

# MRI and MRS on preserved samples as a tool in fish ecology

Christian Bock\*, Felizitas C. Wermter, Katja Mintenbeck

*Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research, Bremerhaven, Germany*

\*Corresponding author:

Dr. Christian Bock

Integrative Ecophysiology

Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research

Am Handelshafen 12

27570 Bremerhaven, Germany

Tel.: 49(471)4831-1288

E-mail: [Christian.Bock@awi.de](mailto:Christian.Bock@awi.de)

**Keywords:** Magnetic resonance imaging, NMR spectroscopy, preserved samples, formalin, *Pleuragramma antarctica*, lipids

**Short Title:** MRI and MRS as tools in fish ecology

## 20 **Abstract**

21 Magnetic Resonance Imaging (MRI) and Magnetic Resonance Spectroscopy (MRS) gain  
22 increasing attention and importance as a tool in marine ecology. So far, studies were largely  
23 limited to morphological studies, e.g. for the creation of digital libraries. Here, the utility of  
24 MRI and MRS for ecologists is tested and exemplified using formalin preserved samples of  
25 the Antarctic silverfish, *Pleuragramma antarctica*. As this species lacks a swim bladder,  
26 buoyancy is attained by the deposition of large amounts of lipids that are mainly stored in  
27 subcutaneous and intermuscular lipid sacs. In this study MRI and MRS are not only used to  
28 study internal morphology, but additionally to investigate functional morphology and to  
29 measure parameters of high ecological interest. The data are compared with literature data  
30 obtained by means of traditional ecological methods.

31 The results from this study show that MR scans are not only an alternative to histological  
32 sections (as shown before), but even allow the visualization of particular features in delicate  
33 soft tissues, such as *Pleuragramma's* lipid sacs. 3D rendering techniques proved to be a  
34 useful tool to study organ volumes and lipid content, which usually requires laborious  
35 chemical lipid extraction and analysis. Moreover, the application of MRS even allows for an  
36 analysis of lipids and fatty acids within lipid sacs, which wouldn't be possible using  
37 destructive methods. MRI and MRS, in particular when used in combination, have the  
38 capacity to provide useful data on parameters of high ecological relevance and thus have  
39 proven to be a highly valuable addition, if not alternative, to the classical methods.

40

41

42

## 43 Introduction

44 Modern imaging modalities like Magnetic Resonance Imaging (MRI) and Computed  
45 Tomography (CT) are increasingly gaining attention in the field of zoology, in particular for  
46 morphological studies [1, 2, 3]. These non-invasive imaging techniques can easily picture the  
47 whole body of organisms in 3-dimensional (3D) digital data without the need for dissection.  
48 Supported by imaging graphic software tools, the data sets can be used to create anatomical  
49 and morphological 3D models discriminating between skeleton and organs or displaying the  
50 connectivity of, e.g., the entire cardiovascular system. Recently, MRI and CT have been  
51 successfully applied to create digital atlantes (e.g. of brains [4]) and libraries to document  
52 and store anatomical and morphological data of specimen from Natural Museums'  
53 collections, and to make these data available online for everyone (see  
54 [www.digitalfishlibrary.org](http://www.digitalfishlibrary.org); [5]).

55 The main difference of MRI compared to other non-invasive imaging techniques is its  
56 excellent soft tissue contrast that allows detecting and illustrating structures, which are hard  
57 to unveil with classical dissection techniques [e.g. 6, 7]. The image contrast can be easily  
58 modified by running specific MRI sequences to produce a distinct discrimination between  
59 particular organs or other specific internal structures. In most tissues the contrast of MR  
60 images is primarily based on magnetic properties of water-bound hydrogen. However, in  
61 lipid-rich tissues lipid-bound hydrogen atoms significantly contributes to the image contrast,  
62 which can be utilized to image lipid-rich tissues and structures in specific body parts [e.g. 8].  
63 The MRI, moreover, can be used in combination with Nuclear Magnetic Resonance  
64 Spectroscopy (NMR, MRS) to get localized and analytical information on, e.g., specific

65 metabolites or lipid composition of organs and body structures. This approach has resulted  
66 in a multitude of applications of *in vivo* MRI and MRS in animals (for review see e.g. [9, 10]).  
67 However, alive animals for such studies are not always available. In these cases, preserved  
68 samples, either frozen or chemically preserved, may be studied instead. The most common  
69 chemicals used for sample preservation and long-term storage are ethanol and formalin. In  
70 particular formalin preservation has some clear advantages regarding tissue integrity of a  
71 sample or animal compared to freezing or the use of fresh tissues: fragile structures are not  
72 damaged by forming ice crystals and no water-loss occurs due to post-mortem cell-  
73 degradation or warming. Chemically preserved samples are frequently available in the  
74 collections of museums, research institutes or universities. Often, these collections even  
75 include rare and/or highly valuable animals for which analyses of, e.g., tissue composition or  
76 internal structure using traditional destructive methods is not an option to be considered.  
77 For such samples or animals, non-invasive MRI and MRS represent perfect tools to study  
78 their internal structure without any damage.

79 Compared to the variety of *in vivo* applications, however, the applications of MRI to  
80 chemically preserved animals so far has been largely limited to general anatomical and  
81 morphological studies, such as the work done in the framework of the Digital Fish Library [5].  
82 Here, we intent to move beyond the pure record and representation of general anatomy and  
83 morphology and to open these tools for ecological research. We extend the basic  
84 morphological approach and present a protocol containing standard MR imaging sequences  
85 in combination with MRS for studies on the structure and function of lipid-rich tissues  
86 (functional morphological MR) in preserved organisms. This protocol is simple to apply even  
87 for the non-expert MR user and demonstrated here using preserved samples of an Antarctic  
88 fish as an example.

89 The Antarctic silverfish, *Pleuragramma antarctica* Boulenger, 1902 is one of the few truly  
90 pelagic fish species inhabiting high Antarctic waters and represents a major trophic link in  
91 the food web. As it is extremely difficult, if not almost impossible, to catch this fragile fish  
92 alive, studies usually have to rely on preserved samples. Structural analyses of this species  
93 are largely limited to histological studies of transverse tissue sections [e.g. 11,12,13]. Despite  
94 the lack of a swim bladder, *Pleuragramma* is almost neutrally buoyant; the lack of a swim  
95 bladder is mainly compensated by a reduced and low ossified bone mass (skeleton) and by  
96 large lipid deposits [11, 14, 15, 16]. Fish have unconstricted vertebrae and a persistent  
97 notochord filling the hollow centra of adult vertebrae [14, 17]. The lipids are mainly stored in  
98 intermuscular and subcutaneous lipid sacs [11, 14], which is a rather rare feature in fish.  
99 Whether the functional role of these lipids is exclusively limited to buoyancy, however, is still  
100 under debate [18].

101 In this study, we test for the capacity of MRI and MRS technologies to address and study  
102 ecologically relevant issues and features of fish species, such as *Pleuragramma*, when  
103 applied to preserved animals. As the lipid storage system in this fish species is very  
104 particular, we not only investigate the general anatomy and the potential to determine  
105 organ volumes, but also focus on the distribution and structure of the lipid sacs and the  
106 possibility to analyze the lipid composition. As investigating such measures of ecological  
107 interests using traditional methods (e.g., histological sections, chemical lipid extractions and  
108 analysis) is very time consuming and destructive, the modern tools used in this study might  
109 provide a useful addition or even alternative to draw a comprehensive ecological picture of a  
110 fish species. This non-invasive approach might prove particularly valuable for studying rare  
111 or highly valuable species.

112

## 113 **Materials and Methods**

### 114 **Animal sampling**

115 Individuals of adult *Pleuragramma* were taken in the western Weddell Sea, east off the  
116 Antarctic Peninsula (south of 60°S) during the RV Polarstern expedition ANT XXVII-3 in 2011  
117 [19]. Fish were caught between 64°47,00'S / 60°23,65'W and 65°31,68'S / 61°33,13'W by  
118 means of a standard bottom trawl and a benthopelagic net. The sampling of fish was  
119 conducted according to the ethics and guidelines of the German law, and approved by the  
120 Federal Environment Agency (FEA; Umweltbundesamt, UBA, Wörlitzer Platz 1, 06844  
121 Dessau-Roßlau), reference number I 3.5 - 94003-3/253, on February 1<sup>st</sup> 2011. Individuals  
122 were fixed in 10% formalin buffered with borax (sodium tetraborate) for preservation. To  
123 exemplify the capacities of MRI and MRS, individual animals were randomly chosen from the  
124 bulk of samples.

125

### 126 **MR imaging and spectroscopy**

127 Directly prior to MR scanning, individual fish were carefully removed from formalin to  
128 determine weight and standard length (SL, cm). Subsequently, the specimen was placed  
129 between two wooden skewers within a Perspex chamber to hold the fish in an upright  
130 position without damaging the skin. The Perspex chamber containing the fish was then  
131 positioned inside the magnet. All MRI and MRS studies were performed in a Bruker 4.7T  
132 magnet equipped with Avance III electronics. For high resolution MRI and MRS a 200mT/m  
133 gradient coil insert (BGU 12) together with an 8 cm <sup>1</sup>H-birdcage resonator (Bruker Biospin,  
134 Germany) was used. Whole body analyses (e.g. total lipid content) were conducted using the  
135 standard 50mT/m gradient coil (BGU 26) together with a 20 cm <sup>1</sup>H-birdcage resonator

136 (Bruker Biospin, Germany). Data acquisition and recording were carried out with ParaVision  
137 5.1 (Bruker Biospin, Germany).

