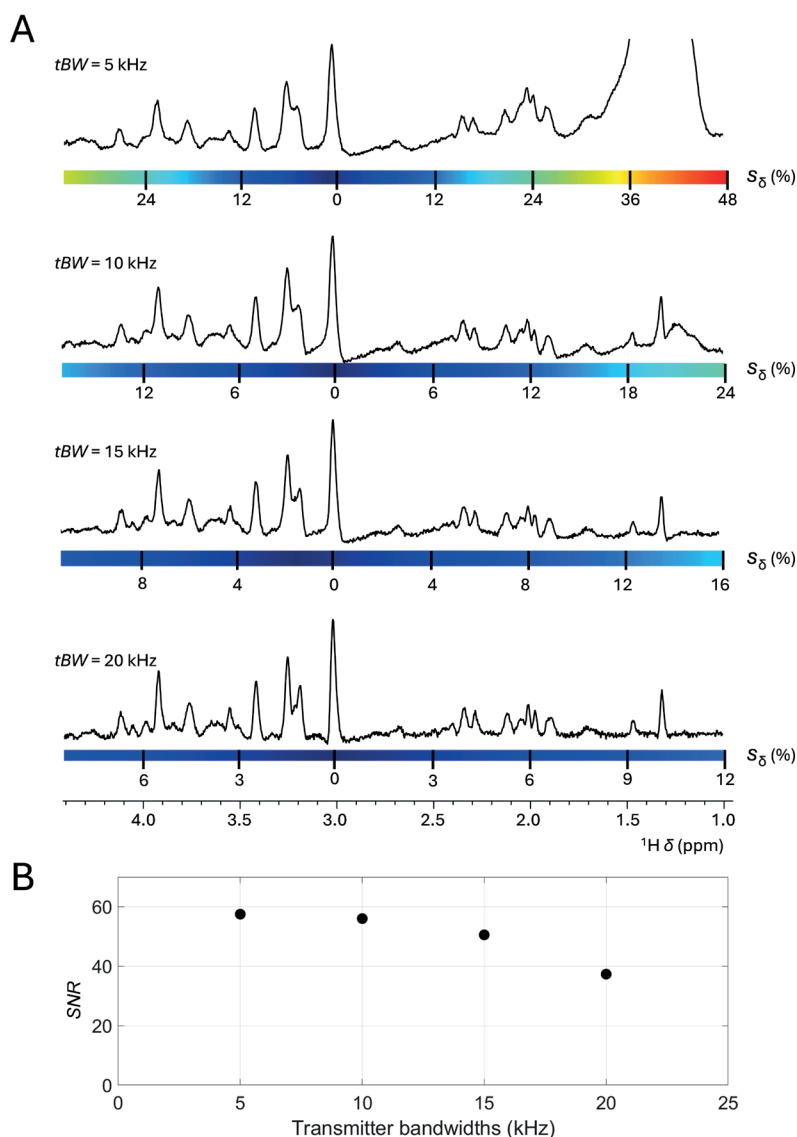


Figure 4.2. Impact of the transmitter RF pulse bandwidth tBW on the acquired single voxel localized ^1H MRS spectra from adult zebrafish mid-brain at 28.2 T. [A] Single voxel localized MRS spectra acquired for various tBW . **[B]** SNR of the tCr signal at $\delta = 3.03 \text{ ppm}$ as a function of tBW . All PRESS spectra were acquired from the same precise location within the zebrafish brain, employing uniform parameters, while adjusting tBW as specified for each spectrum. To facilitate direct comparison, spectral intensities were normalized relative to the tCr signal at $\delta = 3.03 \text{ ppm}$. The S_δ colour bars show the magnitude of chemical shift displacement per transmitter pulse, further elucidated in Supplementary Figure S4.1B. Acquisition details: $TR = 2000 \text{ ms}$, $TE = 15 \text{ ms}$, $ns = 1024$, voxel size $0.8 \times 0.8 \times 0.8 \text{ mm}^3$ with $V_{\text{voxel}} = 512 \text{ nL}$, 2048 acquisition points, acquisition bandwidth $11904 \text{ Hz} / 9.91 \text{ ppm}$, 4 dummy scans, $\nu_1 = 3 \text{ ppm}$. Water suppression and outer volume suppression were applied as specified in the method section. Signal-to-noise ratios estimated based on the tCr signal at $\delta = 3.03 \text{ ppm}$.

Single voxel localized MRS at variable echo times

The applied echo time plays a pivotal role in the acquired metabolic profile and warrants careful consideration during experimental design. A well-established effect is the diminishing of signal intensities at elevated echo times as a consequence of the T_2 relaxation times of metabolites. Thus, shorter echo times are frequently favoured to enhance obtained SNR. Nonetheless, deliberate selection of longer echo times can yield substantial enhancements in metabolite identification and quantification within acquired profiles. Furthermore, applying a range of echo times allows for the estimation of T_2 relaxation times of individual metabolites. Figure 4.3A shows the cerebral metabolite profile at increasing echo times obtained at 17.6 T. A full comparison of the cerebral metabolite profile at both 17.6 T and 28.2 T is shown in Supplementary Figure S4.3. Beyond echo times of 60 ms, the lipid signals around $\delta = 0.9$ ppm and $\delta = 2.0$ ppm become indiscernible amidst the background noise due to the short T_2 relaxation time of macromolecules. This facilitates improved identification and quantification of cerebral metabolites with chemical shifts overlapping with lipid signals, e.g. Lac, Ala, GABA, NAA, and NAAG. Additionally, water exhibits a significantly shorter T_2 relaxation time compared to most presented cerebral metabolites (data not shown). Consequently, the suppression of the broad and predominant water signal is notably improved at higher echo times. In addition to alterations in their relative abundance, the T_2 relaxation times of cerebral metabolites offers insight in changes within their respective environments. For instance, in Alzheimer's disease, a significant increase in the T_2 of NAA was reported, while no significant change was observed in the T_2 of water in the same tissue⁴⁴. Unlike the T_2 of water, which reflects properties of both intracellular and extracellular spaces, NAA is confined solely intracellular, thus furnishing more nuanced and compartment-specific details⁴⁴. The signal intensity of various metabolite

Table 4.2. T_2 relaxation times estimated for brain metabolites at 28.2 T, compared to 17.6 T. Measurements presented were performed once ($n = 1$) as a proof-of-principle, and therefore lack the statistical robustness required for comparison with other values. While this single instance serves to demonstrate the feasibility of the method, reported values should not be extrapolated or compared with other datasets due to the absence of a statistical basis.

