

## METHODS & TECHNIQUES

### Non-invasive lipid measurement in living insects using NMR microscopy

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#### 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<sup>-1</sup>, 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

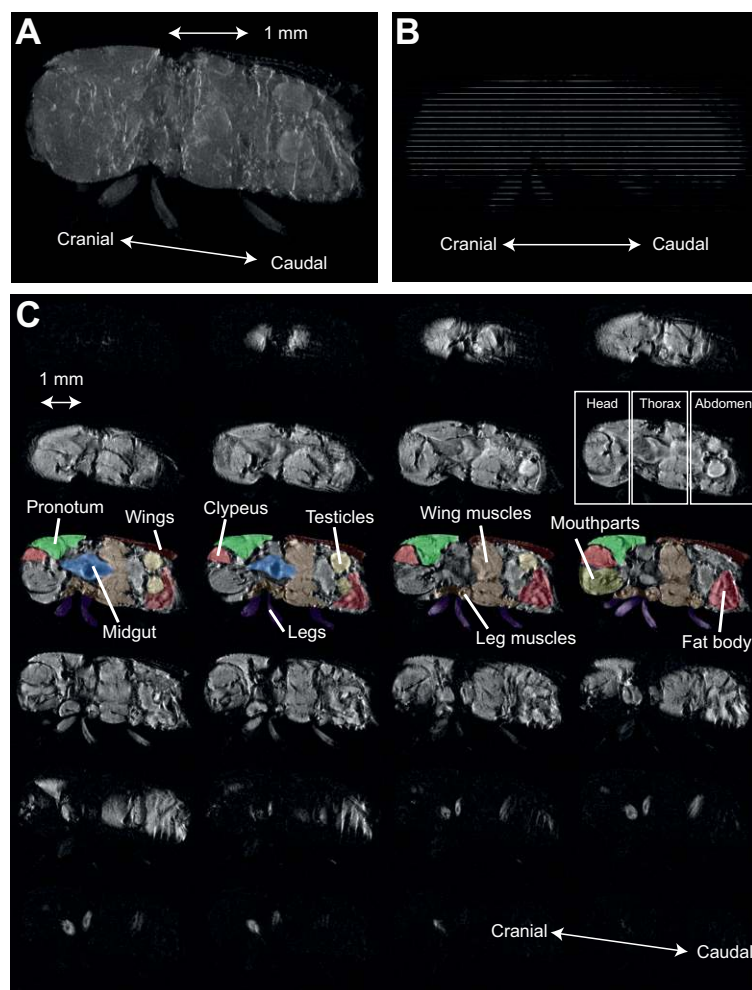


Fig. 1. (A) Maximum-intensity projection of all slices of the bark beetle *Ips typographus*. (B) Orientation of the reconstructed oblique coronal slices. (C) Oblique coronal slices. Several internal structures and organs can be identified: mouthparts, testicles, mid-gut, wing and leg muscles, and legs.

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 Shigemii tube (Shigemii, 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  $3 \text{ T m}^{-1}$ . 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  $31 \times 31 \mu\text{m}$  and a slice thickness of  $150 \mu\text{m}$  were acquired. The repetition time was set to  $T_R=1000 \text{ ms}$ , the echo time was set to  $T_E=9.4 \text{ ms}$ , we used a field-of-view (FOV) of  $0.8 \times 0.8 \text{ cm}$ , the matrix had a size of  $256 \times 256$  and the overall acquisition time was  $t_{\text{acq}}=4 \text{ min } 16 \text{ s}$ . 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

