

## RESEARCH ARTICLE

Metabolic plasticity for subcutaneous fat accumulation in a long-distance migratory bird traced by  $^2\text{H}_2\text{O}$ Ivan Viegas<sup>1,2,\*,\dagger</sup>, Pedro M. Araújo<sup>3,\*</sup>, Afonso D. Rocha<sup>3</sup>, Auxiliadora Villegas<sup>4</sup>, John G. Jones<sup>1</sup>, Jaime A. Ramos<sup>3</sup>, José A. Masero<sup>4</sup> and José A. Alves<sup>5,6</sup>

## ABSTRACT

The migrant black-tailed godwit (*Limosa limosa*) traditionally used natural wetlands in the Iberian Peninsula to prepare for migratory flights by feeding mainly in estuaries. In recent decades, this species has become increasingly dependent on rice fields, thereby relying on a plant-based diet for fuelling. Dietary fatty acids (FA) seem to be determinant to the composition of accumulated subcutaneous fat in migratory birds. It is still unclear whether metabolic plasticity allows for modification and/or synthesis of FA, contributing to a lipid profile that enables a successful migratory performance. Deuterated water was administered to captive black-tailed godwits submitted to two diets (fly larvae versus rice) and the incorporation of deuterium ( $^2\text{H}$ ) into subcutaneous triglycerides was analyzed by NMR. A recently developed localized biopsy method for sampling subcutaneous fat was employed with later successful release of all birds into the wild. The average chemical structure reflected mostly a mixture of saturated and monounsaturated 16- and 18-carbon FA, a profile frequently found in migrant birds. Significantly higher levels of polyunsaturated FA, as well as detectable levels of n-3 FA, were observed in fly-larvae-fed birds. Excess  $^2\text{H}$ -enrichments in FA revealed significantly higher rates of fractional *de novo* lipogenesis and FA desaturation capacity in rice-fed birds. This novel and non-lethal tracer method revealed the capacity of this species to alter its lipid metabolism to compensate for a poorer dietary lipid contribution. Because of its versatility, adapting this method to other scenarios and/or other migratory species is considered feasible and cost-effective.

**KEY WORDS:** NMR, Deuterated water, Lipogenesis, Migration, Lipid accumulation, Godwits

## INTRODUCTION

Bird migration is a complex process involving a network of stimuli, mechanisms and adaptations that encapsulate behavioural, physiologic and metabolic responses (Newton, 2008). Migratory birds become hyperphagic and modify their metabolism, resulting

in increased fuel stores prior to the migratory flight (Wikelski et al., 2003). To endure such exhaustive exercise, birds rely mostly on temporary subcutaneous fat stores in the form of triglycerides (TAG), a light but energy-dense substrate when compared with alternatives such as glycogen or amino acids (Price, 2010). The composition of TAG in terms of fatty acid (FA) chain length, degree of unsaturation and placement of double bonds can affect its rate of mobilization, circulation and oxidation by muscles (Guglielmo, 2010).

The lipid composition of avian tissues is thought to be primarily influenced by dietary FA profile (e.g. Morton and Liebman, 1974; McWilliams et al., 2002; Pierce et al., 2004; Pierce and McWilliams, 2005; Bayly, 2006), which may be amplified by alterations in feeding behaviour prior to migration or during refuelling stopovers. Dietary manipulations have established a preference of several species of migratory songbird for diets with certain FA profiles, particularly unsaturated (UFA) over saturated FA (SFA), and monounsaturated (MUFA) over polyunsaturated FA (PUFA) (Pierce and McWilliams, 2014). This is particularly important because changes in stored subcutaneous fat can potentially alter the performance of the birds in endurance flights (Maillet and Weber, 2006, 2007; Pierce and McWilliams, 2014). However, this pattern of variation in relation to diet is not always consistent (Egeler et al., 2003) with the relative abundance of the most common long-chain UFA (mostly 16:1, 18:1 and 18:2) in migratory birds being rarely modified by diet (McWilliams et al., 2004). FA mobilization for oxidation has previously been shown to be a non-random process (Price et al., 2008), and consequently, the consistency of the metabolic response to sustain migratory flights may rely on this FA profile. This evidence points to a certain degree of metabolic modulation; however, it is unknown to what extent this contributes to the FA composition of subcutaneous fat stores in migratory birds.