Metabolite	Group	δ (ppm)	Multiplicity	T_2 at 28.2 T	T_2 at 17.6 T
tCr	$^2\text{CH}_2$	3.91	s	75.5 ms	112.4 ms
	N(CH ₃)	3.03	s	40.6 ms	111.2 ms
Glx	Glu ^2CH / Gln ^2CH	3.74 / 3.75	dd / t	24.8 ms	33.4 ms
NAAG	$^2\text{CH}_3$	2.04	s	99.8 ms	149.4 ms
NAA	$^2\text{CH}_3$	2.01	s	47.7 ms	51.8 ms
GABA	$^3\text{CH}_2$	1.89	t	25.3 ms	25.2 ms

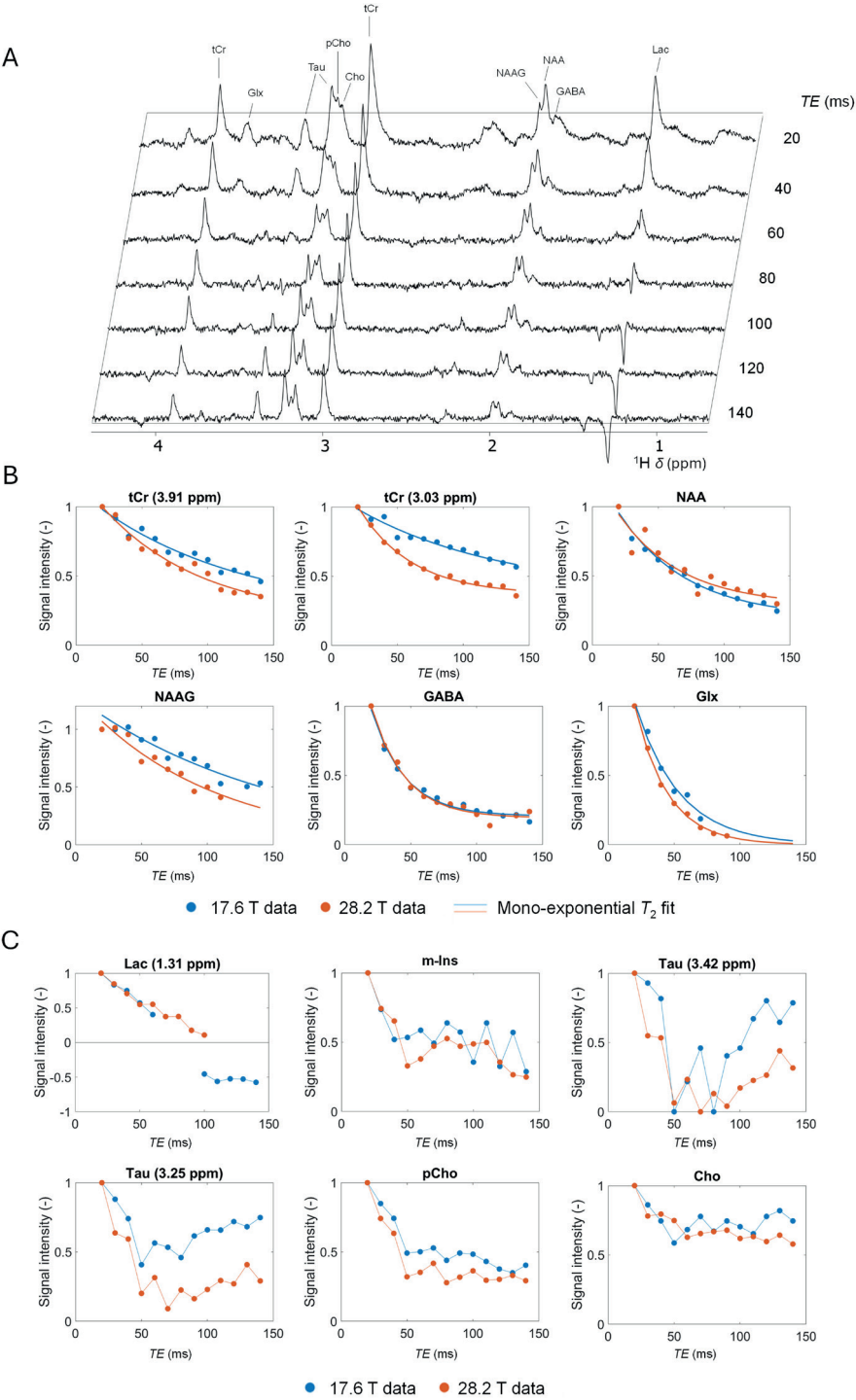


Figure 4.3. Impact of the echo time on the acquired single voxel localized ^1H MRS spectra from adult zebrafish mid-brain at 17.6 T and 28.2 T. [A] Single voxel localized MRS spectra acquired for echo times ranging from 20 to 140 ms at 17.6 T. **[B]** Signal decay observed for various cerebral metabolites with an apparent consistent mono-exponential decrease with echo time. Mono-exponential decay curve from T_2 relaxation time estimation fitted through data. **[C]** Signal decay of cerebral metabolites with a more complex signal intensity evolution, contributed to J-coupling induced signal dephasing. Acquisition details: $TR = 2000$ ms, $TE = 20, 40, 60, 80, 100, 120$, or 140 ms, $ns = 1024$, a voxel size of $0.8 \times 0.8 \times 0.8$ mm³ with $V_{\text{voxel}} = 512$ nL, 2048 acquisition points, acquisition bandwidth 11904 Hz / 9.91 ppm, $tBW = 10$ kHz for all RF pulses, 4 dummy scans, $\nu_1 = 3$ ppm. Water suppression and outer volume suppression were applied as specified in the method section. An extended comparison of spectra with ranging echo times acquired at 17.6 T and 28.2 T is presented in Supplementary Figure S4.3. Figure on the preceding page.

signals, including the tCr signal at $\delta = 3.91$ ppm ($^2\text{CH}_2$) and $\delta = 3.03$ ppm ($\text{N}(\text{CH}_3)$), the NAA signal at $\delta = 2.01$ ppm ($^2\text{CH}_3$), the NAAG signal at $\delta = 2.04$ ppm ($^2\text{CH}_3$), the GABA signal at $\delta = 1.89$ ppm ($^3\text{CH}_2$), and the Glx (Glu + Gln) signal at $\delta = 3.75$ ppm (Glu ^2CH and Gln ^2CH) appeared to consistently decrease exponentially with increasing echo time (see Figure 4.3B) allowing for direct T_2 estimation (see Table 4.2).

Other cerebral metabolite signals show a complex signal intensity evolution with increasing echo times (see Fig 4.3C). Most noticeable, the Lac signal at $\delta = 1.31$ ppm ($^3\text{CH}_3$) shows an anti-phase pattern between 60 ms – 100 ms and a 180° phase shift at echo times above 100 ms. This shift evolution is contributed to the J-coupling of the Lac signals, estimated in the range of 6.9 – 7.3 Hz⁴⁵. Consequently, the Lac signals are negative in-phase at $TE = J^{-1} \sim 140$ ms and anti-phase at $TE = \frac{1}{2} J^{-1} \sim 70$ ms. This characteristic facilitates distinct lactate identification by applying echo times around 140 ms, but also imposes limitations on echo times due to the complex anti-phase behaviour observed within the 60 – 100 ms echo time range. Other metabolites showing a complex echo time evolution include Tau at $\delta = 3.42$ ppm ($^1\text{CH}_2$) and $\delta = 3.25$ ppm ($^2\text{CH}_2$), pCho at $\delta = 3.21$ ppm ($\text{N}(\text{CH}_3)_3$), and Cho at $\delta = 3.19$ ppm ($\text{N}(\text{CH}_3)_3$), contributed to J-coupling induced signal dephasing. For Tau and m-Ins, the presented signal evolution is consistent with previous observation⁴⁶, showing an initial steep signal decline with a local minimum around 50 ms – 80 ms due to anti-phasing ($TE \sim \frac{1}{2} J^{-1}$), after which the signal recovers between 100 ms – 140 ms due to rephasing ($TE \sim J^{-1}$). T_2 relaxation time estimation of singlet resonance signals, e.g. both tCr signals, NAA, and NAAG, is straightforward due to the lack of J-coupling modulation. Estimation of T_2 relaxation times for metabolites with a more complex signal intensity evolution, requires quantum mechanics simulations, as previously reported^{46,47}.