138

### 139 **Functional morphological MR**

140 Modified Driven Equilibrium Fourier Transform (MDEFT) imaging, as recommended for  
141 morphological MRIs [3], was used here to study the internal anatomy of preserved fish.  
142 Using a MDEFT sequence protocol from the ParaVision software, the general internal  
143 anatomy of a fish was investigated (2D) and its total body as well as stomach volume was  
144 determined (3D).

145 Prior to imaging, field homogeneity was optimized using an automated shim routine of the  
146 ParaVision software. The optimal morphological contrast was achieved using a fat  
147 suppression with a Gaussian pulse (700 Hz bandwidth). Parameters were as follows: Echo  
148 time TE = 7.59 ms, Echo repetition time TER = 21.16 ms, segments= 8, segment repetition  
149 time= 3353.75 ms, segmentation duration 1354.24 ms, number of averages: 64, 90° sinc10H  
150 pulse of 2 ms length, matrix size: 512x512, Field of view FOV 60x60 mm, 26 slices, slice  
151 thickness 0.5 mm, maximum achievable resolution 0.117x0.117 mm/pixel. The total  
152 acquisition time varied according to the number of averages from 5 min for an overview  
153 image for the positioning of the voxels used for localized <sup>1</sup>H-NMR spectroscopy up to 12h 24  
154 min for high-resolution images of the lipid sacs.

155 All images were post processed using MeVisLab (MeVis, Germany); to determine total body  
156 and organ volumes, the 3D data were processed with the modules 'region growing' and  
157 'volume rendering'. The stomach was separated from the surrounding tissue with the  
158 selection of a region of interest (ROI). To test whether fish volume data can be used as an

159 equivalent to fish mass, which is together with body size the common measure in fish  
160 ecology, the relationship between individual body weight  $w$  (conventionally measured) and  
161 body volume  $v$  (measured in the MRI) was analyzed.

162 For MR imaging of lipid distribution different approaches were taken into account before  
163 starting the measurements. A search of the current literature on lipid quantification in fishes  
164 revealed that, e.g., the Dixon technique, which is one of the state of the art techniques for  
165 water-fat separation in medical research, yields unpersuasive results when applied to fishes  
166 [20]. The contrast enhancement effect of lipid in fast spin echo sequences [21] (e.g. the rapid  
167 acquisition with relaxation enhancement (RARE)) proved to be the most appropriate  
168 approach and was chosen for the analysis of preserved samples. A standard multi-slice RARE  
169 sequence protocol with the following parameters was used: TE= 14.9 ms, RARE factor: 4,  
170 effective TE<sub>eff</sub>= 29.8 ms, TR= 5s, number of averages 30, 90° hermite pulse of 3 ms length,  
171 180° hermite pulse of 1.9 ms, matrix 512x256, FOV 685x86,4 mm, 14-18 slices, slice  
172 thickness 1.12 mm, total acquisition time 2h 40min.

173 After interpolation of the multi slice data sets to 3D matrices (minimum size 64/512/64  
174 depending on fish length) percent overall lipid content was calculated from volume rendered  
175 MRI scans using MeVis Lab in accordance with Machann and colleagues [22]. The lipid  
176 content of individual *Pleuragramma* was calculated by converting the number of pixels into  
177 a volume  $v$  [mm<sup>3</sup>]. Bright pixels that arose from lipid-bound hydrogen atoms were separated  
178 from other tissues using an operator-controlled threshold of 165 pixel units. The remaining  
179 pixels were counted and summed up and finally divided from the overall sample volume. The  
180 lipid content of individuals is then expressed by the percentage of lipid volume of total fish  
181 body volume (i.e. lipid as % of total body volume).

182



183 The individuals of *Pleuragramma* that were used in this study are part of the institute's  
184 collections and are too rare and valuable to be destroyed for a direct validation of these  
185 results using chemical lipid extraction. Instead, previously published data on chemically  
186 determined lipid content (and composition) of *Pleuragramma* sampled also in the Weddell  
187 Sea were used to indirectly verify our measurements. For the comparison with these  
188 literature data, which are usually given in % dry weight, a linear regression model based on  
189 *Pleuragramma* tissue wet weights and dry weights analyzed as part of Mintenbeck and  
190 colleagues [23] was used with a conversion factor of 0.154 to estimate initial dry weights of  
191 the specimens used in this study.

192

### 193 **<sup>1</sup>H-NMR spectroscopy and lipid composition**

194 Localized <sup>1</sup>H-NMR spectroscopy was conducted for the analysis of lipid composition, i.e. fatty  
195 acid patterns in triacylglycerides [24, 25, 26]. Prior to spectroscopy, field homogeneity for  
196 the specific volume of interest (voxel) was optimized using FASTMAP [27]. A standard <sup>1</sup>H  
197 Point Resolved Spectroscopy (PRESS) sequence with the following parameters was used:  
198 TE=21ms, TR = 5000ms, number of sampling points 2k, 256 averages, acquisition time 21m  
199 40s. Individual voxels were placed inside specific regions of interest of the intermuscular  
200 lipid layers according to previous acquired multi-slice MRIs (see above). Size of individual  
201 voxel varied among samples and ranged from 3x3x3 to 2x2x8 mm, usually including 3-4 lipid  
202 sacs within one voxel, resulting in a volume of interest between 27 and 32 mm<sup>3</sup>. Localised  
203 <sup>1</sup>H-MR spectra from lipids within the lipid sacs were measured in preserved individuals  
204 sampled at two different locations in the Weddell Sea (Station A: area of the former Larsen A  
205 ice shelf; Station B: area of the former Larsen B shelf ice). Prior to the main analysis, we  
206 tested for within-fish and within-sampling location variability. There were no differences in

207 lipid composition within lipid sacs from different positions within one individual fish.  
208 Nevertheless, for the main analysis, the voxels for the localized MR spectroscopy in the lipid  
209 sacs were in each individual fish placed at the same position behind the first dorsal fin. The  
210 variability in lipid composition among individuals from one sampling location (Station A or B)  
211 was low.

212 Lipid composition was determined from the localized  $^1\text{H}$ -MR spectroscopy using the  
213 approach recently described by Machann et al. [22]. The signals in the  $^1\text{H}$ -NMR spectra were  
214 assigned to the respective triacylglyceride resonances according to Berglund et al. [26] and  
215 Petterson & Månsson [28].

216

## 217 **Results and Discussion**

### 218 **General internal anatomy**

219 The Antarctic silverfish *Pleuragramma antarctica* possesses some very special anatomical  
220 features such as the lipid sacs, the less ossified skeleton and the persistent gelatinous  
221 notochord [14, 17]. Images of this species are not (yet) part of the Digital Fish Library [5] and  
222 it was uncertain whether morphological data could be pictured in sufficient quality from  
223 preserved samples of this species due to the high total lipid content and the heterogeneous  
224 lipid distribution; both might theoretically induce artifacts in MR images due to susceptibility  
225 changes resulting from local inhomogeneity between different tissues and chemical shift  
226 effects between lipid and water. To test the applicability of MRI to preserved tissues of the  
227 Antarctic silverfish for the first time and to gain a first overview of the general internal  
228 anatomy, high-resolution 2D and 3D MR imaging, as proposed by Ziegler et al. [3], were  
229 applied.

230 Fig. 1 shows an example of a high-resolution morphological MR image of a multi-slice MR  
231 set. The excellent soft tissue contrast of the MR imaging technique allowed a clear  
232 distinction of muscle (M), notochord (N) and different organs such as brain (B), liver (Lv), and  
233 stomach (S) in the sagittal morphological section (Fig. 1A). There was no sign of any B0 or B1  
234 inhomogeneity, indicating a very homogeneous excitation profile. Between outer skin and  
235 muscles, series of lipid sacs (ILS) can be identified in the dorsal part of the body along the  
236 back and in the ventral part of the body behind the abdomen (Fig. 1A). Fig. 1B shows a  
237 transversal MRI section taken from a 3D-morphological data set of a sample from this study  
238 in comparison to a histological cross (transversal) section of *Pleuragramma* from Eastman &  
239 DeVries [14] shown in Fig. 1C. Both transversal sections (Fig. 1B & C) are from the posterior  
240 one-fifth of the body of an adult individual [cf. 14, 17]. In the MRI slice muscle tissue is  
241 reflected in grey, whereas the lipids (ILS, SLS, IAT; Fig. 1B) generate a dark to almost no  
242 contrast. The details on the MR image are in well agreement with the histological image;  
243 even the thin collar of vertebral bone surrounding the notochord is visible. Besides of the  
244 large muscles, also the dorsal (DFM) and anal fin muscles (AFM) as shown in Johnston et al.  
245 [13] in histological sections (not shown here) can be identified on the MR image. Due to the  
246 lower resolution of MR images compared to histological images, very small details such as  
247 single muscle fibers or vacuoles and myocellular lipids [14, 17] can't be identified. However,  
248 despite of the lower resolution, the morphological imaging data sets obtained from non-  
249 destructive MRI technique provide almost the same array of information without the  
250 necessity to dissect and destroy the animal. In addition, despite of the high lipid content and  
251 heterogeneous structure, the MR images of preserved samples of *Pleuragramma* are of the  
252 same quality as, e.g., images from other (Antarctic) fish species such as *Notothenia coriiceps*  
253 as shown in the Digital Fish Library (see [www.digitalfishlibrary.org](http://www.digitalfishlibrary.org)).

