J. Avian Biol. 40: 457–460, 2009
doi: 10.1111/j.1600-048X.2008.04564.x

Received 24 April 2008, accepted 15 November 2008

Magnetic resonance imaging versus chemical fat extraction in a small passerine, the willow warbler *Phylloscopus trochilus*: a fat-score based statistical comparison

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The amount of fat of a bird is a fundamental metric commonly used by avian biologists in studies of migration, winter energy management and condition. The methods used for estimating fat content range from simply measuring body mass and subtracting the estimated lean mass, visual scoring of subcutaneous fat deposits, to destructive extraction using a Soxhlet apparatus. We used magnetic resonance imaging (MRI) for non-invasive estimation of fat content in willow warblers *Phylloscopus trochilus*. According to an indirect comparison, made using a standardized fat scoring system as a common measure, the MRI method gave very similar fat amount as Soxhlet extraction and we discuss advantages of using MRI over alternative methods. It was also shown that the commonly used fat scoring system yielded a nonlinear relationship between fat mass and fat score.

Fat is the main energy source used by birds to fuel their flight engine during migration. Hence, gauging the patterns of fat deposition and subsequent consumption during flight is imperative to an understanding of adaptive migration strategies (Alerstam and Lindström 1990). Estimating fat deposition is also of great interest in studies of breeding and winter biology of birds. Therefore, ornithologists have since long tried different approaches to estimate the amount of fat of live caught birds (Rogers 2003). Methods to estimate fat content are measuring body mass, estimating abdominal profiles, and visual fat scores on caught birds. Because also non-fat components may vary with fat load (Piersma 1998) and lean mass depends on structural size, hybrid methods involving multiple regression techniques may improve the accuracy. For determining true fat content the conventional method is chemical extraction of fat from homogenized carcasses using petroleum ether and a Soxhlet apparatus. Even though this method has provided valuable information (Odum 1960, Odum et al. 1961, 1964, Connell et al. 1960, Åkesson et al. 1992), it cannot be used for longitudinal studies where the same individual is measured repeatedly. A more recent method measures total-body electrical conductivity (TOBEC), which is related to lean mass and hence allows the fat content to be deduced (Walsberg 1988, Scott et al. 2001). This method was originally devised to measure the fat content of food, but has proven useful also to investigate animals (Van Loan et al. 1987). However, fat estimation from TOBEC requires initial calibration against carcasses and does not provide information on the spatial distribution of fat.

However, using magnetic resonance imaging (MRI), the problems outlined above could be potentially overcome. In this study we estimated fat content by MRI in willow warblers *Phylloscopus trochilus*, and related these measurements to a commonly used scoring system of subcutaneous fat deposits. We also had access to fat scores and Soxhlet-based fat estimates from a group of previously investigated willow warblers (Lundgren et al. 1995). Hence, if there is a correlation between the amount of fat extracted by a Soxhlet apparatus, and the visual fat score as well as between the MRI estimated amount of fat and the visual fat score, we argue that the MRI estimate is likely to be closely correlated with true body fat content. We also evaluated the functional relationship between the visual fat scoring system and true fat content in the willow warbler.

Methods

Five willow warblers were trapped at Ottenby Bird Observatory (56°12′N, 16°24′E), and kept in captivity during the study period in $48 \times 28 \times 28$ cm^3 cages with unrestricted access to fresh water. Between measurements, the fat level was changed by employing different feeding schedules, *ad libitum* or 80%, 50%, 20% and 5% of daily food intake, respectively. The aim was to create a range of different fat loads within individuals in order for each individual to show a different fat load at each MRI measurement. The mean body mass was 9.0 g ± 1.5 (SD) and the range was 6.4–12.8 g. Visual fat loads were scored daily according to a 9-grade scale (cf. Kaiser 1993). This scale extends the 7-grade scale (0–6) presented by Pettersson and Hasselquist (1985) with scores 7–8 to account for extremely high fat loads. However, none of the willow warblers in this study reached a score higher than 6, so effectively the 7-grade scale was used. The fat score is based on visible subcutaneous fat at the abdomen and the furcular depression as seen when blowing aside the feathers covering these tracts. The score 0 is a bird with no fat at the abdomen or the furcular depression, while a score 6 bird has the whole abdomen and part of the flight muscle covered with fat and a broad convex swelling of fat covering the furcular depression and surrounding areas. Birds fatter than this are assigned scores 7–8 (Kaiser 1993). For a detailed description the fat scoring system used the reader is referred to Pettersson and Hasselquist (1985) and Kaiser (1993).