Voxel volume and averaging strategies

One of the main advantages of performed localized MRS is the ability to non-invasively study the cerebral metabolic profile in specific brain regions. However, the tiny size of the

zebrafish brain, in combination with the uneven shape, limits the utilization of voxel sizes. Here, we explore the limits of acquisition of highly resolved single voxel localized MRS spectra in the zebrafish brain in terms of the voxel size and number of averages required for cerebral metabolite identification and quantification. Figure 4.4A shows spectra acquired with $ns = 1024$ in approximately 34 minutes with V_{voxel} ranging between 1000 μL to 125 nL, obtained from

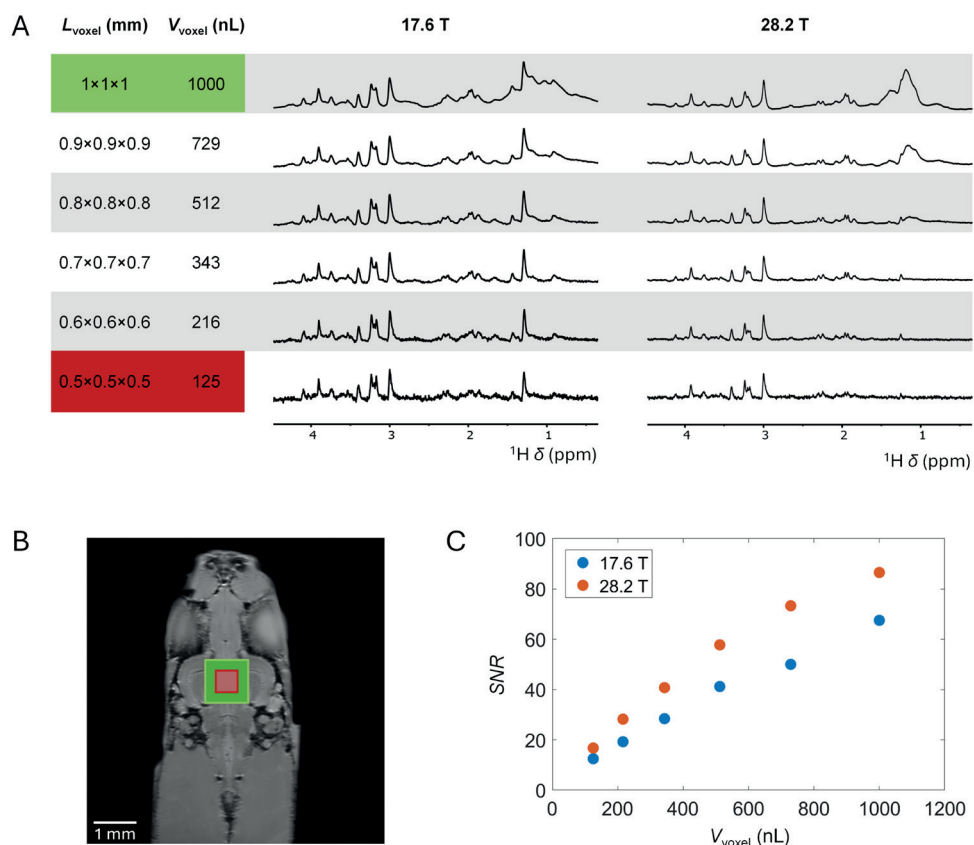


Figure 4.4. Effect of the voxel volume V_{voxel} on localized ^1H MRS spectra from adult zebrafish mid-brain at 17.6 T and 28.2 T. [A] Cerebral metabolic spectra obtained with localized ^1H MRS at 28.2 T compared to 17.6 T at various V_{voxel} , ranging from 1000 nL to 125 nL. **[B]** Anatomical RARE image, coronal slice, of zebrafish brain regions to indicate position of selected PRESS voxels. $V_{\text{voxel}} = 1000$ nL (green) and $V_{\text{voxel}} = 125$ nL (red) are highlighted. The remaining voxels are situated between these indicated voxels. **[C]** SNR of the $\text{N}(\text{CH}_3)_2 \text{tCr}$ signal at $\delta = 3.03$ ppm as a function of V_{voxel} at 17.6 T and 28.2 T. On average, an SNR increase of 40% was observed at 28.2 T compared to 17.6 T. Acquisition details: $TR = 2000$ ms, $TE = 15$ ms, $ns = 1024$, 2048 acquisition points, acquisition bandwidth 7462 Hz / 9.95 ppm at 17.6 T and 11904 Hz / 9.91 ppm at 28.2 T, 4 dummy scans, $v_1 = 3$ ppm, $tBW = 10$ kHz for all RF pulses. Water suppression and outer volume suppression were applied as specified in the method section.

the zebrafish midbrain (Figure 4.4B) at 17.6 T and 28.2 T. Overall, a noteworthy 40% increase in SNR was observed at 28.2 T compared to 17.6 T (Figure 4.4C). However, it is important to note that during this analysis, the impact of increased magnetic field strength on elevated T_1 relaxation times was not accounted for⁴⁸⁻⁵⁰. Hence, it is plausible that a more significant enhancement of the SNR could be achieved by adjusting the repetition time to accommodate the increase in T_1 . Given the small size of the zebrafish brain and its predominantly lipid-rich surrounding, elucidated in Supplementary Figure S4.2, voxels with isotropic dimensions surpassing beyond the brain tissue show broad and predominant lipid signals. These lipid signals are clearly visible around $\delta = 1.3$ ppm and $\delta = 2.0$ ppm and overlap with various cerebral metabolites, posing challenges for precise spectral analysis. In our examination of the midbrain, we observed that beyond a voxel volume of 512 nL, corresponding to a voxel size of $0.8 \times 0.8 \times 0.8$ mm³, interference from lipid signals became noticeable. Conversely, in the forebrain and hindbrain, which are both smaller and more elongated compared to the midbrain, we found for a voxel volume of 216 nL, corresponding to a voxel size of $0.6 \times 0.6 \times 0.6$ mm³, before interference from lipid signals became noticeable. We did not explore the potential benefits of non-isotropic voxel dimensions, which could potentially expand the brain coverage and thus enhance the signal obtained.