254 As the lipid sacs in *Pleuragramma* are a rare feature among fish, their structure was studied  
255 in more detail. Within one particular fish and among individuals from the same sampling  
256 area, the shape of the single lipid sacs was similar. This was not the case among individuals  
257 from different areas. In Fig. 2 the high-resolution MRI scans of two individuals from different  
258 sampling regions are shown. The yellow boxes in both examples display almost identical  
259 positions within the fish. In the scan on the left, the lipid sacs are well separated from each  
260 other, while in the scan on the right the sacs are much more densely packed and display a  
261 much brighter image contrast (in particular within the yellow box). The reason for these  
262 differences still needs to be clarified. However, such morphological features of fragile  
263 objects like the lipid sacs get lost when using destructive techniques, but can be perfectly  
264 identified and analyzed in chemically preserved samples with non-invasive digital imaging  
265 approaches such as MRI.

266

### 267 **Organ volume**

268 Beside the representation of anatomic structures and details, the morphological MRI can  
269 produce 3D MR imaging data sets, e.g. from the abdominal region, which can be used to  
270 determine the volume of the specimen and specific organs of interest, such as the stomach  
271 (Fig. 3). The gastric wall exhibited a bright contrast in this MDEFT image set (see Fig. 3A-C),  
272 which could be easily separated from the other tissues using volume rendering tools (see  
273 Material & Methods). The rendered volume of one stomach is exemplarily shown in Fig. 3D.  
274 To show that the volume data measured in the MRI are comparable with conventional,  
275 ecological data, which are usually in units of weight (or size), the relationship between  
276 individual body weight (conventionally measured) and body volume (measured in the MRI) is  
277 shown in Fig. 4. According to these data, the relationship between both measures is almost

278 1:1 ( $y=0.998x$ ,  $R^2=0.979$ ), validating the comparability of body volume and body weight (i.e.  
279 1 ml  $\approx$  1 g). Given that body volume  $v$  is a function of body weight  $w$  and body density  $\rho$   
280 ( $v=w/\rho$ ), the slope close to 1 is not surprising: *Pleuragramma* is almost neutrally buoyant,  
281 hence, its body density should be close to the density of the seawater, which on the high  
282 Antarctic shelf is about 1.03 g cm<sup>-3</sup> (35 psu, -1,8°C). Accordingly, a body density  $\rho$  close to 1 g  
283 cm<sup>-3</sup> can be expected for *Pleuragramma*, which means that for this species  $v\approx w$ .

284 The volume of a stomach and the thickness of the gastric wall vary strongly depending on  
285 stomach fullness. Both, volume and wall thickness, are thus valuable indicators for the  
286 nutritional state of a fish and may be particularly useful for temporal or spatial comparisons  
287 of individuals in studies on a species' feeding habits. At present, in fish ecology, stomach  
288 fullness is determined gravimetrically or using rather subjective indices [29], both requiring  
289 dissection of the animals of interest and a lot of time.

290 Another important but time-consuming measure in fish ecology, beside stomach fullness, is  
291 the analysis of stomach content composition. Here, the limited gradient power (maximum of  
292 200 mT/m) of the MR imaging system used in this study did not allow (with some  
293 exceptions) for identifying details of the stomach content. However, current animal scanners  
294 equipped with more powerful gradient systems of field strengths above 1000mT/m can  
295 easily reach an in plane resolution below 100  $\mu$ m. Using such systems, the identification of  
296 particular prey organisms should be feasible (unless they are too heavily digested).

297 Here, the use of 3D MR imaging data sets for the determination of organ volumes was  
298 exemplified using stomachs of fish. The same methodology can be also applied to, e.g., the  
299 liver. In fish, the liver serves as an energy storage and is therefore smaller (and less heavy) in  
300 poor environments. Accordingly, in fish ecology, the ratio of liver to body weight  
301 (Hepatosomatic Index, HSI) is used as a measure of fish condition [30]. Intraspecific

302 comparison of liver volume using morphological MRI can therefore provide valuable  
303 information on conditions and prey availability in the area/season of capture.

304

### 305 **Lipid content**

306 Besides the excellent soft tissue contrast for morphological or anatomical observations, MR  
307 techniques can clearly separate tissue water from lipid and are frequently used for fat-water  
308 separation in medical research [for a review see 30]. Accordingly, MR techniques are  
309 frequently used for the analysis of body-fat content *in vivo* [10]. Recently, such MR analyses  
310 were also applied to fresh and defrosted fish tissue for an assessment of, e.g., food quality  
311 [20, 31, 32]. However, to our knowledge, these techniques to analyse body lipid content  
312 have never been applied to chemically preserved samples, before. As lipids in fish play an  
313 important role as energy reserve and in some species, such as *Pleuragramma*, also  
314 contribute to buoyancy, the proportion of lipids in the body is a measure of high interest in  
315 fish ecology. This particularly applies in the case of adult *Pleuragramma*, where the  
316 functional role of lipids is still under debate [18].

317 In species using lipids primarily as energy reserve, total body lipid content is significantly  
318 positively correlated to fish condition [33]. In *Pleuragramma* overall lipid content was shown  
319 to vary with fish body size and life cycle [34, 35, 36, 37]; in *Pleuragramma* larvae total lipid  
320 content was used as a marker for the nutritional condition [38].

321 In Fig. 5B, the blue areas show the lipid fractions depicted with volume rendering and  
322 contrast thresholds (see Material & Methods) from the RARE imaging data. The distribution  
323 of lipids obtained from the RARE images is in very well agreement with the distribution in  
324 the histological sections from Eastman [15, 17] and Eastman & DeVries [14]. Even the thin

325 lipid layer surrounding the vertebral thin collar and the notochord can be identified on the  
326 MR image (Fig. 5B). The notochord itself does not contain lipids (as shown by the lack of blue  
327 color). This is a clear improvement to the morphological data acquired with the MDEFT  
328 sequence, where the notochord and lipids were not clearly distinguishable (see Fig. 1B).

329 The total lipid content of *Pleuragramma*, as determined from the RARE scans, averaged  $9.9$   
330  $\pm 1.7$  % of wet weight (WW) and  $51 \pm 9$  % of dry weight (DW; converted), respectively ( $n = 5$ ;  
331 mean individual body weight:  $15 \pm 1$  g WW; mean standard length, SL:  $13.7 \pm 0.2$  cm). These  
332 results are in very good agreement with literature data on lipid content analyses based on  
333 chemical extraction from similar-sized *Pleuragramma* from the Weddell Sea (mean SL: 15.5  
334 cm, mean lipid contents: 10.2 % WW and 47 % DW; see [34]) and demonstrate the suitability  
335 of RARE imaging for lipid determination in preserved fish samples.

336

### 337 **Lipid composition**

338 Triacylglycerides and wax esters are the most common lipid classes in marine organisms  
339 including fish. Both lipid classes are used as energy reserve and buoyancy aid. Lipid  
340 composition of fish is influenced by the diet, because the fatty acid profile of a consumer  
341 reflects the fatty acids of its prey and its nutrition value [39, 40, 41]. Accordingly, lipid class  
342 composition and fatty acid signatures are commonly used markers to analyze consumers'  
343 trophic ecology [42, 43, 44]. Recently, it has been shown that the lipid composition of  
344 adipose tissue and lipid stores can be determined in humans and mammals using localized  
345  $^1\text{H-NMR}$  spectroscopy (see Material & Methods). The ratios of the  $^1\text{H-NMR}$  signals of the  
346 fatty acids can be used for the calculation of mean chain length, unsaturation status and the  
347 mono- and poly-unsaturation fractions of triacylglycerides, as shown for instance by

348 Berglund et al. [26]. Here, localized  $^1\text{H-NMR}$  spectroscopy was applied to analyze the  
349 composition of lipids stored in the intermuscular sacs (ILS) of *Pleuragramma*.

350 Figure 6 presents the localized  $^1\text{H-NMR}$  spectra from two individuals sampled at two  
351 different locations in the Weddell Sea (Station A and Station B, see Material and Methods for  
352 details). The measured spectra show a typical lipid pattern and the particular signals could  
353 be assigned to hydrogen atoms bound in specific positions of fatty acids according to the  
354 literature [22, 24, 2454]. The signal intensities reflect the relative concentration of hydrogen  
355 atoms bound to specific lipid groups and the signal of the  $\text{CH}_3$ -group can be used as an  
356 internal standard for calibration [22]. According to the spectra in Fig. 6 the dominating lipid  
357 class in the lipid sacs of *Pleuragramma* is triacylglyceride, which is in accordance with results  
358 on muscle tissue and overall lipid content determined using classical chemical lipid analyses  
359 [11, 45, 46]. Interestingly, distinct differences are also obvious between both spectra: e.g.  
360 the signals from the  $-(\text{CH}_2)-$  and the  $-\text{CH}_2-\text{CH}=\text{CH}-\text{CH}_2-$  group are much more pronounced in  
361 the individual from Station A in Fig. 6. These differences are confirmed by the detailed  
362 results from the  $^1\text{H-NMR}$  spectra summarized in Table 1. While the average chain length was  
363 similar in individuals from both sampling stations (A & B), the lipids of individuals from the  
364 two locations significantly differed in their unsaturated and polyunsaturated degree  
365 ( $p < 0.05$ ). Though the actual causes for these differences remain unsolved, so far, such  
366 differences might indicate potential functional changes within the lipid sacs induced by  
367 specific changes in energy/lipid metabolism of the organism.