The migratory black-tailed godwit, particularly its western European population (*Limosa limosa limosa* Linnaeus 1758), has traditionally used natural wetlands during the non-breeding season, but is currently increasingly dependent on rice fields outside its breeding grounds (Gill et al., 2007; Alves et al., 2010). The reduction or modification of natural wetlands as a consequence of human population increase and land conversion into urban and agricultural areas has been accompanied by an expansion of agricultural wetlands for rice production across its distribution range (Sutherland et al., 2012). In the Iberian Peninsula, rice fields provide lodging and artificial foraging habitat for large numbers of waterbirds, and particularly shorebirds, throughout the winter, including black-tailed godwits (Elphick et al., 2010; Lourenço et al., 2010; Navedo et al., 2015). This is particularly evident during their extended stopover in Iberian rice fields between January and February, when these birds efficiently forage almost exclusively on rice kernels, thereby relying on a carbohydrate-rich diet based on

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**List of abbreviations**

<sup>2</sup> H	deuterium
<sup>2</sup> H <sub>2</sub> O	deuterated water
FA	fatty acid
FSR	fractional synthetic rate
MS	mass spectrometry
MUFA	monounsaturated fatty acid
NMR	nuclear magnetic resonance
PUFA	polyunsaturated fatty acid
SFA	saturated fatty acid
TAG	triglycerides
UFA	unsaturated fatty acid

plant material for refueling (Masero et al., 2009; Santiago-Quesada et al., 2009; Alves et al., 2010; Lourenço et al., 2010). However, the Icelandic population of black-tailed godwits (*Limosa limosa islandica*), which also occurs in Iberia at the same time of the year, feeds predominantly on a protein-rich diet mainly consisting of bivalves, polychaetes and other macroinvertebrates by foraging in estuarine tidal flats in the vicinity of the rice fields (Alves et al., 2010, 2013). Despite the habitat segregation, there is interchange between rice field and estuarine feeding black-tailed godwits (Alves et al., 2010; Lopes et al., 2013).

We therefore unravel the metabolic plasticity of migrating black-tailed godwits with respect to selective FA storage and/or transformation (*de novo* lipogenesis, desaturation and elongation) associated with different diets. As part of this study, deuterated water (<sup>2</sup>H<sub>2</sub>O) was administered and the incorporation of deuterium (<sup>2</sup>H) into subcutaneous TAG was followed and quantified by <sup>2</sup>H NMR for the first time in wild birds.

**MATERIAL AND METHODS****Bird capture and experimental setup**

Black-tailed godwits were captured with mist nets in rice fields at a major wintering site on the East Atlantic Flyway, the Tagus estuary (38°44'N, 8°59'W), in January 2015. Black-tailed godwits ( $n=12$ ) were measured and ringed, then transported to University of Extremadura facilities (Badajoz, Spain; 2 h drive from the Tagus estuary) and randomly split into two separate outdoor aviaries (5×2.5×2 m, 6 birds per cage). Both groups were allowed to acclimate for 8 days with *ad libitum* access to drinking water and food (live fly larvae *Protophormia terraenovae*). Each group was then subjected to one of two different dietary treatments for the following 14 days: six birds were kept on the same fly larvae diet while the remaining six were provided exclusively with unprocessed rice (both having *ad libitum* drinking water). Both diets were analyzed for proximate composition and lipid profile (Table 1). On day 23, all black-tailed godwits were injected intraperitoneally with 99.8%-enriched <sup>2</sup>H<sub>2</sub>O (CortecNet, Voisins-Le-Brettonneux, France; ~7% volume per gram body mass), 0.9% saline and supplied with 5%-enriched drinking <sup>2</sup>H<sub>2</sub>O to maintain body water <sup>2</sup>H-enrichment. After 24 h, the birds were weighed and sampled for blood and subcutaneous fat. Blood was collected from the brachial vein, pierced with a 26-gauge needle, collected into a heparinized capillary tube (Microvette CB 300; Sarstedt AG & Co., Germany) and centrifuged at 10.062 g for 10 min. Plasma was stored at –20°C until analysis of body water <sup>2</sup>H-enrichments by <sup>2</sup>H nuclear magnetic resonance (NMR) analysis, quantification of plasma glucose (glucose oxidase method, using a glucose analyser-YSI Model 1500 Sport) and quantification of plasma triglycerides (Spinreact, ref: 1001314). Subcutaneous fat biopsies (~13.0±

**Table 1. Proximate composition and lipid species as determined from <sup>1</sup>H NMR spectra of the two experimental diets provided to black-tailed godwits in captivity**

Proximate composition	Fly larvae*	Rice†
Dry matter (DM; %)	30.8	88.7
Ash (%)	3.9	3.6
Gross energy (kJ g <sup>-1</sup> dry mass)	22.6	17.1
Crude protein (% DM)	52.3	7.5
Crude fat (% DM)	20.8	2.0
Crude fibre (% DM)	6.6	9.6
Starch (% DM)	0.0	56.1
Lipid species (%)		
SFA	18.6	11.1
UFA	81.4	88.9
PUFA	32.5	38.4
MUFA	48.9	50.5
n-3	4.4	n.d.