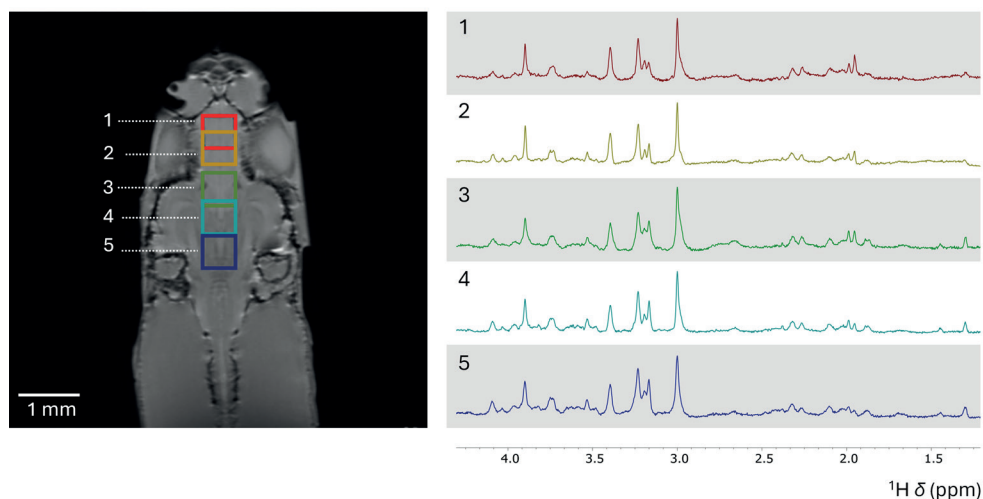


Figure 4.5. Optimized single voxel localized ^1H MRS spectra obtained from various locations in the adult zebrafish brain at 28.2 T. Anatomical RARE image, coronal slice, of zebrafish brain regions to indicate position of selected PRESS voxels. Highly resolved spectra were obtained from 5 distinct brain locations. Here, location 1 and 2 are situated in the forebrain, while 3 – 5 are situated in the zebrafish mid-brain. Acquisition details: TR = 2000 ms, TE = 15 ms, ns = 4096, a voxel size of $0.6 \times 0.6 \times 0.6$ mm³ with $V_{\text{voxel}} = 216$ nL, 2048 acquisition points, acquisition bandwidth 11904 Hz / 9.91 ppm, tBW = 15 kHz, 4 dummy scans, $v_1 = 3$ ppm. Water suppression and outer volume suppression were applied as specified in the method section.

Both at the UHF strength of 17.6 T and 28.2 T, spectra were acquired conducive to the identification and quantification of several cerebral metabolites, even at $V_{\text{voxel}} = 125 \text{ nL}$, corresponding to a voxel size of $0.5 \times 0.5 \times 0.5 \text{ mm}^3$. The ability to obtain high-resolution spectra from such small PRESS voxels allows for the identification and quantification of cerebral metabolites in various small regions in the zebrafish brain. This is elucidated in Figure 4.5, where at 28.2 T the cerebral metabolic profiles were obtained from five distinct voxels with $V_{\text{voxel}} = 216 \text{ nL}$, corresponding to a voxel size of $0.6 \times 0.6 \times 0.6 \text{ mm}^3$, utilizing $ns = 4096$, $tBW = 15 \text{ kHz}$, and $v_1 = 3 \text{ ppm}$. Highly resolved spectra were obtained. By focussing on specific regions of the zebrafish brain, insight can be gained on metabolic processes occurring in that particular area, providing valuable information about its function. Furthermore, it allows for monitoring metabolic changes in specific brain regions during neurodegenerative processes by utilizing pathological models.

Figure 4.6 shows that at 28.2 T, PRESS with $V_{\text{voxel}} = 512 \text{ nL}$, corresponding to a voxel size of $0.8 \times 0.8 \times 0.8 \text{ mm}^3$, acquired in approximately 2 minutes allow the identification and quantification of predominant cerebral metabolites, including tCr ($\delta = 3.02 \text{ ppm}$ and $\delta = 3.91 \text{ ppm}$), Tau ($\delta = 3.42 \text{ ppm}$ and $\delta = 3.25 \text{ ppm}$), Glx ($\delta = 3.75 \text{ ppm}$), and Lac ($\delta = 1.3 \text{ ppm}$). Increasing the number of scans significantly improves the spectral quality. This augmentation enables the identification and quantification of additional metabolites, while increasing the SNR in alignment with $SNR \sim \sqrt{n}$, as illustrated in Figure 4.6B. At 17.6 T we achieved adequate SNR for identifying our target metabolites with $V_{\text{voxel}} = 512 \text{ nL}$, and $ns = 1024$, resulting in approximately 34 minutes scan time. For $V_{\text{voxel}} = 216 \text{ nL}$, $ns = 2048$ was utilized, resulting in approximately 1 hour and 8 minutes total scan time. With the enhanced SNR at 28.2 T of approximately 40%, see Figure 4.4C, approximately half of the measurement time was required to obtain adequate SNR for each voxel volume (data not shown).

Metabolite identification and quantification at UHF

Based on presented results, single voxel localized MRS acquisition parameters were optimized to acquire a spectrum with extended measurement time of approximately 4.5 hours. Figure 4.7 shows the highly resolved spectrum obtained at 28.2 T from a PRESS voxel with $V_{\text{voxel}} = 512 \text{ nL}$, $tBW = 15 \text{ kHz}$ and $v_1 = 3 \text{ ppm}$. Compared to previous results obtained from *in vivo* analysis at 400 MHz³⁰, significantly improved spectra were obtained. This is most evident in the region between $\delta = 1.5 - 3.0 \text{ ppm}$, where important cerebral metabolite signals are present, e.g. GABA, NAA, NAAG, Glu, and Gln. Previously, the use of lower field strength required a larger PRESS voxel, i.e. $V_{\text{voxel}} = 3375 \text{ nL}$, to acquire ample signal from cerebral metabolites for quantification. Consequently, the PRESS voxel surpassed beyond the brain tissue. This led to significant overlap of lipid- and cerebral metabolite signals. However, in our current analysis, improvements in the SNR acquired by utilization of UHF systems allow for smaller