368 Because of the fragile nature of the lipid sacs, an analysis of localized lipid composition  
369 inside the structures seems almost impossible using traditional, destructive methods. Here,  
370 the composition inside this particular lipid storage system was analysed for the first time  
371 using  $^1\text{H-NMR}$  spectroscopy.



372

373 **To be considered when working with preserved samples**

374 All kinds of sample preservation affect tissues of organisms in one or the other way.  
375 Potential effects differ among preservation method and may involve, e.g., alterations in  
376 biochemical composition, shrinkage due to water extraction or cell disruption. Formalin and  
377 its derivatives may induce changes in size and weight, as shown for instance in mice brain [46].  
378 Formalin fixation thus may result in an underestimation of measured organ volume of a  
379 preserved sample, though the reported changes are only in the range of a few percentages.  
380 Formalin (as well as methanol) may cause hydrolysis of tissue lipids and degradation of  
381 polyunsaturated fatty acids and phospholipids [48, 49], while neutral lipids are obviously not  
382 affected [50]. Most changes apparently occur during a short time frame directly after  
383 exposure to the preservation medium, within the first few hours or days; during this period,  
384 measurements and analyses of preserved samples should be avoided [51]. In some studies  
385 no significant effect of formalin on biochemical composition of tissue and samples,  
386 respectively, was found [52, 53]; other studies considered a potential effect negligible, at  
387 least as long as the lipid containing tissues are intact [54, 55].

388 In the present study, whole, undamaged animals were used, without any defects in the skin  
389 that might have facilitated leakage of lipids from the lipid sacs. The MRI studies were started  
390 about one year after fixation in 10% buffered formalin and repeated in part after another  
391 year of formalin storage. No significant difference in weight, volume or shape could be  
392 detected between the two time points, nor was there a difference in measured total lipid  
393 content (personal observation). The risk of lipid loss is expected to be increased in tissues  
394 samples (e.g. liver) that are directly exposed to the preservative. Whole organisms, with  
395 intact intestines and tissues, are most likely less affected. However, some formalin-induced

396 short term release of e.g. phospholipids can't be completely excluded. As lipids in  
397 *Pleuragramma* are mainly represented by neutral triacylglycerides (in particular inside the  
398 lipid sacs) while the amount of phospholipids is very low [37, 46], a potential effect of the  
399 preservation on overall lipid content and composition is considered negligible, here; in  
400 particular because treatment, preservation and storage were identical for all individuals.  
401 Nevertheless, for future MRI studies such potential effects of the preservation methods  
402 needs to be taken into account, particularly when different preservation methods are used  
403 or preserved samples are compared to fresh material.

404

## 405 **Conclusions**

406 As shown in this study, MRI and MRS on preserved fish samples may not only contribute  
407 anatomical and morphometric 3D data to digital libraries and atlantes, but also provide  
408 valuable insights into functional morphology (organ volumes, lipid distribution), energetics  
409 (lipid content) and biochemical composition (lipid composition). Using MRI, lipid content and  
410 organ volumes can be reliably determined. Unexpected and unknown differences in the  
411 shape of lipid sacs among individuals, that would have been hardly found using traditional  
412 methods, could be detected. <sup>1</sup>H-NMR spectroscopy proved to be a valuable tool to analyze  
413 lipid composition, which usually requires destruction of the animal and complex chemical  
414 lipid exaction and analysis. The localized spectroscopy allowed for the first analysis of lipid  
415 composition inside the lipid sacs and revealed differences in lipid composition among  
416 individuals from different sampling stations.

417 Lipid distribution, content and composition as well as organ volumes are all parameters of  
418 high ecological relevance. For the study of preserved samples, MRI and MRS proved to be a

419 highly valuable addition, if not alternative, to the classical methods. The advantage that  
420 these modern techniques are non-invasive and non-destructive opens an array of  
421 opportunities for studies of preserved samples from natural history museums' collections.

422

### 423 **Acknowledgements**

424 We like to thank Prof. Dr. Rascher-Friesenhausen for providing a MeVis Lab software licence  
425 and the crew and officers of FS Polarstern during ANTXXVII/3 for their professional support.

426 We also acknowledge the American Society of Ichthyologists and Herpetologists (ASIH), in  
427 particular the Editor of Copeia, Christopher Beachy, for granting permission to reprint Fig. 2A  
428 from Eastman & DeVries [14] (Fig. 1C in this manuscript). Two anonymous reviewers  
429 provided helpful comments on an earlier version of the manuscript. This work including  
430 animal sampling and analyses were funded by the German Research Foundation (DFG, SSP  
431 1158), grant MI 1391/1-1.

432

433

434

435

436

437

438

439 **References**

- 440 [1] Baker M. (2010) The whole picture. *Nature*. 463: 977-980.
- 441 [2] Lauridsen H, Hansen K, Wang T, Agger P, Andersen JL, Knudsen PS, Rasmussen  
442 AS, Uhrenholt L, Pedersen M (2012) Inside out: modern imaging techniques to reveal  
443 animal anatomy. *PloS One* 6: e17879.
- 444 [3] Ziegler A, Kunth M, Mueller S, Bock C, Pohmann R, Schröder L, Faber C, Giribet  
445 G (2011) Application of magnetic resonance imaging in zoology. *Zoomorphology*  
446 130: 227-254.
- 447 [4] Simões JM, Teles MC, Oliveira RF, Van den Linden A, Verhoye M (2012) A  
448 three-dimensional stereotaxic MRI brain atlas of the cichlid fish *Oreochromis*  
449 *mossambicus*. *PLoS One* 7: e44086.
- 450 [5] Berquist RM, Gledhill KM, Peterson MW, Doan AH, Baxter GT, Yopak KE, Kang  
451 N, Walker HJ, Hastings PA, Lawrence RF (2012) The digital fish library: using MRI to  
452 digitize, database, and document the morphological diversity of fish. *PLoS One* 7: 1-  
453 16.
- 454 [6] Ziegler A, Faber C, Mueller S, Bartolomaeus T (2008) Systematic comparison  
455 and reconstruction of sea urchin (Echinoidea) internal anatomy: a novel approach  
456 using magnetic resonance imaging. *BMC Biol* 6: 33.
- 457 [7] Bock C, Frederich M, Wittig RM, Pörtner HO (2001) Simultaneous observations  
458 of haemolymph flow and ventilation in marine spider crabs at different  
459 temperatures: a flow weighted MRI study. *Magn Reson Imaging* 19: 1113-1124.
- 460 [8] Orgiu S, Lafortuna CL, Rastelli F, Cadioli M, Falini A, Rizzo G (2015) Automatic  
461 muscle and fat segmentation in the thigh from T1-weighted MRI. *J Magn Reson*  
462 *Imaging*. DOI: 10.1002/jmri.25031.

- 463 [9] Van der Linden A, Verhoye M, Pörtner HO, Bock C (2004) The strengths of in-  
464 vivo Magnetic Resonance Imaging (MRI) to study environmental adaptational  
465 physiology in fish. *MAGMA* 17: 236-248.
- 466 [10] Chatham JC, Blackband SJ (2008) Nuclear magnetic resonance spectroscopy  
467 and imaging in animal research. *ILAR J* 42: 189-208.
- 468 [11] DeVries AL, Eastman JT (1978) Lipid sacs as a buoyancy adaptation in an  
469 Antarctic fish. *Nature* 271: 352-353.
- 470 [12] Eastman JT (1988a) Ocular morphology in antarctic notothenioid fishes. *J*  
471 *Morphol* 196:283-306.
- 472 [13] Johnston IA, Camm J-P, White M (1988) Specialisations of swimming muscles  
473 in the pelagic Antarctic fish *Pleuragramma antarcticum*. *Mar Biol* 100: 3-12.
- 474 [14] Eastman JT, DeVries AL (1982) Buoyancy studies of notothenioid fishes in  
475 McMurdo Sound, Antarctica. *Copeia* 2: 385-393.
- 476 [15] Eastman JT (1985) The evolution of neutrally buoyant notothenioid fishes:  
477 Their specialization and potential interactions in the Antarctic marine food web. In:  
478 Siegfried WR, Condy PR, Laws RM, editors. *Antarctic Nutrient Cycles and Food Webs*.  
479 Springer Berlin Heidelberg. Pp. 430-436.
- 480 [16] Eastman JT (1997) Phyletic divergence and specialization for pelagic life in the  
481 Antarctic nototheniid fish *Pleuragramma antarcticum*. *Comp Biochem Physiol* 118A:  
482 1095-1101.
- 483 [17] Eastman JT (1988b) Lipid storage systems and the biology of two neutrally  
484 buoyant Antarctic Notothenioid fishes. *Comp Biochem Physiol* 90B: 529-537.
- 485 [18] Maes J, Van de Putte A, Hecq JH, Volckaert FAM (2006) State-dependent  
486 energy allocation in the pelagic Antarctic silverfish *Pleuragramma antarcticum*:

