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METHODS & TECHNIQUES

Non-invasive lipid measurement in living insects using NMR microscopy

Franz Schilling[1,2,*,†], Kai Dworschak[3,†,‡], Reinhard Schopf[3], Ralph Kühn[4], Steffen J. Glaser[2] and Axel Haase[1]

[1]Institute of Medical Engineering (IMETUM), Technische Universität München, Boltzmannstrasse 11, 85748 Garching, Germany, [2]Department of Chemistry, Technische Universität München, Lichtenbergstrasse 4, 85748 Garching, Germany, [3]Animal Ecology, Department of Ecology and Ecosystem Management, Technische Universität München, Hans-Carl-von-Carlowitz-Platz 2, 85354 Freising, Germany and [4]Molecular Zoology, Department of Animal Science, Technische Universität München, Hans-Carl-von-Carlowitz-Platz 2, 85354 Freising, Germany

*Author for correspondence (fschilling@tum.de)
†These authors contributed equally to this work
‡Current address: Ecological Networks, Department of Biology, Technische Universität Darmstadt, Schnittspahnstrasse 3, 64287 Darmstadt, Germany

SUMMARY

Nuclear magnetic resonance (NMR) microscopy allows us to image and quantify the distribution of NMR-active nuclei in living specimens. Using high-field NMR microscopy at a magnetic field strength of 14.1 T and strong gradients up to 3 T m^{-1}, we show that separation of fat and water nuclear resonances in living insects can be achieved. In contrast to destructive conventional photometric and mass measurements, we demonstrate exemplarily in the European spruce bark beetle that NMR can be efficiently used to quantify absolute fat and water content in living insects. Additionally, anatomic images with a spatial in-plane resolution up to 10 μm and with high soft tissue contrast were acquired. We demonstrate that fat distribution and fat consumption of living insects can be obtained by magnetic resonance imaging (MRI). This enables future research to address questions where single individuals have to be measured several times, which is not possible with conventional destructive methods.

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INTRODUCTION

Lipids in the form of triglycerides, which are stored in the diffuse fat body, are the main energy reserves in most insects (Arrese and Soulages, 2010). These reserves are crucial for processes such as insect flight (Nespolo et al., 2008; Williams and Robertson, 2008) or the survival success of overwintering insects (Hahn and Denlinger, 2011). Lipid reserves are regularly used as fitness parameters for animals (Anderbrant and Schlyter, 1989; May, 1992; Peig and Green, 2009). Classic lipid measurement methods such as chromatography and vanillin or ferric perchlorate assays require chemical extraction and thus the killing of animals (Williams and Robertson, 2008). This fact disables researchers to test fluctuations in the amount of lipid(s) in the same individuals with changing conditions over time. For this purpose, only indirect measures such as correlations with body mass adjusted for size or measuring reference individuals from the same population remain. These indirect methods are much more inaccurate and in many cases may be inappropriate (Green, 2001; Peig and Green, 2010).

In recent years, zoology has adopted non-invasive methods widely used in human medicine. Anatomy of fossil amber arthropods was reconstructed and visualized using computed tomography (Dunlop et al., 2011; Pohl et al., 2010), morphology of an octopus was studied using ultrasound (Margheri et al., 2011) and quantitative nuclear magnetic resonance (NMR) was used to quantitatively measure the body composition of small rodents (Nixon et al., 2010; Tinsley et al., 2004). NMR is a non-destructive and non-invasive technique used to analyse and study the internal morphology of living specimens (Callaghan, 1992). In every living animal, there are many water protons whose NMR-specific parameters, such as the proton spin density, chemical shift, and T1 and T2 relaxation times, can be spatially resolved by magnetic resonance imaging (MRI) (Kuhn, 1990). With the advent of high magnetic field strengths and strong magnetic gradients, spatial resolution of up to one micrometer resolution is achievable (Lee et al., 2001). The first NMR microscopy images were obtained by Aguayo et al. in 1986, when they studied ova from the toad *Xenopus laevis* (Aguayo et al., 1986). Since then, a new dimension of investigating animals opened up and many different species have been characterized by NMR microscopy: e.g. the development of a locust embryo (Gassner and Lohman, 1987), pH metabolism of living insects (Skibbe et al., 1995), development and metamorphosis of lepidopteran pupae (Behr et al., 2011; Goodman et al., 1995), metamorphosis of the silkworm (Mapelli et al., 1997) and the morphology of diving beetles (Wecker et al., 2002) and bees (Haddad et al., 2004; Tomanek et al., 1996). However, because of the high cost of MRI machines, only few zoological studies have been performed. Given its unique properties, 'MRI could in fact be used more widely in zoology' (Ziegler et al., 2011).