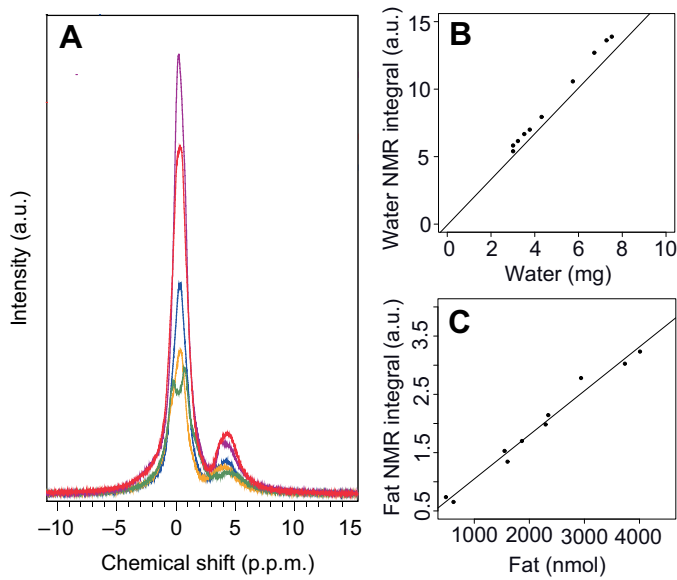


Fig. 2. (A) Proton nuclear magnetic resonance (NMR) spectra of five bark beetles (indicated by different colors) showing a clear separation between the fat peak (0 p.p.m.) and the water peak (4.7 p.p.m.). The spectra were acquired using a conventional pulse-acquire 1-D NMR experiment with 16 scans. (B) 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 (Abramoff 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 (Abramoff et al., 2004) were produced.

High-resolution three-dimensional imaging with an in-plane resolution of  $12 \times 12 \mu\text{m}$  and a slice thickness of  $120 \mu\text{m}$  of the bark

beetles was performed to study the insects' morphology. The repetition time was set to  $T_R = 1000 \text{ ms}$ , the echo time was set to  $T_E = 7.3 \text{ ms}$ , we used an FOV of  $0.6 \times 0.6 \text{ cm}$ , the matrix had a size of  $512 \times 512$  and the overall acquisition time was  $t_{\text{acq}} = 7 \text{ h } 7 \text{ min}$ .

#### 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  $60^\circ\text{C}$  for 24 h.

For conventional destructive fat measurement, beetles were killed in a freezer at  $-20^\circ\text{C}$ . 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  $30^\circ\text{C}$  in an ultrasonic bath. The chloroform samples were dried overnight at  $65^\circ\text{C}$ . Fat reserves were measured photometrically as fatty acid ester equivalents based on the method of Snyder and Stephens (Snyder and Stephens, 1959), modified after Krauß-Opatz et al. (Krauß-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  $5.0 \mu\text{mol}$  in 0.5  $\mu\text{mol}$  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

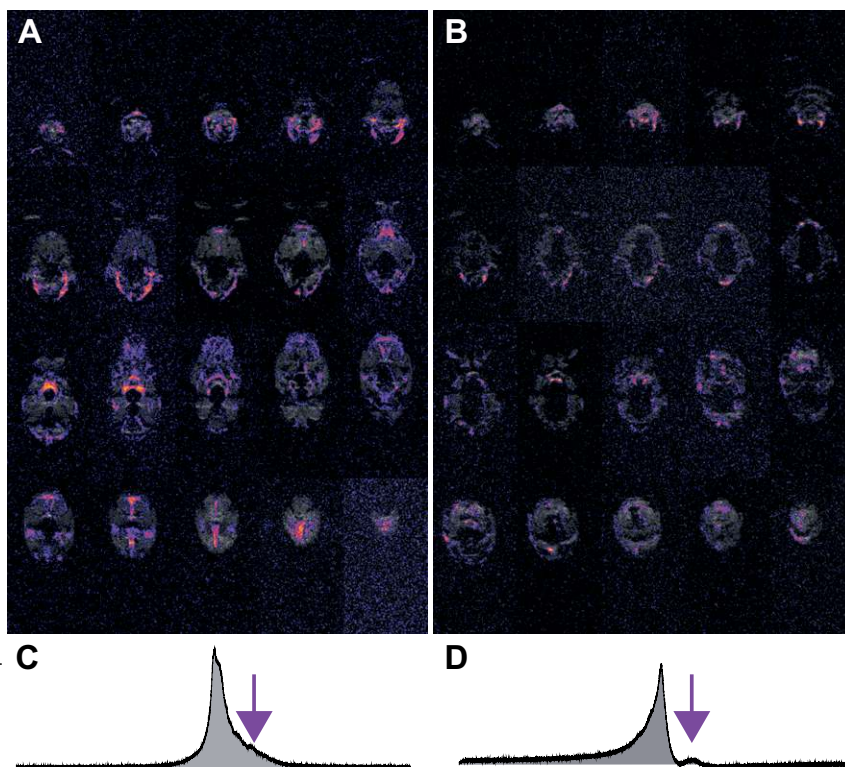


Fig. 3. (A) Oblique sagittal slices of fat distribution (darker color resembles lower fat amount and brighter color resembles higher fat amount) and proton density (grey tones) before flight in *Ips typographus*. (B) Oblique sagittal slices after flight. (C) NMR spectrum before flight. (D) NMR spectrum 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