- 487 trade-off between winter reserves and buoyancy. *Mar Ecol Prog Ser* 326: 269-282.
- 488 [19] Knust R, Gerdes D, Mintenbeck K, editors (2012) The Expedition ANTARKTIS  
489 XXVII/3 CAMBIO) of RV "Polarstern" in 2011. *Rep Polar Mar Res* 644. 200pp.
- 490 [20] Brix O, Apablaza P, Baker A, Taxt T, Grüner R (2009). Chemical shift based MR  
491 imaging and gas chromatography for quantification and localization of fat in Atlantic  
492 mackerel. *J Exp Mar Biol Ecol* 2009;376(2):68–75.
- 493 [21] Henkelman RM, Hardy PA, Bishop JE, Poon CS, Plewes DB (1992). Why fat is  
494 bright in RARE and fast Spin-Echo imaging. *J Magn Reson Imag* 2: 533-540.
- 495 [22] Machann J, Stefan N, Schabel C, Schleicher E, Fritsche A, Würslin C, Häring H-  
496 U, Claussen CD, Schick F (2012) Fraction of unsaturated fatty acids in visceral adipose  
497 tissue (VAT) is lower in subjects with high total VAT volume – a combined 1H MRS  
498 and volumetric MRI study in male subjects. *NMR Biomed* 26: 232-236.
- 499 [23] Mintenbeck K, Brey T, Jacob U, Knust R, Struck U (2008) How to account for  
500 the lipid effect on carbon stable-isotope ratio ( $\delta^{13}C$ ): sample treatment effects and  
501 model bias. *J Fish Biol* 72: 815-830.
- 502 [24] Ye Q, Danzer CF, Fuchs A, Vats D, Wolfrum C, Rudin M (2012) Longitudinal  
503 evaluation of hepatic lipid deposition and composition in ob/ob and ob/+ control  
504 mice. *NMR Biomed* 29: 1079-1088.
- 505 [25] Lee Y, Jee H-J, Noh H, Kang G-H, Park J, Cho J, Cho J-H, Ahn S, Lee C, Kim O-H,  
506 Oh B-C, Kim H (2013) In vivo 1H-MRS hepatic lipid profiling in nonalcoholic fatty liver  
507 disease: An animal study at 9.4 T. *Magn Reson Med* 70: 620-629.
- 508 [26] Berglund J, Ahlström H, Kullberg J (2012) Model-based mapping of fat  
509 unsaturation and chain length by Chemical Shift Imaging – phantom validation and in  
510 vivo feasibility. *Magn Reson Med* 68: 1815-1827.

- 511 [27] Gruetter R (1993). Automatic, localized *in vivo* adjustment of all first and  
512 second-order shim coils. *Magn Reson Med* 29:804-811.
- 513 [28] Petterson P, Månsson S (2013) Simultaneous quantification of fat content and  
514 fatty acid composition using MR imaging. *Magn Reson Med* 69: 688-697.
- 515 [29] Dalpado P, Gjørseter J (1988) Feeding ecology of the lanternfish *Benthosema*  
516 *pterotum* from the Indian Ocean. *Mar Biol* 99: 555-567.
- 517 [30] Lloret J, Gil de Sola L, Souplet A, Galzin R (2002) Effects of large-scale habitat  
518 variability on condition of demersal exploited fish in the north-western  
519 Mediterranean. *ICES J Mar Sci* 59: 1215-1227.
- 520 [31] Toussaint C, Fauconneau B, Médale F, Collewet G, Akoka S, Haffray P, Davenel  
521 A (2005) Description of the heterogeneity of lipid distribution in the flesh of brown  
522 trout (*Salmo trutta*) by MR imaging. *Aquaculture* 243: 255-267.
- 523 [32] Picaud J, Collewet G,\*, Idier J. (2016). Quantification of mass fat fraction in  
524 fish using water-fat separation MRI. *Magn Reson Imag* 34: 44-50.
- 525 [33] Herbinge CM, Friars GW (1991) Correlation between condition factor and  
526 total lipid content in Atlantic Salmon, *Salmo salar* L., parr. *Aquacult Fish Manage* 22:  
527 527-529.
- 528 [34] Friedrich C., Hagen W. (1994). Lipid contents of five species of notothenioid  
529 fish from High-Antarctic waters and ecological implications. *Polar Biol* 14: 359–369.
- 530 [35] Hagen W, Kattner G, (2013). The role of lipids in the Antarctic silverfish  
531 *Pleuragramma antarcticum*. Book of abstracts; XIth SCAR biology symposium. 15-  
532 19.07.13, Barcelona, Spain, P-2.10: 224.
- 533 [36] Mayzaud P, Chevallier J, Tavernier E, Moteki M, Koubbi P (2011) Lipid  
534 composition of the Antarctic fish *Pleuragramma antarcticum*. Influence of age class.

- 535 Polar Sci 5: 264-271.
- 536 [37] Reinhardt SB, van Vleet ES (1985) Lipid composition of Antarctic midwater  
537 fish. *Ant J US* 19:144-145.
- 538 [38] Giraldo C, Mayzaud P, Tavernier E, Irisson J-O, Penot F, Becciu J, Chartier A,  
539 Boutoute M, Koubbi P (2013) Lipid components as a measure of nutritional condition  
540 in fish larvae (*Pleuragramma antarcticum*) in East Antarctica. *Mar Biol* 160: 877-887.
- 541 [39] Kamler E, Krasicka B, Rakusa-Suszczewski S (2001) Comparison of lipid content  
542 and fatty acid composition in muscle and liver of two notothenioid fishes from  
543 Admiralty Bay (Antarctica): an eco-physiological perspective. *Polar Biol* 24:735-743.
- 544 [40] Lea M-A, Nichols PD, Wilson G (2002) Fatty acid composition of lipid-rich  
545 myctophids and mackerel icefish (*Champsocephalus gunnari*) - Southern Ocean  
546 food-web implications. *Polar Biol* 25: 843-845.
- 547 [41] Phleger CF, Nelson MM, Mooney BD, Nichols PD (1999) Wax esters versus  
548 triacylglycerols in myctophid fishes from the Southern Ocean. *Antarct Sci* 11: 436-  
549 444.
- 550 [42] Iverson S J, Field C, Bowen WD, Blanchard W (2004) Quantitative fatty acid  
551 signature analysis: A new method of estimating predator diets. *Ecol Monogr* 74: 211-  
552 235.
- 553 [43] Nyssen F, Brey T, Dauby P, Graeve M (2005) Enhanced analysis of trophic  
554 position of Antarctic amphipods revealed by a 2-dimensional biomarker assay. *Mar*  
555 *Ecol Prog Ser* 300: 135-145.
- 556 [44] Alfaro AC, Thomas F, Sergent L, Duxbury M (2006) Identification of trophic  
557 interactions within an estuarine food web (northern New Zealand) using fatty acid  
558 biomarkers and stable isotopes. *Estuar Coast Shelf Sci* 70: 271-286.



- 559 [45] Hubold G, Hagen W (1997) Seasonality of feeding and lipid content of  
560 *Pleuragramma antarcticum* (Nototheniidae) in the southern Weddell Sea. In:  
561 Battaglia B, Valencia J, Walton DWH, editors. Antarctic communities: species,  
562 structure and survival. Cambridge University Press, Cambridge. Pp. 277-283.
- 563 [46] Hagen W, Kattner G, Friedrich C (2000) The lipid compositions of high-  
564 Antarctic notothenioid fish species with different life strategies. Polar Biol 23: 785-  
565 791.
- 566 [47] Weisbecker V (2012) Distortion in formalin-fixed brains: using geometric  
567 morphometrics to quantify the worst-case scenario in mice. Brain Struct Funct 217:  
568 677-685.
- 569 [48] Morris RJ (1972) The preservation of some oceanic animals for lipid analysis. J  
570 Fish Res Board Can 29: 1303-1307.
- 571 [49] Halliday N (1939) The effect of formalin fixation on liver lipids. J Biol Chem  
572 129: 65-69.
- 573 [50] Kapisris K, Miliou H, Moraitou-Apostolopoulou M (1997) Effects of  
574 formaldehyde preservation on biometrical characters, biomass and biochemical  
575 composition of *Acartia clausi* (Copepoda, Calanoida). Helgolander Meeresun 51: 95-  
576 106.
- 577 [51] Steedman HF, editor (1976) Zooplankton fixation and preservation. The  
578 Unesco Press, Paris. 350p.
- 579 [52] Danovaro R, Dell'Anno A, Martorano D, Parodi P, Marralle ND, Fabiano M  
580 (1999) Seasonal variation in the biochemical composition of deep-sea nematodes:  
581 bioenergetic and methodological considerations. Mar Ecol Prog Ser 179: 273-283.
- 582 [53] Wakeham SG, Hedges JI, Lee C, Pease TK (1993) Effects of poisons and

583 preservatives on the composition of organic matter in a sediment trap experiment. J  
584 Mar Res 51: 669-696.

585 [54] Davies KTA, Ryan A, Taggart CT (2012) Measured and inferred gross energy  
586 content in diapausing *Calanus* spp. in a Scotia shelf basin. J Plankton Res 34: 614-  
587 625.

588 [55] Heulett ST, Weeks SC, Meffe GK (1995) Lipid dynamics and growth relative to  
589 resource level in juvenile Eastern Mosquitofish (*Gambusia holbrooki*: Poeciliidae).  
590 Copeia 1: 97-104.

591

592

593

594

595 **Figure legends:**

596 **Figure 1:** Example of a high-resolution morphological MRI scan obtained from a formalin preserved sample of  
597 *Pleuragramma* using MDEFT. The figure shows a sagittal section (A) and a transversal (cross) section (B) of an  
598 individual analyzed in this study in comparison to (C) a histological transversal section of *Pleuragramma* from  
599 Eastman & DeVries [14] (with kind permission from Copeia and the American Society of Ichthyologists and  
600 Herpetologists, ASIH). B, brain; IAT, intermuscular adipose tissue; ILS, intermuscular lipid sacs (dorsal and  
601 ventral); Lv, liver; M, muscle; N, notochord; NV, notochordal vesicles; RMLS, red muscle fibres of the lateralis  
602 superficialis (according to Eastman & DeVries [14]); S, stomach; SLS, subcutaneous lipid sacs; DFM, dorsal fin  
603 muscles and AFM, anal fin muscles according to Johnston et al. [13].