\*Live fly larvae from *Protophormia terraenovae*-Comercial Las Grullas, Badajoz, Spain.

†Unprocessed rice seeds harvested in Extremadura, Spain. n.d., not detected.

1.5 mg,  $n=12$ ; Table 2) were obtained by a small incision in the furcular zone according to Rocha et al. (2016). This area consists primarily of loose connective tissue and subcutaneous yellow fat tissue which was clearly visible under the skin and accessible after laterally moving the feathers from the breast area. Fat samples were kept in methyl tert-butyl ether (MTBE; Sigma, Spain), TAG were extracted according to Matyash et al. (2008) and stored at –20°C until NMR analysis. All experimental procedures complied with the guidelines of the European Union (Directive 2010/63/EU) and were approved by the national authorities (Instituto da Conservação da Natureza e das Florestas; permit 04/2015/CAPT). The birds recovered immediately after tissue sampling but were kept under observation for the following 10 days with food and water provided *ad libitum*. With no alterations to behaviour or welfare being observed, the birds were successfully released into the wild at the capture site.

**Diet analysis**

Diet proximate composition was analyzed in duplicate following the methods described by the Association of Official Analytical Chemists (2006): dry content was calculated after samples were oven-dried at 70°C until constant weight; ash content was calculated after incineration in a muffle furnace for 6 h at 550°C (NÜVE MF110); protein content ( $N \times 6.25$ ) was obtained by the Kjeldahl method after acid digestion using a Leco N analyzer (model FP-528; Leco Corporation, St Joseph, MI, USA); fat content was obtained by petroleum ether extraction (40–60°C) using a Soxtec 2055 Fat Extraction System (Foss, Hillerød, Denmark); fibre content was obtained from the defatted samples by difference in weight after

**Table 2. Somatic and blood parameters from black-tailed godwits submitted to two different dietary treatments in captivity**

	Fly larvae	Rice
Initial mass (g)	250.0±19.7	253.8±14.6
Final mass (g)	265.8±18.4	258.0±16.1
Blood glucose (mg dl <sup>-1</sup> )	223.3±27.2	205.0±12.9
Triglycerides (mg dl <sup>-1</sup> )	69.1±17.4	63.6±7.6
Fat sample mass (mg)	12.9±2.6	13.1±1.8
Body water <sup>2</sup> H-enrichment (%)	7.5±0.5	8.8±0.3

Means±s.e.m. are presented ( $n=6$ ). No differences between dietary treatments were found for any of the parameters (*t*-test,  $P>0.05$ ).

calcination; starch content was obtained by enzymatic digestion with glucoamylase; and gross energy was measured in an adiabatic bomb calorimeter (Werke C 2000 basic; IKA, Staufen, Germany).