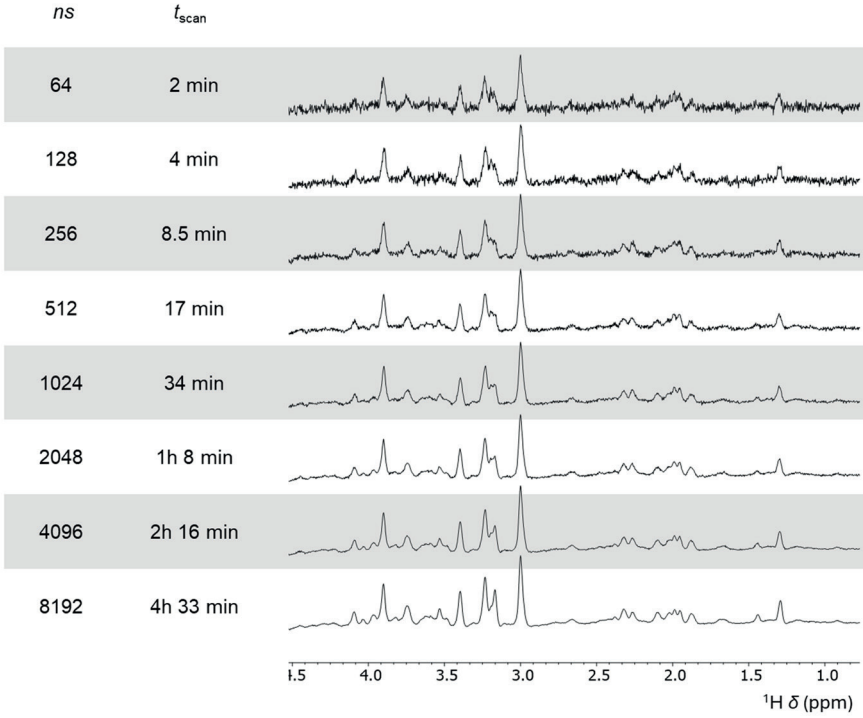
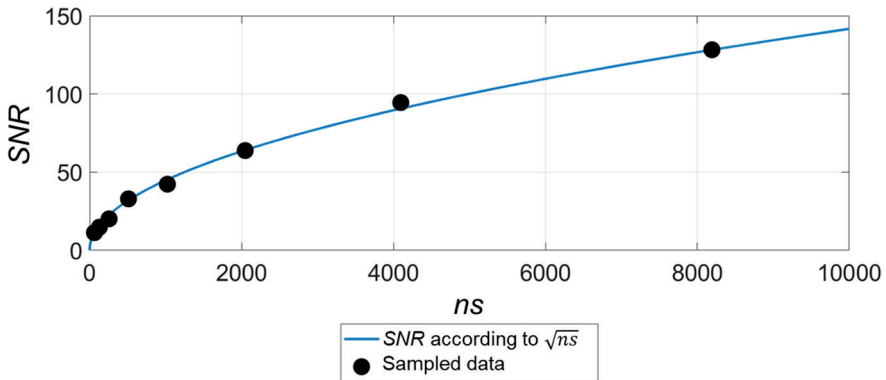
A**B**

Figure 4.6. Impact of ns in localized ^1H MRS from adult zebrafish mid-brain at 28.2 T. [A] Single voxel localized MRS spectra acquired from the mid-brain of an adult zebrafish, acquired with increasing ns [B] SNR of the tCr signal at $\delta = 3.03$ ppm as a function of ns . Acquisition details: $TR = 2000$ ms, $TE = 15$ ms, a voxel size of $0.8 \times 0.8 \times 0.8$ mm³ with $V_{\text{voxel}} = 512$ nL, 2048 acquisition points, acquisition bandwidth 11904 Hz / 9.91 ppm, tBW = 15 kHz, 4 dummy scans, $v_1 = 3$ ppm. Water suppression and outer volume suppression were applied as specified in the method section.

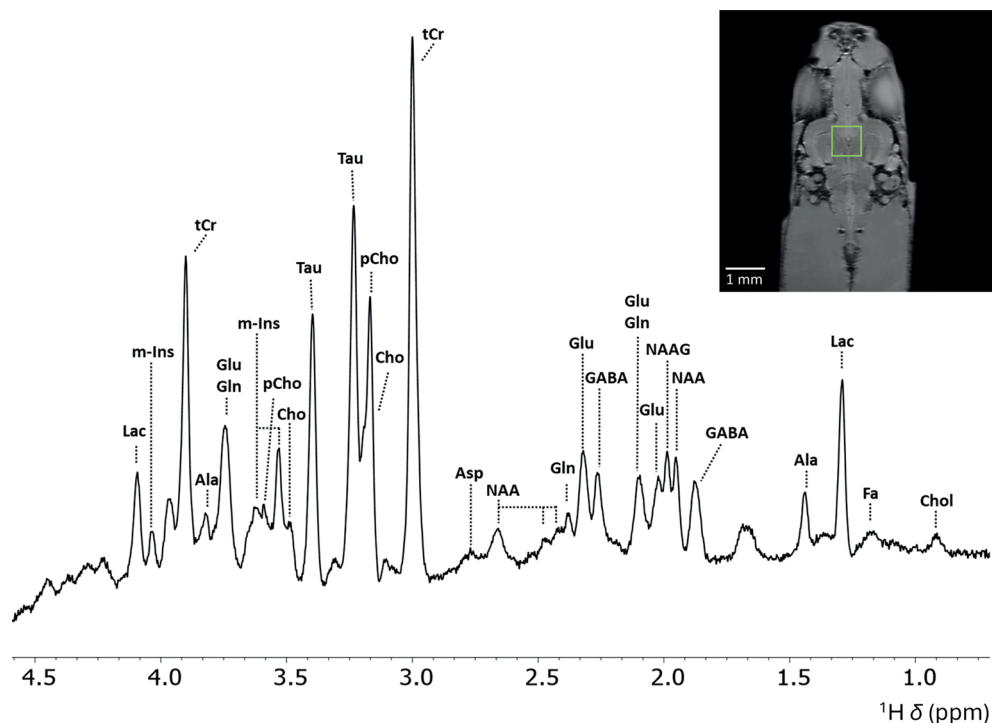


Figure 4.7. Optimized single voxel localized ^1H MRS spectrum from adult zebrafish mid-brain at 28.2 T. Anatomical RARE image, coronal slice, of zebrafish brain regions to indicate position of selected PRESS voxel. Acquisition details: $TR = 2000$ ms, $TE = 15$ ms, a voxel size of $0.8 \times 0.8 \times 0.8 \text{ mm}^3$ with $V_{\text{voxel}} = 512 \text{ nL}$, $ns = 8192$, scan time 4 hours 33 minutes, 2048 acquisition points, acquisition bandwidth 11904 Hz / 9.91 ppm at 28.2 T, 4 dummy scans, $v_1 = 3$ ppm, $tBW = 15$ kHz. Water suppression and outer volume suppression were applied as specified in the method section. Abbreviations; Lac – Lactate, m-Ins – myo-inositol, tCr – total creatine, Ala – alanine, Glu – glutamate, Gln – glutamine, pCho – phosphocholine, Cho – choline, Tau – taurine, Asp – aspartate, NAAG – N-acetylaspartyl-glutamate, NAA – N-acetylaspartate, GABA – γ -aminobutyric acid, FA – fatty acids, Chol – cholesterol.

PRESS voxels and the lipid signals are no longer detected. Moreover, the $^2\text{CH}_3$ signals of NAA and NAAG are resolved, and are separated by $\Delta\delta = 0.04 \text{ ppm}^{39}$. Frequently, due to spectral overlap, the $^2\text{CH}_3$ signals of NAA and NAAG are reported as the tNAA⁵¹ signal or simply as NAA^{6,7,9,10}, given the predominant contribution of NAA in the human brain. Post-processing techniques enable to resolve these signals⁵², or alternatively, quantification can be achieved through carefully designed sequences specifically for NAA and NAAG detection, such as the MEGA-PRESS sequence by Edden *et al.*⁵³. Previously the separation of NAA and NAAG signals was achieved using high-resolution localized two-dimensional COSY MRS in mouse brain⁵⁴. As demonstrated here, direct separation of NAA and NAAG signals can be achieved through

single voxel localized one-dimensional MRS at UHF strengths. Furthermore, we observe a very clear separation of the GABA, Glu and Gln signals, providing a solid basis for comparing levels of compounds involved in the glutamate/GABA – glutamine brain cycle.