Beetle no.	Fat (nmol)		Total fat consumption (nmol)	Water (mg)		Total water loss (mg)	Flight distance (km)
	Before flight	After flight		Before flight	After flight		
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 Shigemitsu tubes. In addition, the Shigemitsu 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. <sup>1</sup>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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### REFERENCES

- Abràmoff, M. D., Magalhaes, P. J. and Ram, S. J. (2004). Image processing with ImageJ. *Biophoton. Int.* **11**, 36-42.
- Aguayo, J. B., Blackband, S. J., Schoeniger, J., Mattingly, M. A. and Hintermann, M. (1986). Nuclear magnetic resonance imaging of a single cell. *Nature* **322**, 190-191.
- Anderbrant, O. and Schlyter, F. (1989). Causes and effects of individual quality in bark beetles. *Holarctic. Ecol.* **12**, 488-493.
- Arrese, E. L. and Soulages, J. L. (2010). Insect fat body: energy, metabolism, and regulation. *Annu. Rev. Entomol.* **55**, 207-225.
- Baker, W. V. and Estrin, C. L. (1974). The alimentary canal of *Scolytus multistriatus* (Coleoptera: Scolytidae): a histological study. *Can. Entomol.* **106**, 673-686.
- Behr, V. C., Müller, S. C. and Mahsberg, D. (2011). A morphological study of the metamorphosis of butterflies using MR-microscopy at 17.6T. *Proc. ESMRMB*, #290, Leipzig, Germany.
- Calder, A. A. (1990). Gross morphology of the soft parts of the male and female reproductive systems of Curculionioidea (Coleoptera). *J. Nat. Hist.* **24**, 453-505.
- Callaghan, P. T. (1992). *Principles of Nuclear Magnetic Resonance Microscopy*. Oxford: Oxford University Press.
- Díaz, E., Arciniega, O., Sánchez, L., Cisneros, R. and Zúñiga, G. (2003). Anatomical and histological comparison of the alimentary canal of *Dendroctonus micans*, *D. ponderosae*, *D. pseudotsugae*, *D. rufipennis*, and *D. terebrans* (Coleoptera: Scolytidae). *Ann. Entomol. Soc. Am.* **96**, 144-152.
- Dunlop, J. A., Penney, D., Dalüge, N., Jäger, P., McNeil, A., Bradley, R. S., Withers, P. J. and Preziosi, R. F. (2011). Computed tomography recovers data from historical amber: an example from huntsman spiders. *Naturwissenschaften* **98**, 519-527.
- Gassner, G. and Lohman, J. A. B. (1987). Combined proton NMR imaging and spectral analysis of locust embryonic development. *Proc. Natl. Acad. Sci. USA* **84**, 5297-5300.
- Goodman, B. A., Gordon, S. C., Chudek, J. A., Hunter, G. and Woodford, J. A. T. (1995). Nuclear magnetic resonance microscopy as a non-invasive tool to study the development of lepidopteran pupae. *J. Insect Physiol.* **41**, 419-424.
- Green, A. J. (2001). Mass/length residuals: measures of body condition or generators of spurious results? *Ecology* **82**, 1473-1483.