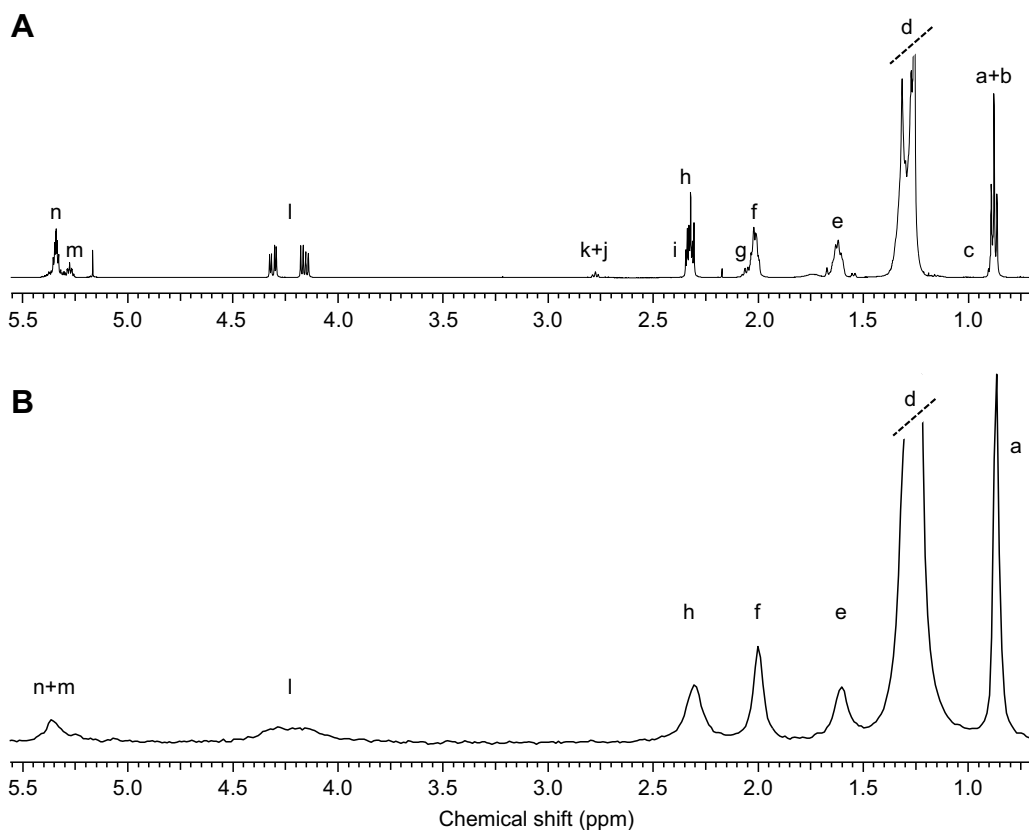
### <sup>1</sup>H and <sup>2</sup>H NMR analysis

Body water <sup>2</sup>H-enrichments were determined from 10 µl aliquots of bird plasma by <sup>2</sup>H NMR as described in Jones et al. (2001), where water content was assumed to be 92% of total plasma. NMR spectra of TAG samples were obtained at 25°C with a Bruker Avance III HD system with an UltraShield Plus magnet (11.7 T, <sup>1</sup>H operating frequency 500 MHz) equipped with a 5-mm <sup>2</sup>H-selective probe with <sup>19</sup>F lock and <sup>1</sup>H-decoupling coil. TAG were reconstituted in chloroform containing a pyrazine standard. <sup>1</sup>H NMR spectra were acquired with a 90 deg pulse, 3 s of acquisition time and 8 s of delay, for 16 scans. <sup>2</sup>H NMR spectra were acquired with a 90 deg pulse, 0.67 s of acquisition time and 8 s of delay, with the number of scans ranging from 1500 to 2500, corresponding to approximately 5 h of collection time.

The FA profile (in percentage) was estimated by <sup>1</sup>H NMR because lipid species such as n-3 FA (0.90 ppm), MUFA (1.90 ppm) and PUFA (2.00 ppm) provide distinguishable peaks in specific regions of the spectrum (assigned from literature values), which can then be estimated relative to the pyrazine standard, while the SFA were calculated by difference (Duarte et al., 2014). All FA were considered polymers of methylenic (CH<sub>2</sub>) and/or olefinic (HC=CH) subunits [i.e. <sup>-</sup>OOC-(CH<sub>2</sub>)<sub>x</sub>-(HC=CH)<sub>y</sub>-CH<sub>3</sub>], so that

an average chemical structure of FA could also be estimated by <sup>1</sup>H NMR (Viegas et al., 2016).

TAG <sup>2</sup>H-enrichments were quantified from the <sup>1</sup>H and <sup>2</sup>H NMR spectra by measuring the <sup>1</sup>H and <sup>2</sup>H intensities of selected signals relative to the <sup>1</sup>H and <sup>2</sup>H intensities of a pyrazine standard according to Duarte et al. (2014). Briefly, (1) by determining the <sup>2</sup>H-enrichment in the FA terminal methyl site for TAG-bound FA derived from *de novo* lipogenesis (Fig. 1B, a); (2) by determining the <sup>2</sup>H-enrichment in the sn-1,3 glycerol site for newly synthesized TAG-bound glycerol (Fig. 1B, l) and (3) by determining the <sup>2</sup>H-enrichment in the MUFAs' allylic protons for desaturation of SFA (Fig. 1B, f). Moreover, while the terminal methyl site is enriched with <sup>2</sup>H during the first round of FA synthesis, the α protons incorporate <sup>2</sup>H in the last round of elongation. Therefore, if elongation occurs on pre-existing (unlabelled) FA, the α- and methyl protons will be differentially labelled and will inform of the fractional contribution of elongation to lipid synthesis. Excess TAG positional <sup>2</sup>H-enrichments were calculated after systematic subtraction of the values with 0.0156%, taken as the mean background <sup>2</sup>H-enrichment based on the Vienna Standard Mean Ocean Water (δ<sup>2</sup>H). Fractional synthetic rates (FSR; in % day<sup>-1</sup>) were estimated by dividing these positional TAG enrichments by that of body water. Spectra were processed by applying exponential multiplication to the free-induction decay (<sup>1</sup>H: 0.1 Hz; <sup>2</sup>H: 1.0 Hz), and analyzed using the curve-fitting routine supplied with ACD Labs 1D NMR processor software 2.4.