604

605 **Figure 2:** Magnification of high-resolution MRI scans (MDEFT) of the dorsal lipid sac region from two different  
606 *Pleuragramma* individuals sampled at different areas. Note the different shape of the lipid sacs between both  
607 examples (see yellow boxes).

608

609 **Figure 3:** Examples of 3D MR image sets (MDEFT) taken in the abdominal region of preserved *Pleuragramma*.  
610 Shown are a sagittal (A), a coronal (B) and a transversal section (C). On the right, the 3D surface-rendered  
611 stomach (D), as used for volume determination, is shown.

612

613 **Figure 4:** Relationship between individual body weight [g] and body volume [ml] in *Pleuragramma* (N=6).  
614 Dashed line represents the linear relationship described by  $y=0.997x$  ( $R^2=0.98$ ). Note the excellent correlation  
615 that allows a direct conversion from volume into weight.

616

617 **Figure 5:** Example of a whole body 3D MRI data set acquired with RARE of *Pleuragramma* used for the analysis  
618 of body lipid content. In (A) a sagittal, a coronal and a transversal section are shown; lipids are bright/white,  
619 other tissues are grey. In (B) an example of a transversal MRI after volume rendering is shown, illustrating the  
620 muscle/water (grey) and lipid (blue) distribution in *Pleuragramma*.

621

622 **Figure 6:**  $^1\text{H}$ -NMR spectra measured in the dorsal intermuscular lipid sacs (ILS) of two different individual fish  
623 from two sampling locations (Stations A and B). The ROI (volume from where the spectrum was acquired) are  
624 marked by the red square. Both NMR spectra display a “typical” triacylglyceride/lipid pattern, but show clear  
625 differences in specific signal intensities.

Figure 1  
[Click here to download high resolution image](#)

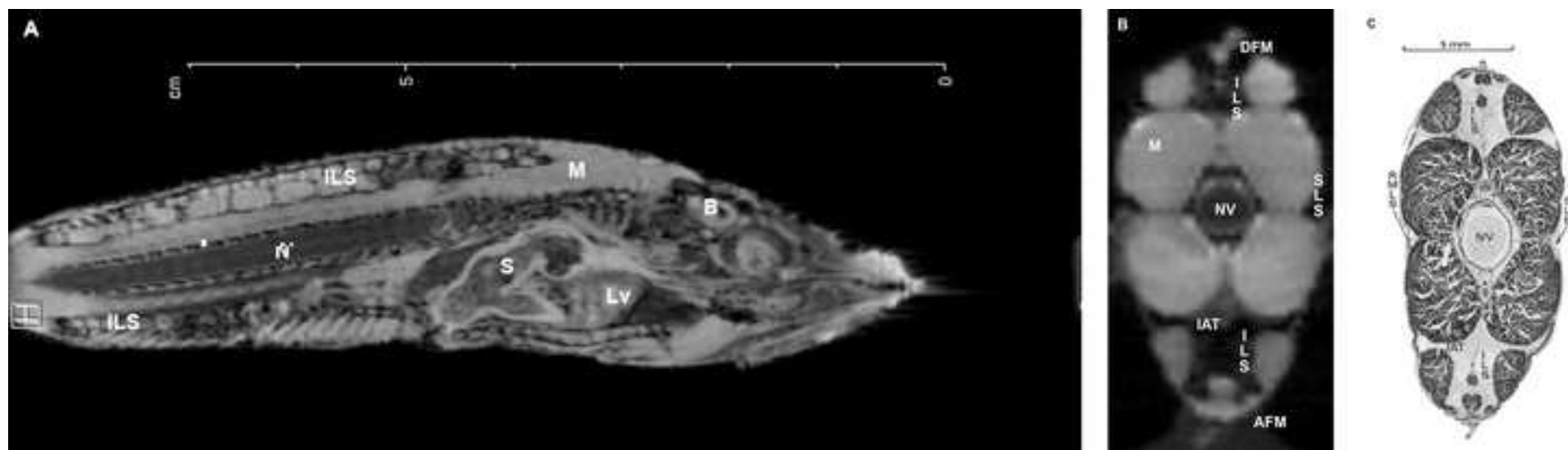
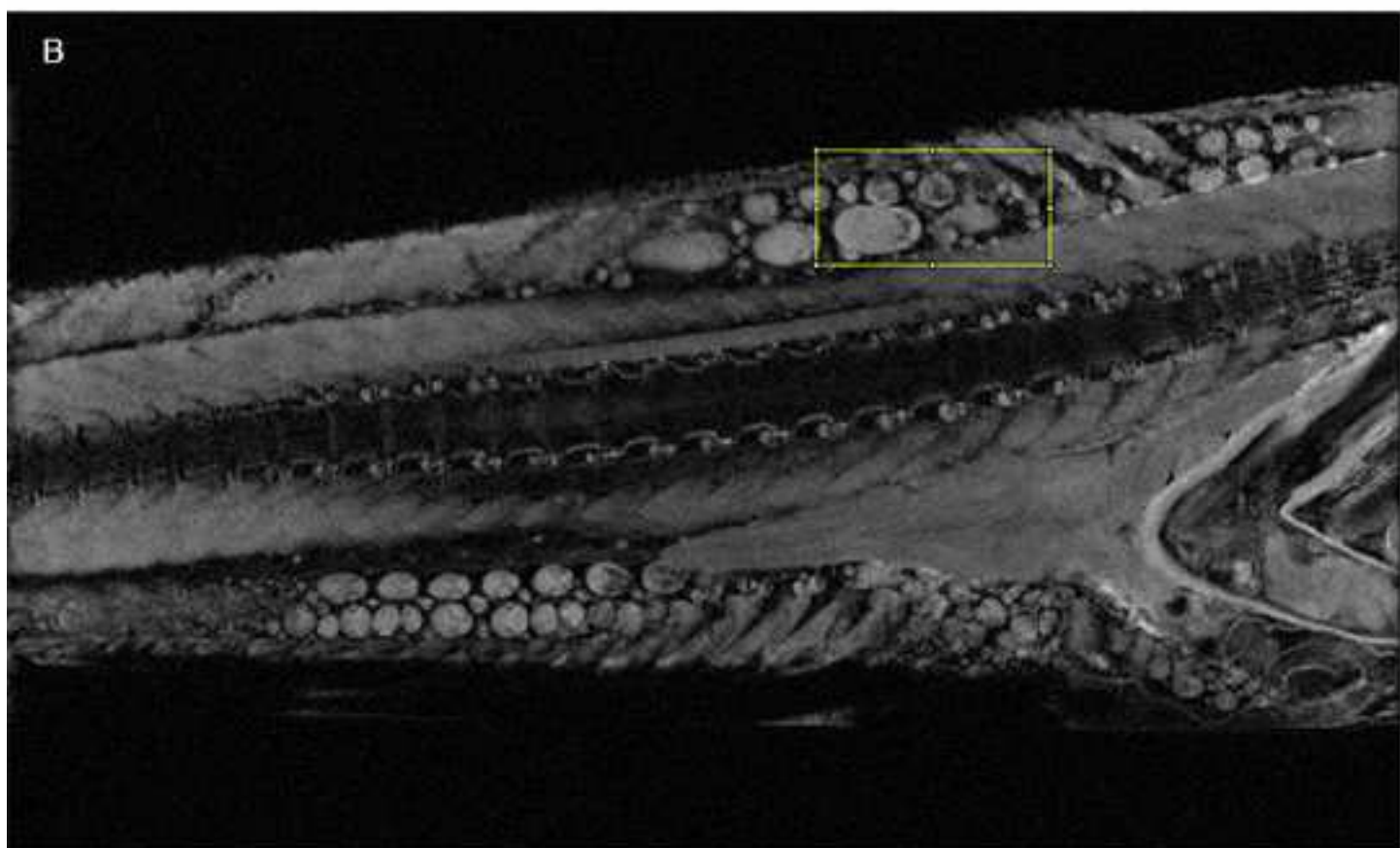
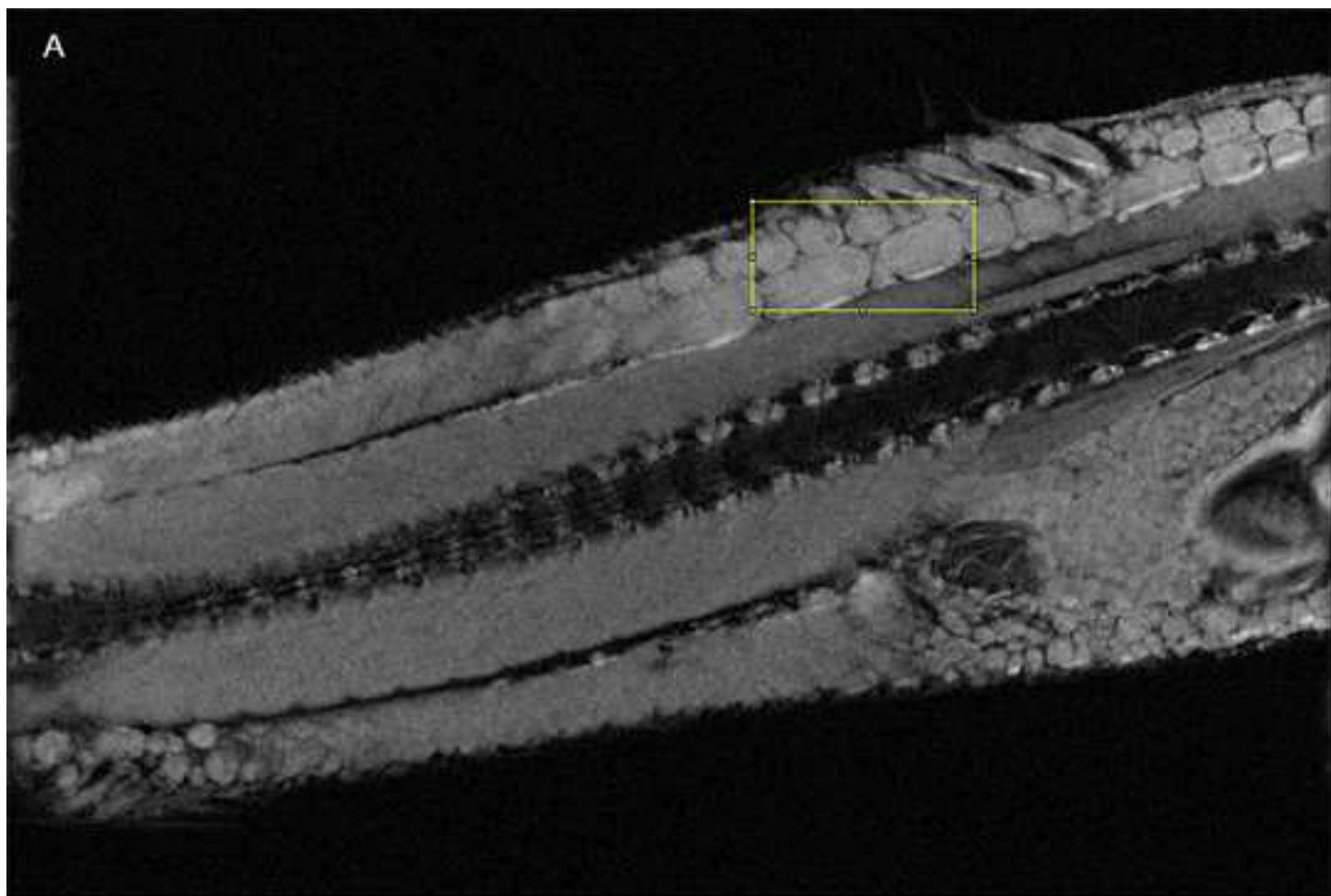


