Metabolic plasticity for subcutaneous fat accumulation in a long distance migratory bird traced by $^{2}H_{2}O$

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Summary Statement

A novel and non-lethal tracer method using deuterated water revealed alteration in lipid metabolism of migrant black-tailed godwits submitted to different diets.

Abstract

The migrant black-tailed godwit (*Limosa limosa*) traditionally used natural wetlands in the Iberian Peninsula preparing 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 fueling. 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 for a lipid profile that enables a successful migratory performance.

Deuterated water was administered to captive black-tailed godwits submitted to two diets (fly larvae *vs.* rice) and the incorporation of deuterium (^{2}H) into subcutaneous triglycerides was analysed by NMR. A recently developed localized biopsy method for sampling subcutaneous fat was employed with ulterior 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}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. Due to its versatility, adapting this method to other scenarios and/or other migratory species is considered feasible and cost-effective.

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 birds' 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 stop-overs. Dietary manipulations have established a preference of several species of migratory songbird for diets with certain FA profile, particularly unsaturated over saturated FA, and monounsaturated (MUFA) over polyunsaturated FA (PUFA) (Pierce and McWilliams, 2014). This is particularly important since 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 unsaturated FA (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., 2007) and consequently, on this FA profile may rely the consistency of the metabolic response to sustain migratory flights. These evidences point to a certain degree of metabolic modulation, however it is unknown to which 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 stop-over 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 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; Alves et al., 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 (^2H_2O) was administered and the incorporation of deuterium (^2H) into subcutaneous TAG was followed and quantified by ^2H 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 (38º 44' N, 8º 59' W) in January of 2015. Black-tailed godwits (n=12) were measured and ringed, then transported to University of Extremadura facilities (Badajoz, Spain; two hour drive from 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 eight days with *ad libitum* access to drinking water and food (live fly larvae *Protophormia terraenovae*). Each group was then subjected to two dietary different 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 analysed for proximate composition and lipid profile (Table 1). On day 23 all black-tailed godwits were injected intraperitoneally with 99.8%-enriched ^2H_2O (CortecNet, Voisins-Le-Bretonneux, France; ~7% volume per body weight), 0.9% saline and supplied with 5%-enriched drinking ^2H_2O to maintain body water ^2H-enrichment. After 24h, the birds were weighed and sampled for blood and subcutaneous fat. Blood was collected from the brachial vein, pierced with a 26 G needle, collected into a heparinized capillary tube (Microvette CB 300; Sarstedt AG & Co., Germany) and centrifuged at 10,000 rpm for 10 min. Plasma was stored at -20ºC until analysis of body water ^2H-enrichments by ^2H NMR, 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±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 on methyl tert-butyl ether (MTBE; Sigma, Spain), TAG extracted according to Matyash et al. (2008), and stored at -20ºC 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 (ICNF; 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 *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 analysis was analyzed in duplicate following the methods described by the Association of Official Analytical Chemists (2006): dry content was calculated after oven-dried at 70°C until constant weight; ash content after incineration in a muffle furnace for 6 h at 550°C for (NÜVE MF110); protein content (N×6.25) obtained by the Kjeldahl method after acid digestion using a Leco N analyser (model FP-528; Leco Corporation); fat content obtained by petroleum ether extraction (40–60°C) using a Soxtec™ 2055 Fat Extraction System (Foss); fiber content from the defatted samples by difference in weight after calcination; starch by enzymatic digestion with glucoamylase; and gross energy was measured in an adiabatic bomb calorimeter (Werke C 2000 basic; IKA).

^{1}H and ^{2}H NMR analysis

Body water ^{2}H-enrichments were determined from 10 µL aliquots of bird plasma by ^{2}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 25ºC with a Bruker Avance III HD system with UltraShield Plus™ magnet, 11.7 T (^{1}H operating frequency 500 MHz) equipped with a 5-mm ^{2}H-selective probe with ^{19}F lock and ^{1}H-decoupling coil. TAG were reconstituted in chloroform containing a pyrazine standard. ^{1}H NMR spectra were acquired with a 90º pulse, 3 s of acquisition time and 8 s of delay, for 16 scans. ^{2}H NMR spectra were acquired with a 90º 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.

FA profile (in percentage) was estimated by ^{1}H NMR since lipid species such as n-3 (0.90 ppm), MUFA (1.90 ppm) and PUFA (2.00 ppm) FA 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 saturated FA (SFA) were calculated by difference (Duarte et al., 2014). All FA were considered polymers of methylenic (CH_2) and/or olefinic (HC=CH) subunits [i.e. $^{-}OOC–(CH_2)_x–(HC=CH)_y–CH_3$], so that an average chemical structure of FA could also be estimated by ^1H NMR (Viegas et al., 2016).