**Fig. 1. Representative nuclear magnetic resonance (NMR) spectra of subcutaneous fat triglycerides from black-tailed godwits submitted to two different dietary treatments in captivity and to <sup>2</sup>H<sub>2</sub>O administration for 24 h. (A) <sup>1</sup>H; (B) <sup>2</sup>H. Assigned peaks are as follows: (a) non n-3 methyls; (b) partial n-6 methyls; (c) n-3 methyls; (d) aliphatic chain methylenes; (e) β methylenes; (f) monounsaturated allylic hydrogens; (g) polyunsaturated allylic hydrogens; (h) α methylenes; (i) docosahexaenoic acid (DHA) α and β methylenes; (j) linoleic acid bisallylic hydrogens; (k) other bisallylic hydrogens; (l) sn-1,3 of triglyceride-bound glycerol; (m) sn-2 of triglyceride-bound glycerol; and (n) olefinic hydrogens. Off the spectra: chloroform (solvent; singlet at 7.25 ppm) and pyrazine (internal standard; singlet at 8.50 ppm).**

### Statistical analysis

Data are presented as means±s.e.m. Student's two-tailed unpaired *t*-test was used to compare means between dietary treatments. Analyses were performed in GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). Differences were considered statistically significant at  $P<0.05$ .

### RESULTS

The two diets had distinctive nutrient profiles (Table 1): while fly larvae had high protein and substantial moisture content, unprocessed rice consisted mostly of starch and fibre with less hydration and lower energy density. Moreover, fly larvae had 10-fold more lipid compared with rice. While the overall FA composition of the two diets was similar, n-3 FA, which were present in low abundance in the fly larvae, were undetected in rice. After 14 days of dietary treatment, no differences in weight gain, blood glucose and blood TAG levels were observed. Twenty-four hours after  $^2\text{H}_2\text{O}$  injection, no differences were detected in terms of body water  $^2\text{H}$ -enrichment (Table 2), suggesting that there was no significant difference in lean body mass, and therefore adiposity, between the two groups.

Following extraction, TAG from subcutaneous fat biopsies originated well-characterized  $^1\text{H}$  and  $^2\text{H}$  NMR spectra (Fig. 1). The FA/glycerol ratio was consistent and  $\sim 3$  for all birds, as expected from pure TAG preparations (Table 3). TAG composition did not differ in terms of total SFA and UFA for the two groups, but within the UFA, rice-fed birds had less PUFA and more MUFA compared with those fed with fly larvae.  $^1\text{H}$  NMR spectra of TAG from birds fed on fly larvae had quantifiable n-3 FA signals, but these were absent in spectra from rice-fed birds (as in Fig. 1A). TAG-bound FA from birds fed on rice had on average a higher number of carbons compared with the fly-larvae-fed group. Both groups had similar levels of FA desaturation with an overall average of 1.5 double bonds per FA.

Excess  $^2\text{H}$ -enrichments of FA terminal methyl hydrogens, representing *de novo* lipogenesis activity, were significantly lower in the birds fed with fly larvae compared with those fed with rice ( $0.51\pm 0.24\%$  versus  $3.85\pm 1.47\%$ , respectively;  $P=0.04$ ), resulting in a significantly lower rate of fractional *de novo* lipogenesis in fly-larvae-fed birds (Fig. 2). For TAG-bound glycerol, excess

$^2\text{H}$ -enrichments in the sn-1,3 glycerol site were not significantly different ( $0.25\pm 0.12\%$  versus  $0.38\pm 0.19\%$  for fly-larvae- and rice-fed birds, respectively), translating to similar rates of fractional TAG-glycerol synthesis (Fig. 2). Excess  $^2\text{H}$ -enrichment of MUFA allylic protons showed a similar pattern to the FA methyls, i.e. lower levels for fly-larvae-fed compared with rice-fed birds ( $0.04\pm 0.02\%$  versus  $0.32\pm 0.12\%$ , respectively;  $P=0.04$ ), which translated to significantly lower fractional desaturation rates ( $0.49\pm 0.24\%$  versus  $3.82\pm 1.47\%$ , respectively). Finally, based on the comparison of FA  $\alpha$ - and methyl hydrogen enrichments, there was no significant FA elongation activity associated with either diet.