Figure 2  
[Click here to download high resolution image](#)



**Figure 3**  
[Click here to download high resolution image](#)

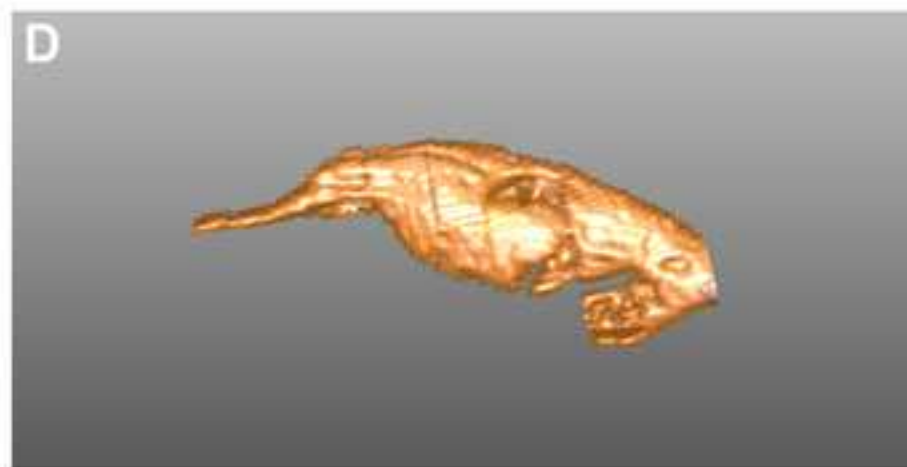
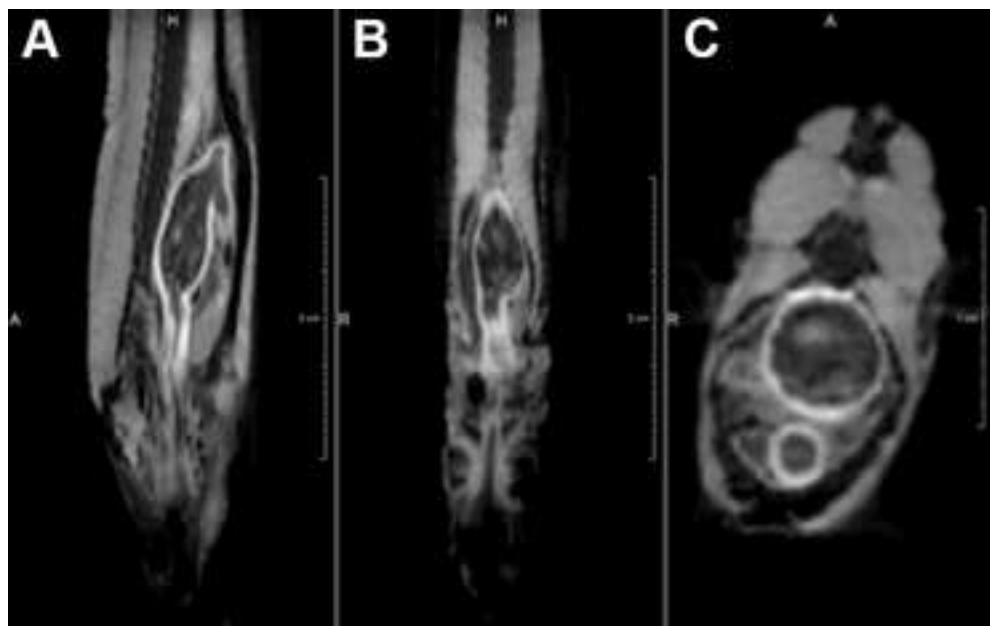
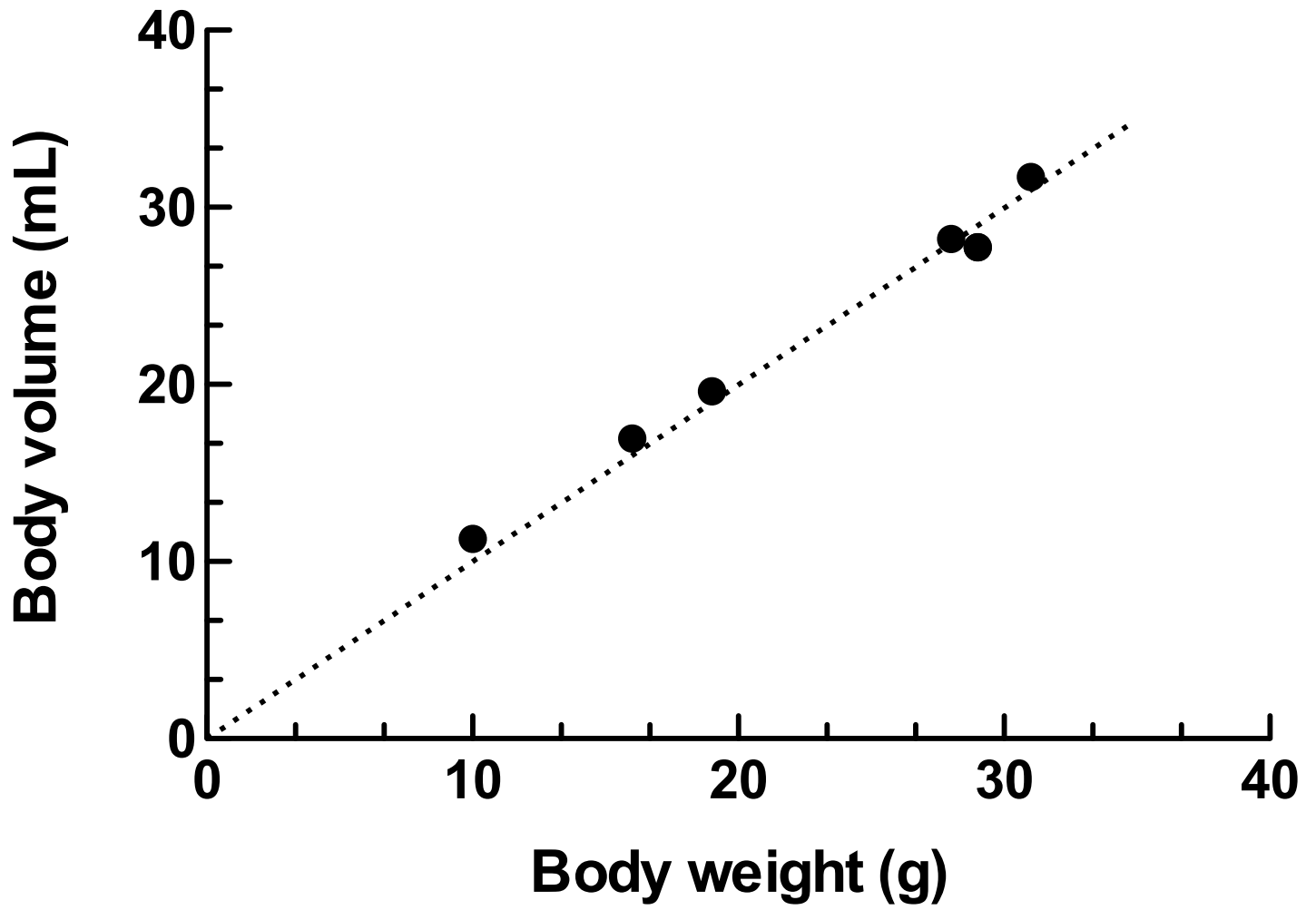