In Figure 4.8, we present statistical analysis of the cerebral metabolic profile obtained from different regions in the zebrafish brain, acquired at 28.2 T ($n = 3$). In the midbrain, PRESS was performed with $V_{\text{voxel}} = 512$ nL and a voxel size of $0.8 \times 0.8 \times 0.8$ mm³. In the forebrain

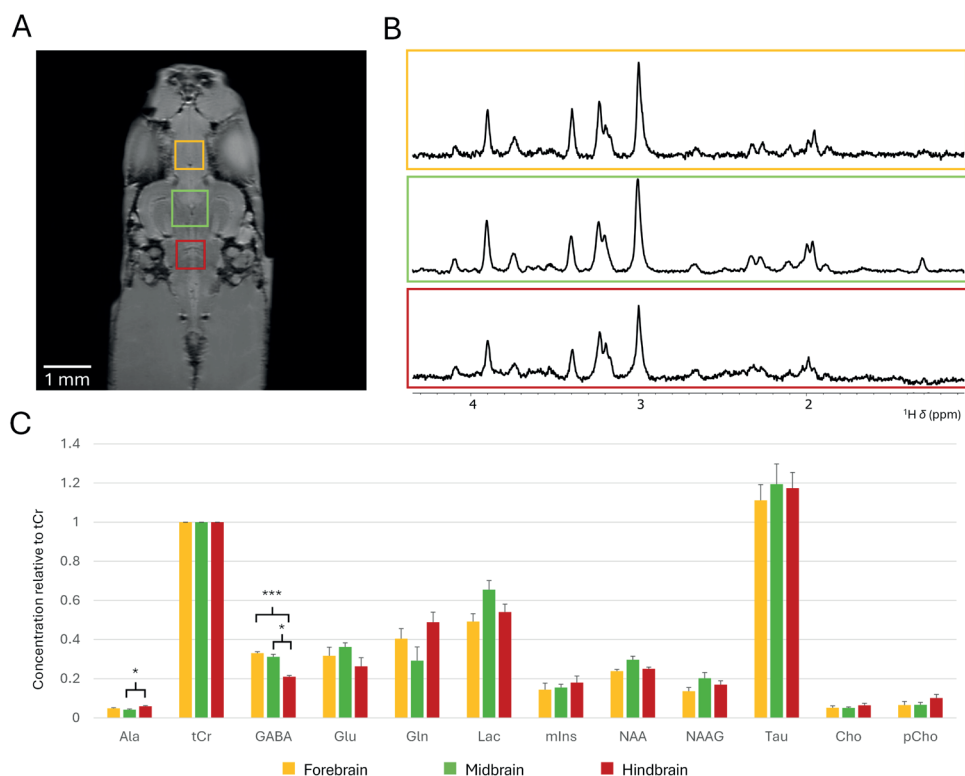


Figure 4.8. Comparison of cerebral metabolites levels in the forebrain, midbrain, and hindbrain of adult zebrafish at 28.2 T [A] Anatomical RARE image, coronal slice, of zebrafish brain regions to indicate position of selected PRESS voxels in the forebrain, midbrain, and hindbrain [B] Single voxel localized ¹H MRS spectra obtained from the forebrain, midbrain, and hindbrain. [C] Comparison of cerebral metabolite levels relative to tCr signal at $\delta = 3.03$ ppm. Statistically significant differences in metabolite levels between different brain regions obtained by unpaired t-test, assuming Gaussian distribution, $n = 3$, with $p < 0.05$ considered to indicate a significant difference. * $p < 0.05$, *** $p < 0.05$. 28.2 T. In all images, yellow label indicates the forebrain, green the midbrain, and red the hindbrain. Abbreviations; Ala – alanine, tCr – total creatine, GABA – γ -aminobutyric acid, Glu – glutamate, Gln – glutamine, Lac – Lactate, m-Ins – myo-inositol, NAA – N-acetylaspartate, NAAG – N-acetylaspartyl-glutamate, Tau – taurine, Cho – choline, pCho – phosphocholine

and hindbrain, PRESS was performed with $V_{\text{voxel}} = 216 \text{ nL}$ and a voxel size of $0.6 \times 0.6 \times 0.6 \text{ mm}^3$ (see Figure 4.8A). Highly resolved spectra were obtained from each region (see Figure 4.8B). Quantification of metabolites was performed with Chenomx. As indicated, chemical shift displacement effects necessitate quantification utilizing consistent chemical groups of a specific metabolite for all analysis, as utilizing different signals can lead to different quantitative outcomes (see Table 4.1). A full overview of the signals used for quantification is given in Table 4.3. We observed minimal variation in metabolite levels across the studied brain regions. However, significantly lower levels of GABA were found in the hindbrain compared to the forebrain ($p < 0.005$) and midbrain ($p < 0.05$). This discrepancy can be attributed to voxel placement. Throughout the zebrafish brain, GABAergic neurons are not uniformly distributed. Regions including the optic tectum, the subpallium, and the corpus cerebelli exhibit higher densities of GABAergic neurons^{55,56}. In our analysis, PRESS voxels in the forebrain and midbrain predominantly overlapped with these GABAergic neuron rich regions. However, the hindbrain voxel, situated just posterior to the cerebellar corpus, appears to have been positioned within a region characterised by lower levels of GABAergic neurons, thereby elucidating the observed difference in GABA levels.

Table 4.3. Estimated concentrations of brain metabolites at 28.2 T, relative to the tCr signal at 3.03 ppm, in the forebrain, midbrain, and hindbrain in young adult zebrafish ($n = 3$). Annotation of the molecular groups is consistent with those used by Govindaraju et al.³⁹.

Metabolite	Group used for quantification (ppm)	Forebrain Conc. rel. tCr ($\pm SE$)	Midbrain Conc. rel. tCr ($\pm SE$)	Hindbrain Conc. rel. tCr ($\pm SE$)
Ala	$^3\text{CH}_3$ ($\delta = 1.47 \text{ ppm}$)	0.049 ± 0.008	0.042 ± 0.004	0.059 ± 0.003
GABA	$^3\text{CH}_2$ ($\delta = 1.89 \text{ ppm}$)	0.331 ± 0.029	0.312 ± 0.013	0.21 ± 0.007
Glu	$^4\text{CH}_2$ ($\delta = 2.45 \text{ ppm}$)	0.317 ± 0.025	0.362 ± 0.021	0.263 ± 0.044
Gln	^2CH ($\delta = 3.75 \text{ ppm}$)	0.405 ± 0.006	0.293 ± 0.07	0.489 ± 0.051
Lac	^2CH ($\delta = 4.09 \text{ ppm}$)	0.492 ± 0.044	0.655 ± 0.047	0.541 ± 0.04
m-Ins	^4CH ($\delta = 3.61 \text{ ppm}$)	0.144 ± 0.016	0.155 ± 0.017	0.18 ± 0.034
NAA	$^2\text{CH}_3$ ($\delta = 2.01 \text{ ppm}$)	0.239 ± 0.034	0.297 ± 0.018	0.25 ± 0.009
NAAG	$^2\text{CH}_3$ ($\delta = 2.04 \text{ ppm}$)	0.136 ± 0.02	0.203 ± 0.03	0.17 ± 0.019
Tau	$^2\text{CH}_2$ ($\delta = 3.25 \text{ ppm}$)	1.111 ± 0.017	1.145 ± 0.138	1.174 ± 0.081
Cho	$\text{N}(\text{CH}_3)_3$ ($\delta = 3.19 \text{ ppm}$)	0.052 ± 0.004	0.05 ± 0.006	0.064 ± 0.01
pCho	$\text{N}(\text{CH}_3)_3$ ($\delta = 3.21 \text{ ppm}$)	0.065 ± 0.004	0.067 ± 0.012	0.059 ± 0.003