### DISCUSSION

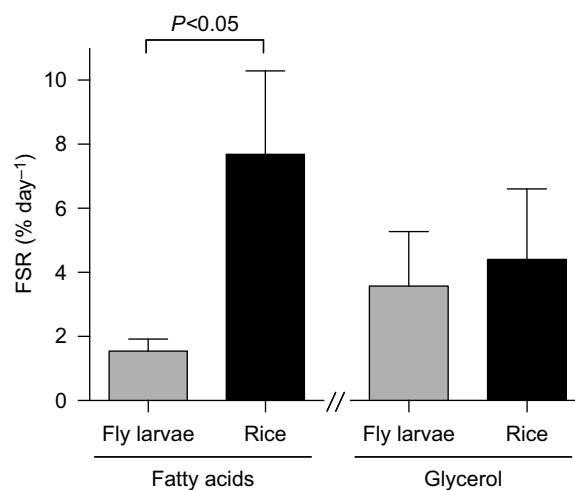
The present study demonstrates a novel and highly practical tracer method for studying the synthesis of subcutaneous TAG stores in wild migratory birds. Thus far, the sole purpose of administering  $^2\text{H}_2\text{O}$  to birds has been to effectively measure body composition (lean versus fat) through the deuterium dilution method (McWilliams and Whitman, 2013). But, by following  $^2\text{H}$  incorporation into subcutaneous fat TAG, this study provides strong evidence for metabolic plasticity in fat accumulation by a long-distance migratory bird, as black-tailed godwits fed with rice increased *de novo* lipogenesis activity. By converting carbohydrate to fat, these birds were able to compensate for the low lipid levels in their diet. To the best of our knowledge, this was quantified for the first time in a free-ranging bird species, using  $^2\text{H}_2\text{O}$  as metabolic tracer, and by applying NMR-based techniques following the methodologies previously described for rodents (Duarte et al., 2014) and fish (Viegas et al., 2016). The localized biopsy method developed by Rocha et al. (2016) allowed for sampling of subcutaneous fat in the furcular zone that provides the bulk of the lipid fuel for these birds. Even if it was not possible to trace the changes in other regional adipose stores, this method allowed the experimental procedures to be performed without observable signs of distress on the birds during the short period held in captivity, resulting in their subsequent successful release into the wild.

Conversion of natural land for agricultural purposes is the primary driver of biodiversity loss throughout the world (Newbold

**Table 3. Lipid species and chemical structure of esterified fatty acids as determined from  $^1\text{H}$  NMR spectra of subcutaneous fat triglycerides from black-tailed godwits submitted to two different dietary treatments in captivity**

	Fly larvae	Rice
Lipid species (%)		
SFA	37.7±3.6	36.8±0.6 <sup>n.s.</sup>
UFA	62.3±3.6	63.2±0.6 <sup>n.s.</sup>
PUFA	15.3±3.0	6.8±0.5*
MUFA	47.0±2.1	56.4±0.3**
n-3	0.4±0.2	n.d.
Chemical structure		
Average number of carbons	17.5±0.4	18.7±0.1*
Average number of protons	31.1±0.4	33.6±0.2***
Olefinic units (HC=CH)	1.6±0.2	1.4±0.0 <sup>n.s.</sup>
Methylene units (CH <sub>2</sub> )	12.6±0.1	13.9±0.1*
FA/glycerol	3.1±0.1	3.0±0.0 <sup>n.s.</sup>

Means±s.e.m. are presented ( $n=6$ ). Differences between dietary treatments are indicated by asterisks (*t*-test, \* $P<0.05$ ; \*\* $P<0.01$ ; \*\*\* $P<0.001$ ; n.s.: not significant; n.d.: not detected). Chemical structure: FA were considered polymers of olefinic (HC=CH) and methylenic (CH<sub>2</sub>) subunits [i.e.  $-(\text{OC}-\text{CH}_2)_x-(\text{HC}=\text{CH})_y-\text{CH}_3$ ], calculated as in Viegas et al. (2016).