- Haddad, D., Schaupp, F., Brandt, R., Manz, G., Menzel, R. and Haase, A. (2004). NMR imaging of the honeybee brain. *J. Insect Sci.* **4**, 7.
- Hahn, D. A. and Denlinger, D. L. (2011). Energetics of insect diapause. *Annu. Rev. Entomol.* **56**, 103-121.
- Krauß-Opatz, B., Köhler, U. and Schopf, R. (1995). The energetic state of *Ips typographus* L. (Coleoptera: Scolytidae) during the life cycle. *J. Appl. Entomol.* **119**, 185-194.
- Kuhn, W. (1990). NMR microscopy – fundamentals, limits and possible applications. *Angew. Chem. Int. Ed. Engl.* **29**, 1-19.
- Lee, S. C., Kim, K., Kim, J., Lee, S., Han Yi, J., Kim, S. W., Ha, K. S. and Cheong, C. (2001). One micrometer resolution NMR microscopy. *J. Magn. Reson.* **150**, 207-213.
- Mapelli, M., Greco, F., Gussoni, M., Consonni, R. and Zetta, L. (1997). Application of NMR microscopy to the morphological study of the silkworm, *Bombyx mori*, during its metamorphosis. *Magn. Reson. Imaging* **15**, 693-700.
- Margheri, L., Ponte, G., Mazzolai, B., Laschi, C. and Fiorito, G. (2011). Non-invasive study of *Octopus vulgaris* arm morphology using ultrasound. *J. Exp. Biol.* **214**, 3727-3731.
- May, P. G. (1992). Flower selection and the dynamics of lipid reserves in two nectarivorous butterflies. *Ecology* **73**, 2181-2191.
- Nespolo, R. F., Roff, D. A. and Fairbairn, D. J. (2008). Energetic trade-off between maintenance costs and flight capacity in the sand cricket (*Gryllus firmus*). *Funct. Ecol.* **22**, 624-631.
- Nixon, J. P., Zhang, M. Z., Wang, C. F., Kuskowski, M. A., Novak, C. M., Levine, J. A., Billington, C. J. and Kotz, C. M. (2010). Evaluation of a quantitative magnetic resonance imaging system for whole body composition analysis in rodents. *Obesity* **18**, 1652-1659.
- Peig, J. and Green, A. J. (2009). New perspectives for estimating body condition from mass/length data: the scaled mass index as an alternative method. *Oikos* **118**, 1883-1891.
- Peig, J. and Green, A. J. (2010). The paradigm of body condition: a critical reappraisal of current methods based on mass and length. *Funct. Ecol.* **24**, 1323-1332.
- Pohl, H., Wipfler, B., Grimaldi, D., Beckmann, F. and Beutel, R. G. (2010). Reconstructing the anatomy of the 42-million-year-old fossil *Mengea tertiaria* (Insecta, Strepsiptera). *Naturwissenschaften* **97**, 855-859.
- Skibbe, U., Christeller, J. T., Eccles, C. D., Laing, W. A. and Callaghan, P. T. (1995). Phosphorus imaging as a tool for studying the pH metabolism in living insects. *J. Magn. Reson. B* **108**, 262-268.
- Snyder, F. and Stephens, N. (1959). A simplified spectrophotometric determination of ester groups in lipids. *Biochim. Biophys. Acta* **34**, 244-245.
- Tinsley, F. C., Taicher, G. Z. and Heiman, M. L. (2004). Evaluation of a quantitative magnetic resonance method for mouse whole body composition analysis. *Obes. Res.* **12**, 150-160.
- Tomanek, B., Jasinski, A., Sulek, Z., Muszynska, J., Kulinowski, P., Kwiecinski, S., Krzyzak, A., Skorka, T. and Kibinski, J. (1996). Magnetic resonance microscopy of internal structure of drone and queen honey bees. *J. Apic. Res.* **35**, 3-9.
- Wecker, S., Hörschemeyer, T. and Hoehn, M. (2002). Investigation of insect morphology by MRI: assessment of spatial and temporal resolution. *Magn. Reson. Imaging* **20**, 105-111.
- Williams, W. I. and Robertson, I. C. (2008). Using automated flight mills to manipulate fat reserves in Douglas-fir beetles (Coleoptera: Curculionidae). *Environ. Entomol.* **37**, 850-856.
- Ziegler, A., Kunth, M., Mueller, S., Bock, C., Pohmann, R., Schröder, L., Faber, C. and Giribet, G. (2011). Application of magnetic resonance imaging in zoology. *Zoomorphology* **130**, 227-254.