In this study we used one of the main features of NMR, namely, that different chemical environments can be separated due to their different chemical shift. As model organism we chose the

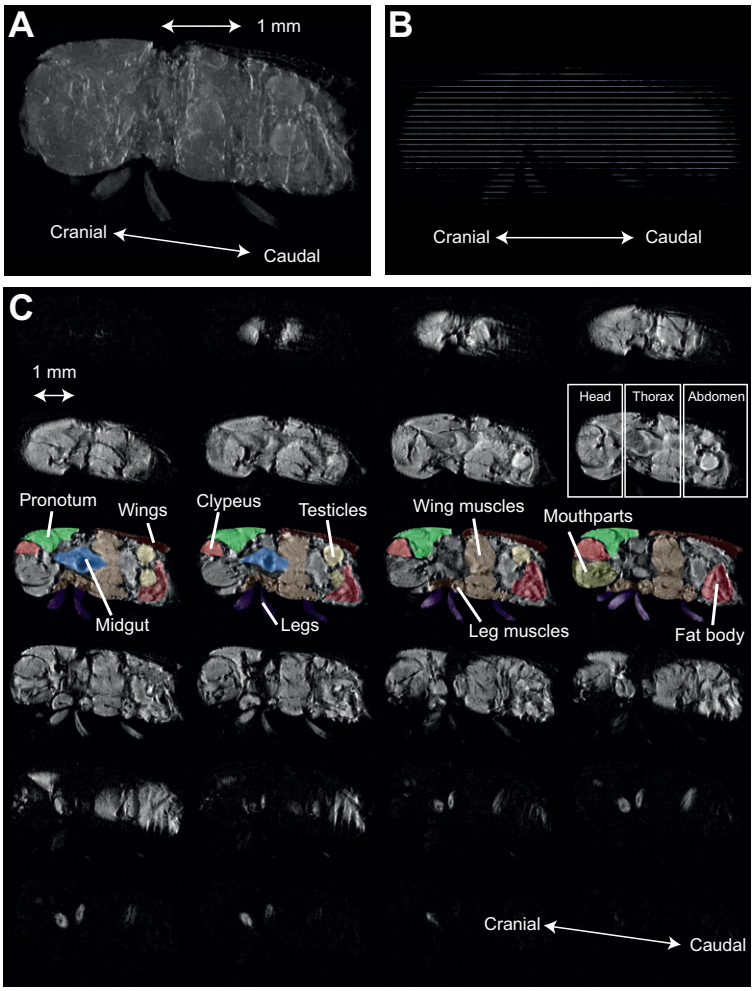

Fig. 1. (A) Maximum-intensity projection of all slices of the bark beetle *Ips typographus*. (B) Orientation of the reconstructed oblique coronal slices. (C) Oblique coronal slices. Several internal structures and organs can be identified: mouthparts, testicles, mid-gut, wing and leg muscles, and legs.

European spruce bark beetle, *Ips typographus* L. (Coleoptera: Curculionidae: Scolytinae). This beetle is 4 to 6 mm long and has a fresh mass of 10 to 14 mg. We show that NMR microscopy can be used to quantify and visualize fat and water distribution in small living insects.

MATERIALS AND METHODS

Animal handling

Freshly emerged *I. typographus* individuals of approximately 5 mm length and 2 mm width were investigated. Beetles originated from the 29th generation of a laboratory rearing. To immobilize beetles, they were cooled to approximately 2°C prior to the experiments. Before transferring the beetles to the NMR microimaging system they were fixed mechanically in their position in a 5 mm Shigemi tube (Shigemi, Allison Park, PA, USA) by glass rods from the top and the bottom. Temperature inside the tube containing the beetle was adjusted to 2°C with a constant nitrogen flow around the glass tube. NMR spectral signal intensities of fat and water of 10 different beetles were correlated to water and lipid amount obtained by conventional measurements (see below).