**Fig. 2. Triglyceride-bound fatty acid and glycerol fractional synthetic rates (FSR) expressed as percent per day of subcutaneous fat triglycerides from black-tailed godwits submitted to two different dietary treatments in captivity and to  $^2\text{H}_2\text{O}$  administration for 24 h. Means±s.e.m. are presented ( $n=6$ ). Differences between dietary treatments are indicated (*t*-test,  $P<0.05$ ).**



et al., 2015). However, some agricultural areas such as rice fields can act as wetland surrogates and have been largely occupied as feeding habitats for many waterbird species (Navedo et al., 2015), thus providing a significant ecological benefit compared with other cultures. Migrant birds engage in a number of adaptations prior to onset of long-distance endurance flights. These include changes in behaviour, such as altering foraging patterns and managing stopover costs in terms of energy, time and predation risk (Newton, 2008). These are accompanied by changes in physiology and nutrient metabolism that maximize lipid storage ahead of the journey and promote its efficient utilization for energy during the flight (Piersma and Lindström, 1997; McWilliams et al., 2004). Despite the wide diversity of food resources and habits, accumulated fat in migratory birds displays a highly conserved mixture of saturated and monounsaturated 16- and 18-carbon FA (Egeler and Williams, 2000; McWilliams et al., 2004; Pierce et al., 2004; Pierce and McWilliams, 2005). In the present study, the average chemical structure estimated by  $^1\text{H}$  NMR analysis captured this profile. This pattern is thought to be a compromise between effective lipid storage on the one hand, and FA mobilization and circulation to the working muscles to supply oxidative fuel on the other (Raclot, 2003). While analysis of circulating levels of glucose, TAG, non-esterified FA, very-low density lipoprotein and other substrates can provide a valuable insight into nutritional and metabolic status, this approach does not fully inform the dynamics of lipids. For example, it cannot distinguish between FA molecules that have been synthesized versus those that were absorbed from food. Apart from overall body mass (Jenni-Eiermann and Jenni, 1998; Guglielmo et al., 2005), associations between the levels of circulating substrates and factors such as migration distance and flight performance (Gannes, 2001), fattening/fasting/refuelling (McWilliams et al., 2004), rest/exercise (Jenni-Eiermann et al., 2002) and their interaction, have been difficult to establish. In black-tailed godwits submitted to dietary interventions resembling those of the present study, blood parameters were well correlated with increases in body mass but interactions between plasma TAG, glycerol and diet were non-significant (Albano et al., 2016). As for mammals, plasma TAG and glycerol show a reciprocal relationship during the transition from fed to fasted state, with maximal TAG levels found during postprandial lipid assimilation while glycerol, released by adipose tissue lipolysis, is highest during fasting lipid oxidation (Guglielmo et al., 2005). Despite not having quantified glycerol, the values found for plasma glucose and TAG concentrations in this study were blind to the underlying lipogenic fluxes. Naturally high lipolytic rates as observed in ruff (*Philomachus pugnax*; Vaillancourt and Weber, 2007) and/or the selective upregulation of flux of specific FA for oxidation as observed in white-throated sparrow (*Zonotrichia albicollis*; Price and Guglielmo, 2009) may contribute to the lack of detectable effects on blood concentrations. In this sense, metabolic flux by  $^2\text{H}_2\text{O}$  resulted in a more comprehensive view than concentrations alone.

Preference for lipid-rich diets has been frequently documented in migratory birds (Bairlein and Gwinner, 1994; Egeler and Williams, 2000; Pierce and McWilliams, 2005; Ben-Hamo et al., 2011) and partially drives the composition of subcutaneous fat that is ultimately a determinant for flight performance. Consequently, it has also been suggested that for migrating birds such as *Z. albicollis*, there is some degree of selectivity over which FA are released during lipolysis of stored fat (Price and Guglielmo, 2009). In fact, several bird species increase the proportion of UFA in their fat stores in the period prior to migration (Egeler and Williams, 2000; Pierce