Before MR imaging, which could only be performed once a week due to limited access to the equipment, birds were deprived of food for 3–5 h to empty the gut. Immediately before imaging body mass was measured using a Pesola spring balance, fat score was taken (by the same person throughout the study). Tarsus and wing chord was measured once for the birds. The birds were immobilized with thin layers of foam plastic and positioned in a ventilated perspex cylinder. Ambient light in the scanner room was reduced to minimize stress on the bird. No pharmacological anesthesia or sedation was employed.

Whole-body fat was visualized using a T1-weighted (T1W) spin-echo pulse sequence (echo time 15 ms, repetition time 500 ms, field of view 50×50 mm^2, matrix 256×256 pixels) using a 1.5 T MR unit at Lund University Hospital (Magnetom Vision, Siemens Medical Systems, Erlangen) with a small vendor-supplied flexible surface receiver coil. The slice thickness was 3 mm, with zero distance between adjacent slices. The whole body of the bird was covered by 16 transversal slices. Each individual was measured on 3–4 occasions, resulting in a total of 18 measurements on the five birds included in the study. In the statistical analyses of fat distribution along the body axis, adjacent slices were merged into four segments.

After minimal spatial smoothing, the fat-containing pixels in each T1W image were identified by an empirical segmentation procedure (Scion Image for Windows 4.02), i.e. if a pixel showed signal values above a certain threshold the corresponding volume element (voxel) in the bird was classified as containing fat. The total fat volume (excluding the head of the bird) was calculated as the number of fat-containing pixels (summarized over all relevant slices) multiplied by the nominal voxel volume (given by the pixel area times the slice thickness). Conversion to mass was accomplished by assuming the density 0.94 g/cm^3 for solid adipose tissue and 1.0 g/cm^3 for other soft tissues (Keys and Brožek 1953, Duck 1990).

For comparison we used previously published data on Soxhlet-extracted fat in 8 and 15 individuals collected during spring and autumn, respectively, at Ottenby bird observatory (Lundgren et al. 1995).

Because the study involved repeated MRI measurements of the same individual birds, we used a generalized linear mixed model (GLMM) approach with individual as random effect in addition to the fixed effect being analyzed, and with REML estimation of degrees of freedom. The tests were performed using the package JMP statistical discovery release 6.0 (2005) from SAS institute Inc.

Results

The longitudinal fat distribution is shown in Fig. 1, indicating two minor and one major peak concentrations of fat deposition. Morphologically, these peaks reflect fat deposits in the furcular depression, the flanks and the ventral belly. The fat distribution along the body axis differed from a uniform distribution ($F_{3,61} = 5.2$, $P < 0.01$).

In the GLMM analysis we entered estimated fat, from MRI or Soxhlet extraction, as the dependent variable and fat score, method and season as fixed effects, while individual was random effect. The outcome was that method had no significant effect ($F_{1,25} = 0.066$, $P = 0.8$), as well as season ($F_{1,37} = 2.61$, $P = 0.11$), while fat score had a highly significant effect ($F_{1,36} = 79.5$, $P < 0.001$).

Because the pooled data from MRI and Soxhlet extraction suggested a non-linear relationship when plotted as a function of fat score (Fig. 2), we performed a test for this by analyzing measured fat versus fat score and fat score squared for the combined data, which resulted in a

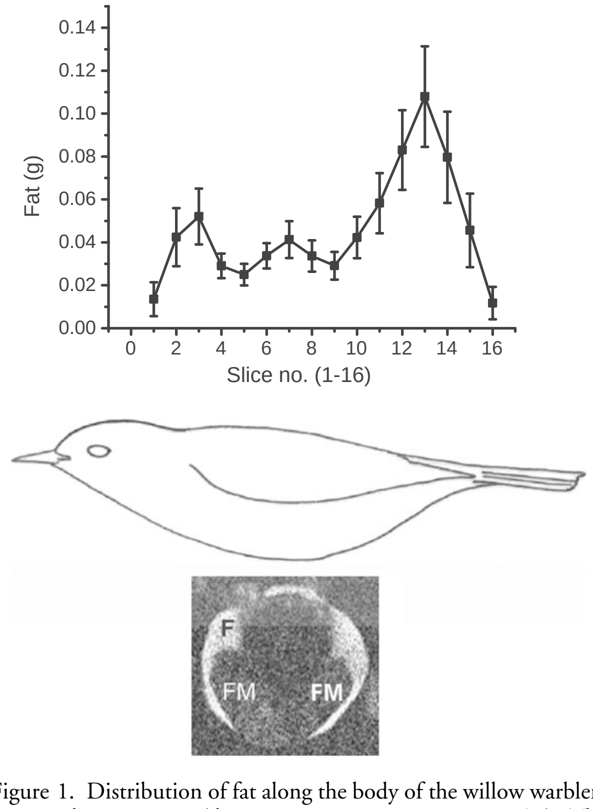

Figure 1. Distribution of fat along the body of the willow warblers measured using MRI (data points are given as mean $\pm$ SE). The silhouette bird indicates the approximate location of the transverse 3 mm thick slices (1–16) imaged during each measurement. The bottom panel shows an example of an image obtained at the position of the wing root, where the hyperintense (white) regions are fat (F), and the two ventral lobes are flight muscle (FM).