In addition to NMR spectroscopy, two beetles were examined by NMR microscopy before and after tethered flight. Cooled beetles were warmed up to room temperature after their initial NMR measurement and attached to flight mills for approximately 20 h. Flight mills consisted of a vertical pivot with a rotary arm attached perpendicularly to it. The pivot consisted of a glass microcapillary. To minimize friction, we attached the tips of insect needles to each end of the capillary. The rotary arm was made of a gas chromatography column and its radius was 10.5 cm. Hence, one rotation of the arm corresponded to a flight distance of 0.66 m. Each full rotation of the arm triggered a photo sensor and was recorded using DIAdem version 10.0 (National Instruments, Austin, TX, USA, 2005).

NMR methods

The beetles were analyzed in a 14.1 T NMR microscopy system (BrukerBioSpin, Rheinstetten, Germany) with gradient strengths up to 3 T m^{-1}. Magnetic field inhomogeneity was improved by manual shimming up to second order. One-dimensional (1-D) proton spectra of the whole insect without any spatial encoding were acquired with a standard pulse-acquire NMR experiment within 16 averages and a repetition time of 4 s.

In addition to spectroscopy, multi-slice spin echo images with an in-plane resolution of 31×31 μm and a slice thickness of 150 μm were acquired. The repetition time was set to T_R=1000 ms, the echo time was set to T_E=9.4 ms, we used a field-of-view (FOV) of 0.8×0.8 cm, the matrix had a size of 256×256 and the overall acquisition time was t_{acq}=4 min 16 s. Optionally, fat saturation using a 90 deg Gaussian pulse at 4.0 p.p.m. offset with respect to the proton resonance (bandwidth=3.5 p.p.m.) was performed before image acquisition. Transverse magnetization was dephased by a spoiler gradient. Before and after flight of the beetles, images with and without fat saturation were taken. Then, the difference of the two pictures yielded the fat distribution. Maps of fat distribution were

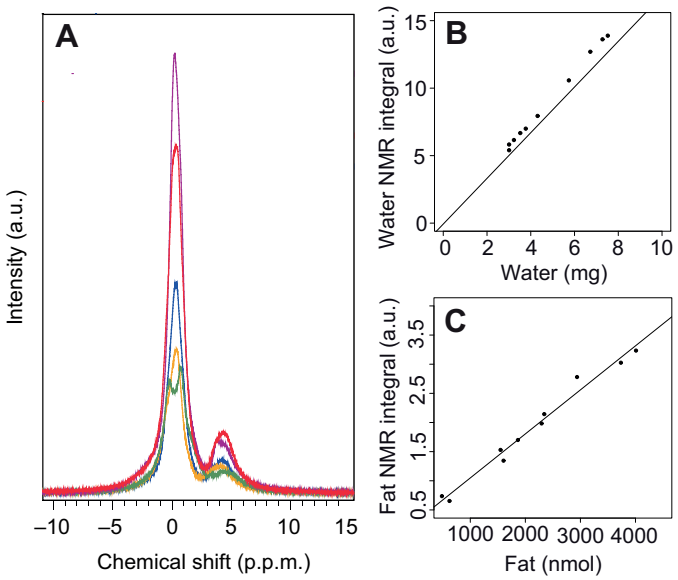

Fig. 2. (A) Proton nuclear magnetic resonance (NMR) spectra of five bark beetles (indicated by different colors) showing a clear separation between the fat peak (0 p.p.m.) and the water peak (4.7 p.p.m.). The spectra were acquired using a conventional pulse-acquire 1-D NMR experiment with 16 scans. (B) Correlation between NMR and conventional water measurement, R^2>0.99. (C) Correlation between NMR and conventional fat measurement, R^2>0.99.

calculated with ImageJ (Abràmoff et al., 2004). The acquisition time for the two images was 8 min 32 s. To identify the spatial distribution of fat more clearly, pictures with a transparent-zero projection (Abràmoff et al., 2004) were produced.