Figure 4



**Figure 5**  
[Click here to download high resolution image](#)

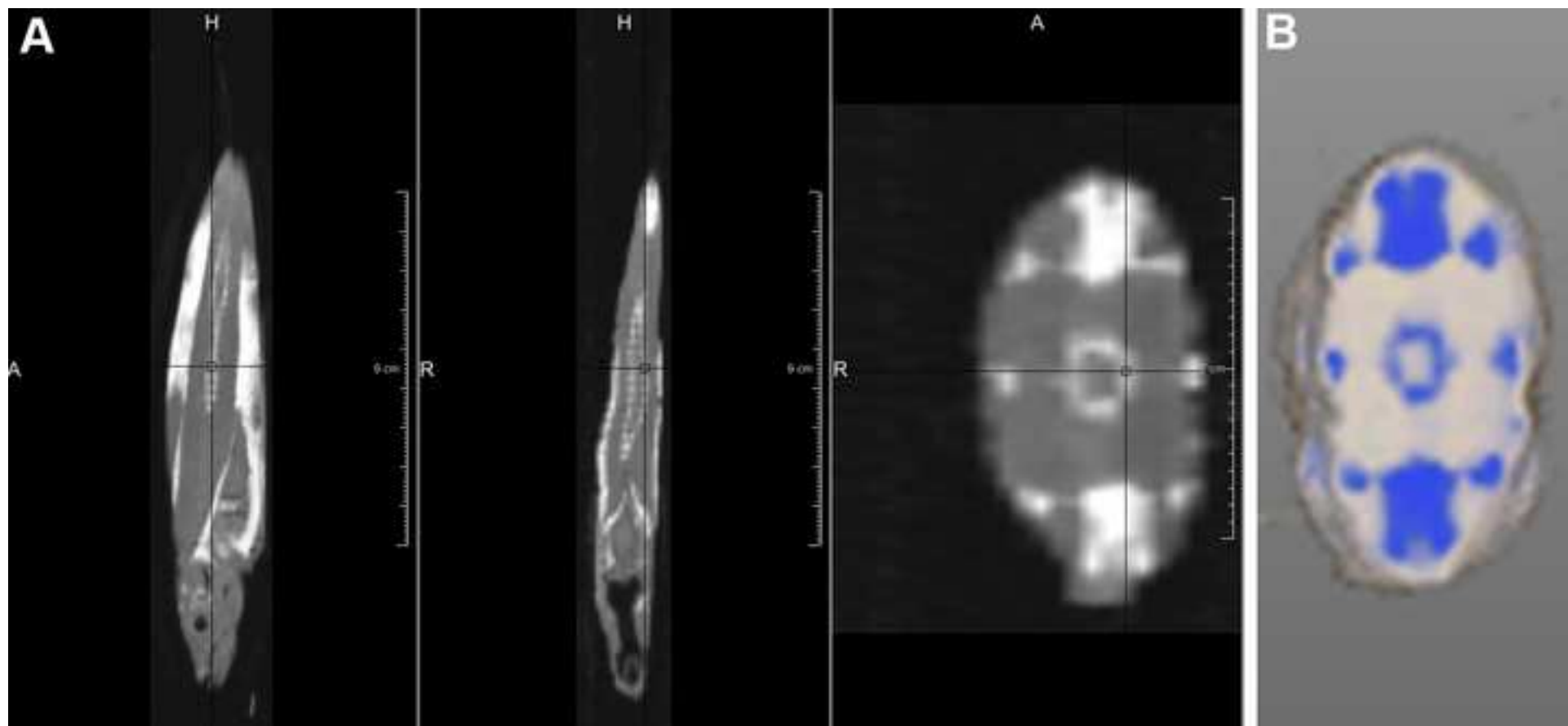
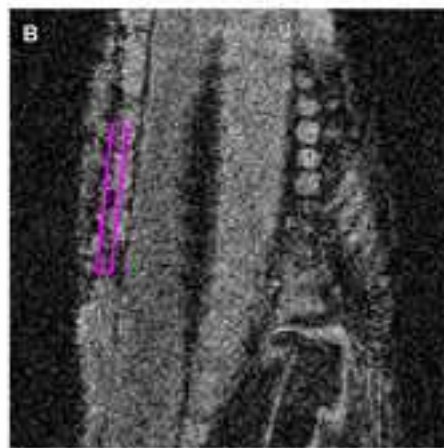
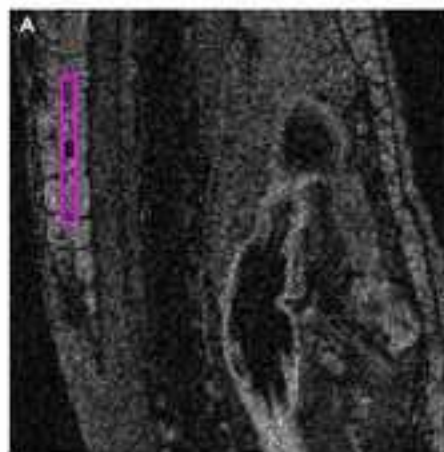
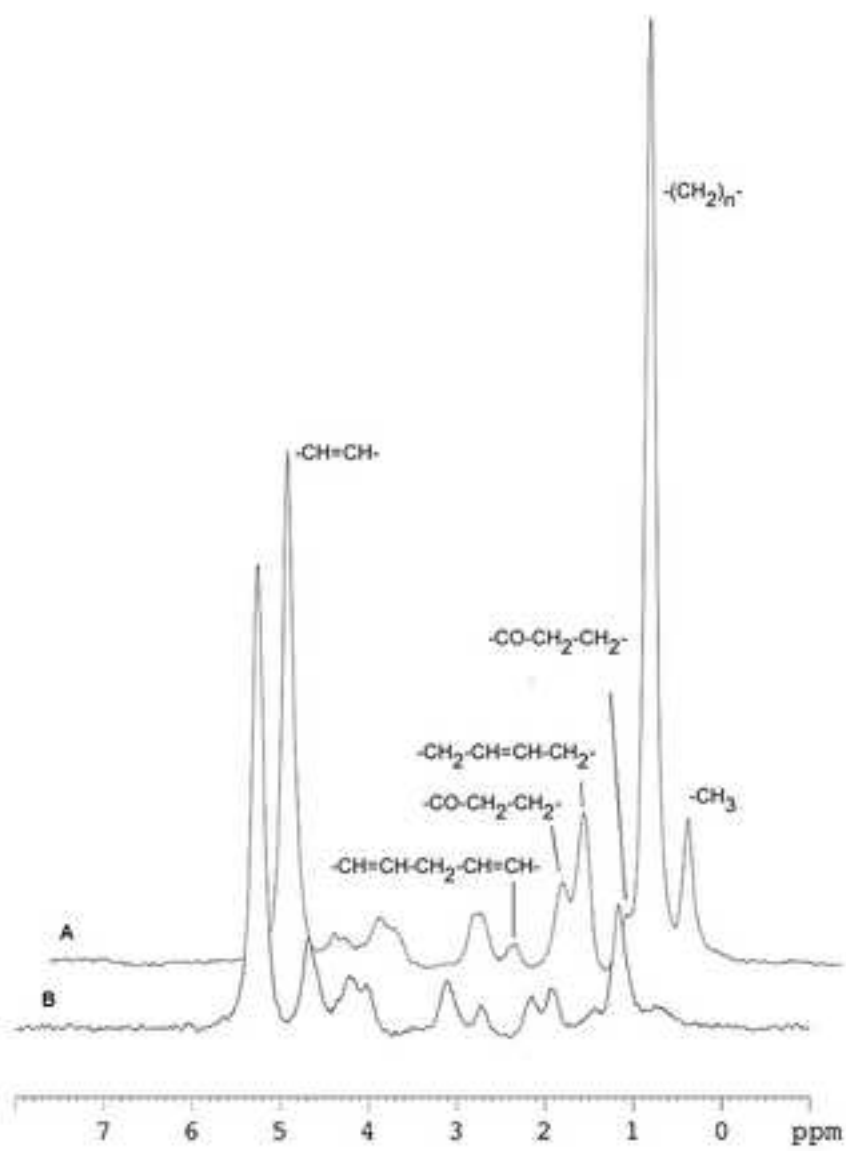




Figure 6mod

[Click here to download high resolution image](#)



**Table 1:** Mean fatty acid (FA) chain length, unsaturation degree and degree of polyunsaturation derived from localized  $^1\text{H-NMR}$  spectra measured within the intermuscular lipid sacs of preserved fish from two different sampling locations, Station A (area of the former Larsen A shelf ice) and Station B (former Larsen B shelf ice). \* significantly different from Station A,  $p < 0.05$ .

	FA chain length	Unsaturation degree	Polyunsaturation degree
Station A (n=4)	7,98±1,98	3,15±1,68	1,45±0,79
Station B (n=5)	6,06±1,14	1,21±0,79*	0,36±0,26*