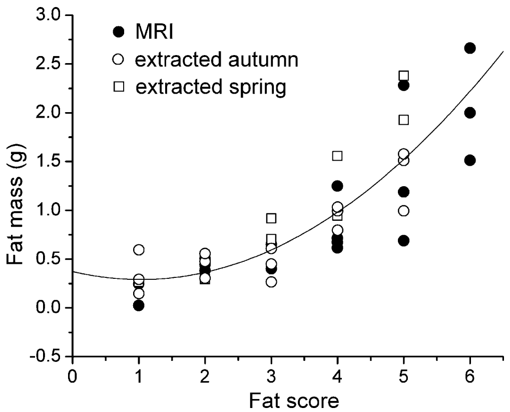

Figure 2. Estimated fat mass in relation to visual fat score in willow warblers using MRI (this study), and Soxhlet extraction during spring and autumn, respectively. The Soxhlet-extracted fat data were obtained from Lundgren et al. (1995).

significant effect of the quadratic term ($F_{1,38} = 9.98$, $P < 0.005$). The equation describing the relationship between estimated fat and fat score (X) was

$$\text{Fat (g)} = 0.37 - 0.16X + 0.078X^2, \quad (1)$$

which is also shown in Fig. 2.

Discussion

By using a visual fat score as a link between data sets of fat mass measured by MRI and Soxhlet extraction (in different populations), this study suggests that the use of MRI for estimating fat content in a small passerine gives similar results as when using a Soxhlet apparatus. It should be noted that the present study is not a direct validation of the MRI method against a Soxhlet apparatus. Such a direct comparison, i.e. an *in vivo* MRI measurement followed by Soxhlet extraction of the sacrificed bird, will indeed be required, in an adequate sample of birds, to further establish the accuracy of the MRI technique. Using MRI clearly overcomes the main problems, both scientific and ethical, of the destructive Soxhlet extraction. The possibility of repeated measurements on live specimens allows for longitudinal studies of fat dynamics by following individual birds over time (cf. Berthold et al. 2001). MRI also allows visualization of the spatial distribution of fat within the body, and it is thus clear that MRI has advantages also in comparison with TOBEC measurements (Scott et al. 2001). Not only fat tissue can be measured by MRI, but structures such as flight muscle volume (Wirestam et al. 2008), reproductive organs (Czisch et al. 2001) and brain tissue can be imaged (Van der Linden et al. 1998), although some measurements may require MRI equipment dedicated for small objects.

The fat was non-uniformly deposited along the body axis, which implies that the body undergoes a change in shape during fat accumulation. In aerodynamic models of bird flight it is often assumed that fat is deposited uniformly so that the body frontal area increases in direct proportion to fat mass (e.g. Hedenström 1992, Dietz et al. 2007). In the real birds studied here, it appears as if fat is deposited primarily towards the front and rear ends of the body, and the maximum projected frontal area will thus increase less pronounced than had fat been deposited uniformly. This may be interpreted as an adaptation to minimize aerodynamic drag with heavy fat loads, a notion that has been confirmed by computational aerodynamic models of shape drag (Olsson 2004).

The non-linear relationship between fat mass and the visual fat scale means that relatively more fat mass is deposited between the higher fat classes than between those at the lower end of the scale (cf. Kaiser 1993, Wirestam et al. 2008). Therefore, the visual fat scale should not be used for direct estimates of potential flight range, but by first establishing a relationship such as Eq. (1) to derive actual fat mass the flight range can be estimated using an aerodynamic model (Pennyuick 2008), or an empirical flight range model (e.g. Delingat et al. 2008). Some of the variation about the curve seen in Fig. 2 could be reduced if the resolution of the fat scoring system is increased as suggested by Kaiser (1993).

On the negative account is that MRI facilities are expensive and those used for human diagnostic imaging are heavily used and therefore accessible to research during limited times only. Dedicated animal MRI units are, however, becoming increasingly common, with improved spatial resolution and higher signal-to-noise ratio than whole-body scanners for clinical use. Therefore, we expect that MRI will become widely used in studies of avian physiology, ecology and behaviour.

Acknowledgements – This study was supported by the Crafoord Foundation, Lund, Sweden. A. H. is a Swedish Royal Academy of Sciences Research Fellow supported by a grant from thr Knut and Alice Wallenberg Foundation. We are grateful for the comments provided by two referees. This is contribution no. 231 from Ottenby bird observatory.

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