High-resolution three-dimensional imaging with an in-plane resolution of 12×12 µm and a slice thickness of 120 µm of the bark beetles was performed to study the insects' morphology. The repetition time was set to T_R=1000 ms, the echo time was set to T_E=7.3 ms, we used an FOV of 0.6×0.6 cm, the matrix had a size of 512×512 and the overall acquisition time was t_{acq}=7 h 7 min.

Conventional water and lipid measurements

We determined the water content of the beetles by calculating the difference between their fresh and dry mass. Therefore, beetles were dried at 60°C for 24 h.

For conventional destructive fat measurement, beetles were killed in a freezer at –20°C. They were then cut into three pieces, making incisions between the head and the thorax and between the thorax and the abdomen. Fatty acid esters were extracted from the dissected beetles three times in 1 ml chloroform at 30°C in an ultrasonic bath. The chloroform samples were dried overnight at 65°C. Fat reserves were measured photometrically as fatty acid ester equivalents based on the method of Snyder and Stephens (Snyder and Stephens, 1959), modified after Kraußе-Opatz et al. (Kraußе-Opatz et al., 1995). This measurement is based on a hydroxylaminolysis, in which an ester group forms a hydroxamic acid when reacting with alkaline hydroxylamine. After addition of acid ferric perchlorate, the hydroxamic acid forms a purple iron–chelate complex. This can be measured photometrically at 530 nm. A standard calibration curve for fatty acid esters was calculated based on 10 samples of methyl oleate (Sigma-Aldrich, St Louis, MO, USA) dissolved in chloroform in concentrations between 0.5 and 5.0 µmol in 0.5 µmol steps (Pearson's product-moment correlation: R^2>0.99, t=41.50, d.f.=8, P<0.0001, N=10).

RESULTS AND DISCUSSION

Anatomy

Using high-resolution spin-echo imaging, the anatomy of living spruce bark beetle individuals was analyzed. In the acquired oblique coronal slices through the beetle's body, the different body segments

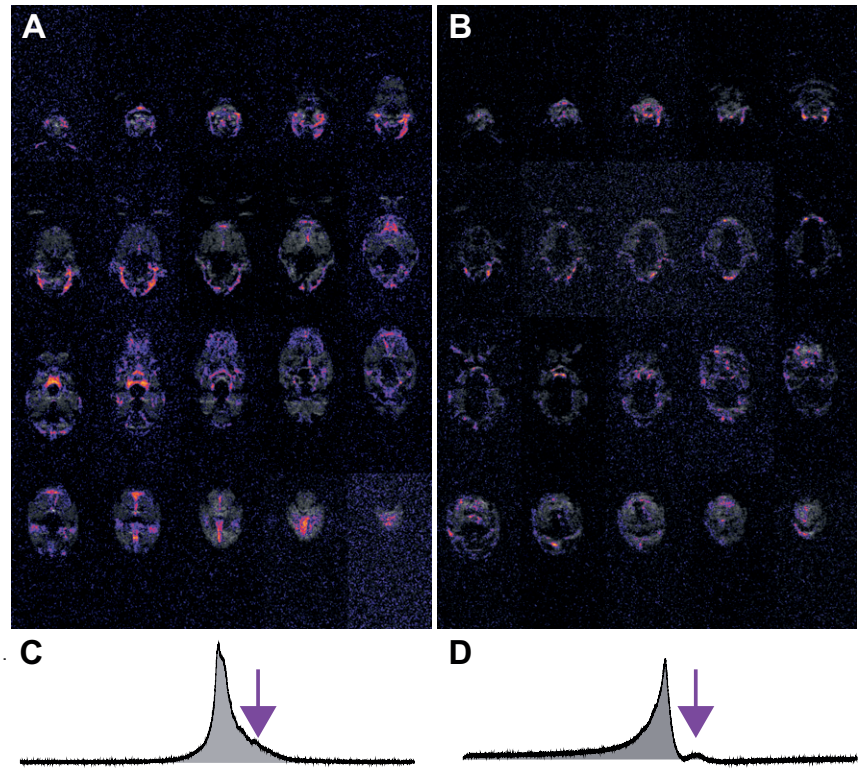

Fig. 3. (A) Oblique sagittal slices of fat distribution (darker color resembles lower fat amount and brighter color resembles higher fat amount) and proton density (grey tones) before flight in *Ips typographus*. (B) Oblique sagittal slices after flight. (C) NMR spectrum before flight. (D) NMR spectrum after flight. The purple arrows indicate the fat saturation pulse used to obtain spectral information in the images. Quantitative analysis is shown in Table 1 (beetle no. 1).