4. CONCLUSION

In conclusion, single voxel localized magnetic resonance spectroscopy at ultra-high magnetic fields provides distinct advantages and challenges for the identification and quantification of cerebral metabolites in the tiny zebrafish brain. Chemical shift displacements are significant and need to be taken into account not only during the experimental design, but also during data processing. However, ultra-high fields provide the necessary signal-to-noise and spectral separation required to identify and quantify a wide range of cerebral metabolites in a volume as small as 125 nL.

In future, our optimized methods, with relatively short measurement times of up to 30 minutes, are well-suited for implementation in *in vivo* studies. *In vivo* measurements enable the utilization of various zebrafish models of human pathological diseases for longitudinal studies by allowing continuous and non-invasive monitoring of metabolic changes over time. This approach facilitates the study of disease progression and the long-term effects of therapeutic interventions within the same subjects, providing a more comprehensive understanding of the underlying mechanisms. Furthermore, various other non-invasive MRS measurements could be tested and optimized for UHF, not discussed in the current work. This includes the optimization and comparison of other available single voxel localized ^1H MRS methods including stimulated echo acquisition mode (STEAM) or semi-localization by adiabatic-selective refocusing (sLASER) sequences. Furthermore, multi-voxel MRS methods including chemical shift imaging (CSI) for whole brain analysis could be optimized. Besides different methods, a metabolic region that was not taken into consideration during the experiments presented here, was the chemical shift region between $\delta = 6.00$ ppm and $\delta = 8.00$ ppm. This chemical shift region contains the signals of various energy metabolites, including ATP, ADP, NADH, NAD $^+$, NADPH, and NADP $^+$. Monitoring these metabolites non-invasively would potentially provide valuable information during metabolic analysis of, for instance, neurodegenerative diseases. However, these metabolites are separated by $\Delta\delta = 3 - 5$ ppm from tCr for quantification, which could potentially make quantification difficult as a consequence of chemical shift displacement effects. Finally, localized MRS utilizing different nuclei might provide valuable information. In particular, localized ^{31}P MRS can provide valuable information on phosphorus-containing (cerebral) metabolites including phosphocholine, glycerophosphocholine, ATP, and NADH.

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5. APPENDIX

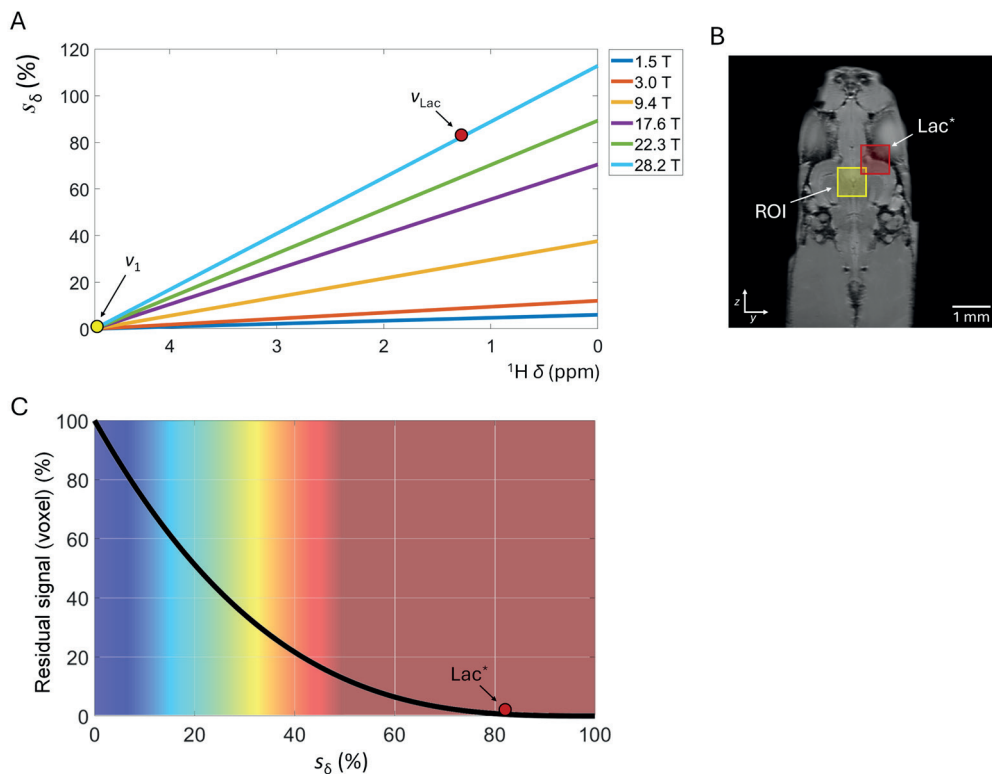


Figure S4.1. Chemical shift displacement effect in single voxel localized MRS [A] s_δ (%) at chemical shift of ^1H (ppm) for increasing magnetic field strengths for PRESS. v_1 , excitation frequency (ppm); v_{Lac} , chemical shift of the $^3\text{CH}_3$ group lactate at $\delta = 1.32$ ppm. [B] Impact of the chemical shift displacement on the selected ROI and the actual location from which the lactate signal is obtained at 28.2 T. In this illustration, only the chemical shift displacement in the z and y direction are shown. [C] s_δ (%) versus the remaining fraction of the signal from the selected voxel (%). Colour code is used to visualize severity of s_δ . Exceeding a chemical shift displacement effect of 50% per transmitter RF pulse, almost 90% of the signal falls beyond the selected PRESS voxel. The theoretical signal loss is determined as the percentage of the signal that extends beyond the selected PRESS voxel due to chemical shift displacement. * Chemical shift displacement of lactate assuming $B_0 = 28.2$ T, tBW = 5 kHz, a voxel size of $0.8 \times 0.8 \times 0.8$ mm³ with $V_{\text{voxel}} = 512$ nL, and $v_1 = 4.7$ ppm.