and McWilliams, 2005), a feature considered adaptive for and also potentially influencing spatial packing, fluidity, ion-leak, signalling pathways and integral protein function of membranes (Guglielmo, 2010; Pierce and McWilliams, 2014). However, in the case of lipid-poor diets, the subcutaneous accumulation of certain UFA such as the n-3 may pose as a physiological challenge, as observed in the rice-fed birds. Methodological constraints regarding the lower sensitivity offered by  $^1\text{H}$  NMR spectroscopy when compared with other analytical techniques such as mass spectrometry (MS) should also not be ignored, as n-3 FA were not detected in the diet as well. Overall, and regardless of the dietary input, the proportion of n-3 PUFA found in subcutaneous fat seems to be lower than in the diets. In contrast, the proportion of n-3 PUFA found in breast muscle was much higher (Pierce et al., 2004; Price and Guglielmo, 2009). Because most of n-3 PUFA are essential for several physiologic functions, including flight exercise, the need to prioritize muscular function surpasses its capacity to accumulate these PUFA under a lipid-poor diet. Mobilization of such FA is facilitated by lipoprotein shuttles, translocases and FA binding proteins (McFarlan et al., 2009) that synergistically accelerate lipid transport and allow for high lipid fluxes (Weber, 2009). In semipalmated sandpipers, *Calidris pusilla*, high intake of n-3 PUFA increased migratory performance by enhancing the functional capacity of membranes and increasing the aerobic capacity of flight muscles (Maillet and Weber, 2006, 2007). The presence of dietary n-3 PUFA was also able to increase the activity of oxidative enzymes by 58–90% in the flight muscle of northern bobwhite (*Colinus virginianus*), a non-migratory bird (Nagahuedi et al., 2009). Regardless of the role of the muscle, it seems evident that between dietary assimilation and subcutaneous storage, there is a metabolically significant modification of the FA profile. In red-eyed vireos, *Vireo olivaceus*, a selective metabolism of stored n-6 FA (20:4n-6, 22:4n-6, and 22:5n-6) from dietary linoleic acid (18:2n-6) was proposed (Pierce et al., 2004). In *C. pusilla*, not all dietary FA were deposited equally into adipose tissue: linoleic acid (18:2n-6) was incorporated into fat stores until the proportion in adipose tissue exceeded that in the diet, while palmitate (16:0), although highly abundant in the diet, was not proportionally incorporated into adipose tissue (Egeler et al., 2003). For the same species, Maillet and Weber (2007) reported that more than half of dietary n-3 PUFA were converted to other FA, mainly to oleic acid (18:1n-9), before storage. For the most part, these studies relied on a FA balance approach, which analyzes the system's compartments (diet, stored fat, circulating FA, muscle membranes) and interprets differences in its proximate composition.

The present method involving the effective delivery of a stable isotope demonstrated unequivocally the capacity of black-tailed godwits to significantly upregulate both *de novo* FA synthesis and modification of existing FA when feeding on a FA-deficient diet (unprocessed rice). In contrast to using labelled FA (e.g.  $^2\text{H}$ - or  $^{13}\text{C}$ -palmitate),  $^2\text{H}_2\text{O}$  allows  $^2\text{H}$ -enrichment to rapidly equilibrate with total body water and distribute homogeneously within tissues. Even if  $^2\text{H}$  atoms can be exchanged with other organic pools beyond the biochemical routes of lipid metabolism, providing the birds with deuterated drinking water between the intraperitoneal injection and tissue sampling assured a consistent labelling capacity as confirmed by the final body water  $^2\text{H}$ -enrichment. Despite the incomparable specificity obtained by direct traceability of a particular substrate (as reviewed by McCue, 2011), its delivery and dosage may also influence the response of these metabolic pathways by being absorbed and/or oxidized differently. Another option would be resolving the  $^2\text{H}$ -enrichment levels of specific FA compared with the presented aggregated analysis of enrichment by  $^2\text{H}$  NMR. This

approach would require further sample treatment and sample processing, as each FA would need to be acquired separately by MS instrumentation. Moreover, MS analyses do not resolve positional hydrogen enrichments of the FA chain, hence processes such as chain elongation would not be directly inferred. The structural variety of FA, the intricate pathways for FA modification and the apparent FA oxidative selectivity (Price and Guglielmo, 2009; McCue et al., 2010) indicate that integrating tracer delivery with conventional blood and tissue biopsy analyses would further our understanding of migratory bird lipid metabolism in a variety of ecological, nutritional and physiological settings.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

I.V., P.M.A., A.D.R. and J.A.A. conceived and designed the experiment; A.D.R. and J.A.A. organized fieldwork and A.V. and J.A.M. kept birds in captivity; P.M.A., A.D.R., A.V., J.A.R., J.A.M. and J.A.A. performed the experiment; I.V., P.M.A. and J.G.J. performed the lab work; I.V., J.G.J. and J.A.A. analysed the data; I.V., P.M.A. and J.A.A. led the writing of the manuscript with substantial inputs from all other authors.

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