Table 1. Covered flight distances and absolute amounts of fat and water content and consumption of two different bark beetles measured by NMR spectroscopy before and after flight

	Fat (nmol)			Water (mg)			
Beetle no.	Before flight	After flight	Total fat consumption (nmol)	Before flight	After flight	Total water loss (mg)	Flight distance (km)
1	1519.55	1137.64	381.91	3.64	2.89	0.74	0.34
2	1688.17	1312.71	375.46	4.92	4.13	0.79	13.45

such as the head including mouthparts, legs and wings and their respective muscles and intestinal (Baker and Estrin, 1974; Díaz et al., 2003) and genital organs (Calder, 1990) have been identified (Fig. 1, see also supplementary material Movie 1). Susceptibility artifacts were greatly reduced by using Shigemi tubes. In addition, the Shigemi tube allowed us to fix the beetle in position and eliminate movement artifacts caused by gradient vibrations. Cooling beetles to 2°C erased all motion artifacts during the measurement without harming them.

Spectroscopy

Fat and water content of living bark beetles were investigated by conventional NMR techniques. ^{1}H-NMR spectra of bark beetles showed a clear chemical shift separation between fat (4.7 p.p.m.) and water (0 p.p.m.), which arises from the different electronic environment of fat and water protons (Fig. 2A). The NMR peak integrals of fat and water of 10 beetles significantly correlated with the data obtained by the destructive standard detection method (Pearson's product-moment correlation: water, R^2>0.99, t=66.04, d.f.=8, P<0.0001, N=10; fat, R^2>0.99, t=19.99, d.f.=8, P<0.0001, N=10; Fig. 2B,C). Such a set of independent measurements can serve as a calibration of NMR integrals on every NMR spectrometer, relating the dimensionless NMR peak integrals to absolute standard units of fat (nmol) and water (mg) content.

Fat and water content of small insects can therefore be quantitatively analyzed by NMR. In contrast to traditional methods, this technique opens up the possibility to measure one specimen repeatedly, which we demonstrated by determining fat content of two bark beetles before and after tethered flight (Fig. 3C,D, supplementary material Fig. S1C,D).

Relative fat distribution

To image the relative spatial distribution of fat in bark beetles, fat saturation was performed before a spin-echo imaging sequence. An image overlay of anatomy images (grey) with the corresponding areas of relative fat distribution (purple) shows where the bark beetles store their energy reserves and where they are used up after flight (Fig. 3A,B, supplementary material Fig. S1A,B). In contrast to standard spectroscopic 1-D methods, signal intensity was not correlated to standard units of fat and water because signal intensities are more prone to errors. Therefore we obtained a relative spatial fat distribution. In addition, absolute quantification of fat and water content, as well as fat and water consumption, was performed by acquiring a standard 1-D proton NMR spectrum (Table 1, Fig. 3C,D; see NMR methods).

We identified one major fat reservoir in the abdomen of the beetle, most likely the diffuse fat body, and one in the center of the body.

Spatial information of the bark beetles has been combined with spectral information, which we can gain because of the spectral separation of fat and water signals. Within 8 min 32 s, two high-resolution images can be acquired, allowing identification of the relative fat distribution within the beetle with regard to its internal morphology before and after flight. This method non-invasively images and, in combination with spectroscopic techniques, quantifies spatial fat consumption in certain areas of interest in a specimen.

Conclusions

Here we demonstrated the potential of NMR microscopy in zoology and especially entomology to follow fat distribution across whole insects and fat consumption in different organs of interest as a function of external parameters over time. It was possible to quantify lipid reserves of living insects giving a direct measure of total stored energy. The method allows monitoring dynamic processes of lipid consumption non-invasively, as we have shown for the energy consumption of bark beetles before and after flight. This study is exemplary for any other non-invasive investigation in zoology and especially entomology, where energy consumption and its spatial distribution in living species are of interest.

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