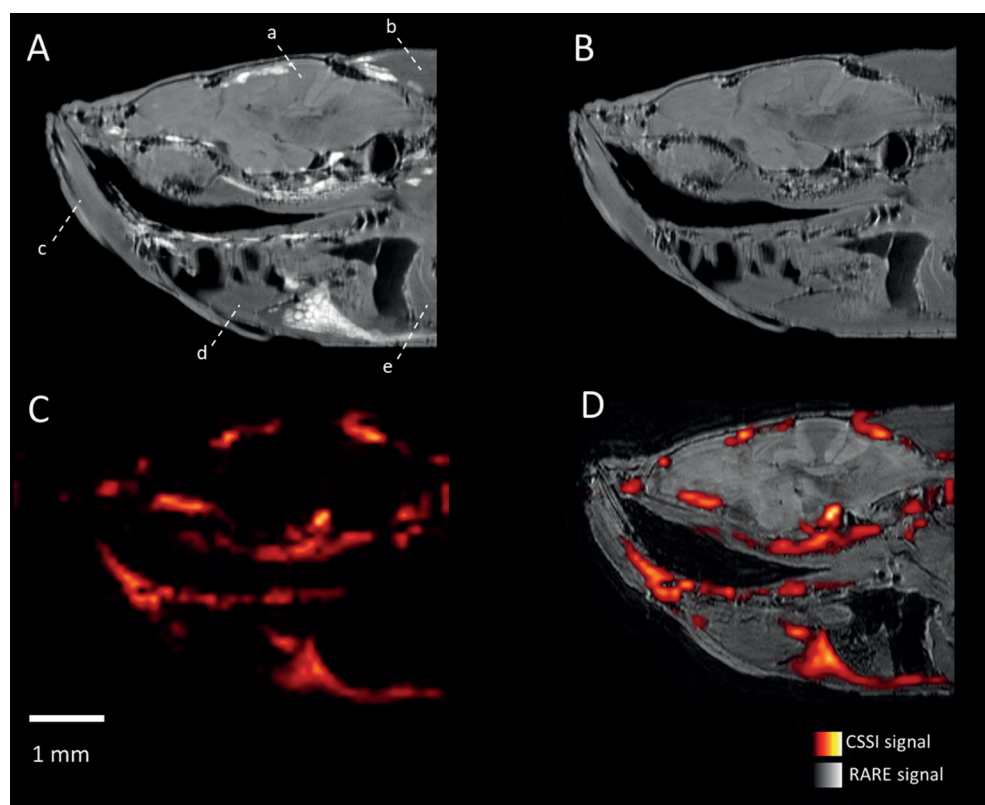


Figure S4.2. Chemical shift selective imaging (cssi) for the visualization of lipid distribution in the zebrafish head at 28.2 T. For these measurements, wild-type three-month-old adult-zebrafish, fixed in 4% buffered paraformaldehyde were utilized as described previously. **[A]** Sagittal 2D RARE image without fat suppression. Chemical shift artifact causes displacement of lipids in the frequency-encode direction, which increases with B_0 . **[B]** Sagittal 2D RARE image with fat suppression. **[C]** Lipid signal obtained by 3D cssi. **[D]** 3D cssi signal (red colour scale) overlaid on 3D RARE image (grey colour scale), showing the distribution of lipids in the zebrafish head. Data acquisition and processing were performed using Paravision 360 v3.3. Acquisition details: 2D RARE images: TR = 3000 ms, TE = 5.6 ms, ns = 64, resolution $23\ \mu\text{m} \times 23\ \mu\text{m} \times 100\ \mu\text{m}$, RARE factor 4. 3D RARE image: TR = 1800 ms, TE = 6.1 ms, ns = 4, resolution $23\ \mu\text{m} \times 23\ \mu\text{m} \times 47\ \mu\text{m}$, RARE factor 16. 3D cssi image: TR = 2000 ms, TE = 4.5 ms, ns = 1, resolution $94\ \mu\text{m} \times 94\ \mu\text{m} \times 188\ \mu\text{m}$, shift selective excitation frequency 1200.7 MHz (1.24 ppm), excitation pulse bandwidth 2000 Hz (1.6 ppm). (a) brain; (b) muscle; (c) dentary; (d) sternohyoid; (e) liver

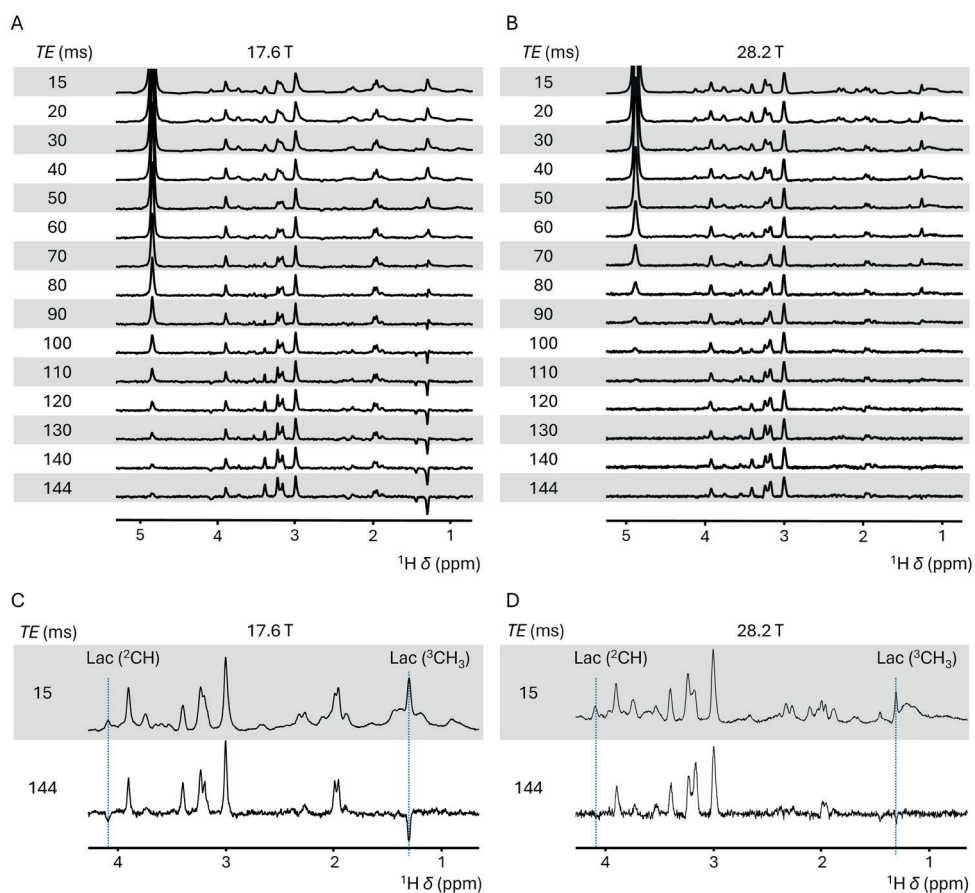


Figure S4.3. Full comparison of echo times in single voxel localized ^1H MRS spectrum from adult zebrafish mid-brain at 17.6 T and 28.2 T, in the range between 15 ms and 144 ms. [A – B] Single voxel localized MRS spectra acquired for echo times at 17.6 T and 28.2 T, respectively. [C – D] Comparison of spectra obtained at a short echo time of 15 ms and a long echo time of 144 ms at 17.6 T and 28.2 T. Particular attention is directed towards the lactate 180° phase shift at 144 ms, contributed to J-coupling induced signal dephasing. This effect is prominently evident at 17.6 T. However, at 28.2 T, due to escalated chemical shift displacement effects, the lactate signal acquired at 144 ms is submerged within spectral noise, highlighting the necessity for compensation strategies to address these effects.