TAG ^2H-enrichments were quantified from the ^1H and ^2H NMR spectra by measuring the ^1H and ^2H intensities of selected signals relative to the ^1H and ^2H intensities of a pyrazine standard according to Duarte et al. (2014). Briefly, i) by determining the ^2H-enrichment in the FA terminal methyl site for TAG-bound FA derived from *de novo* lipogenesis [Fig. 1b) - A]; ii) by determining the ^2H-enrichment in the sn-1,3 glycerol site for newly synthesized TAG-bound glycerol [Fig. 1b) - L] and iii) by determining the ^2H-enrichment in the MUFAs' allylic protons for desaturation of saturated FA [Fig. 1b) - F]. Moreover, while the terminal methyl site is enriched with ^2H during the first round of FA synthesis, the α protons incorporate ^2H 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 ^2H-enrichments were calculated after systematic subtraction of the values with 0.0156%, taken as the mean background ^2H-enrichment based on the Vienna Standard Mean Ocean Water (VSMOW; δ^2H). Fractional synthetic rates (FSR; in % day^{-1}) 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 (^1H: 0.1 Hz; ^2H: 1.0 Hz), and analysed using the curve-fitting routine supplied with ACD Labs 1D NMR processor software 2.4.

Statistical analysis

Data are presented as mean ± 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, Inc.). 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 to 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 ^2H_2O injection, no differences were detected in terms of body water

^{2}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}H and ^{2}H NMR spectra (Fig. 1). The FA/Glycerol ratio was consistent and ~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 to those fed with fly larvae. ^{1}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 to 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}H-enrichments of FA terminal methyl hydrogens, representing *de novo* lipogenesis activity, was significantly lower in the birds fed with fly larvae compared with those fed with rice (0.51±0.24% *vs.* 3.85±1.47%, respectively; p=0.04) resulting in a significantly lower rate of fractional *de novo* lipogenesis in fly larvae-fed (Fig. 2). For TAG-bound glycerol, excess ^{2}H-enrichments in the *sn*-1,3 glycerol site were not significantly different (0.25±0.12% *vs.* 0.38±0.19, for fly larvae- and rice-fed birds, respectively) translating to similar rates of fractional TAG-glyceryl synthesis (Fig. 2). Excess ^{2}H-enrichment of MUFA allylic protons showed a similar pattern to the FA methyls, i.e. lower levels for fly larvae-fed compared to rice-fed birds (0.04±0,02% *vs.* 0.32±0.12%, respectively; p=0.04), which translated to significantly lower fractional desaturation rates (0.49±0.24% *vs.* 3.82±1.47%, for fly larvae- and rice-fed birds, respectively). Finally, based on the comparison of FA α and methyl hydrogen enrichments, there was no significant FA elongation activity associated with either diet.

DISCUSSION

This study demonstrated 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 $^{2}H_2O$ to birds has been to effectively measure body composition (lean *vs.* fat) through the deuterium dilution method (McWilliams and Whitman, 2013). But, by following ^{2}H incorporation into subcutaneous fat TAG, this study provided 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 $^{2}H_2O$ 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 proposes is the primary driver of biodiversity loss throughout the world (Newbold 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 to 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 display 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}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 (NEFA), very-low density lipoprotein (VLDL) 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 to increase 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 $^{2}H_2O$ 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 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}H NMR spectroscopy when compared with other analytical techniques like 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. On the contrary, the proportion of n-3 PUFA found in breast muscle was much higher (Pierce et al., 2004; Price and Guglielmo, 2009). Since most of them are essential for several physiologic functions including flight exercise, the need to prioritize muscular function surpasses its capacity to accumulate them under a lipid poor diet. Mobilization of such FA is facilitated by lipoprotein shuttles, translocases and fatty acid 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*, it was proposed 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) (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. As for the most part, these studies relied on a FA balance approach, which analyses 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}H- or ^{13}C-palmitate), $^{2}H_2O$ allows ^{2}H-enrichment to rapidly equilibrate with total body water and distribute homogeneously within tissues. Even if ^{2}H atoms can be exchanged with other organic pools beyond the biochemical routs of lipid metabolism, providing the birds with deuterated drinking water between the IP injection and tissue sampling assured a consistent labelling capacity as confirmed by the final body water ^{2}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}H-enrichment levels of specific FA compared to the presented aggregated analysis of enrichment by ^{2}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.

Abbreviations

^{2}H, deuterium; $^{2}H_2O$, deuterated water; fractional synthetic rate, FSR; fatty acid (FA); mass spectrometry (MS); nuclear magnetic resonance (NMR); polyunsaturated fatty acid, PUFA; saturated fatty acid, SFA; triglycerides (TAG); unsaturated fatty acid, UFA.

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Competing interests

All authors approved the submission of this manuscript and have no conflicts of interest, financial or otherwise, to declare.

Author contributions

IV, PMA and JAA conceived and designed the experiment; ADR and JAA organized fieldwork and AV and JAM kept birds in captivity; PMA, ADR, AV, JAR, JAM and JAA performed the experiment; IV, PMA and JGJ performed the lab work; IV, JGJ and JAA analysed the data; IV, PMA and JAA led the writing of the manuscript with substantial inputs from all other authors.

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Figures

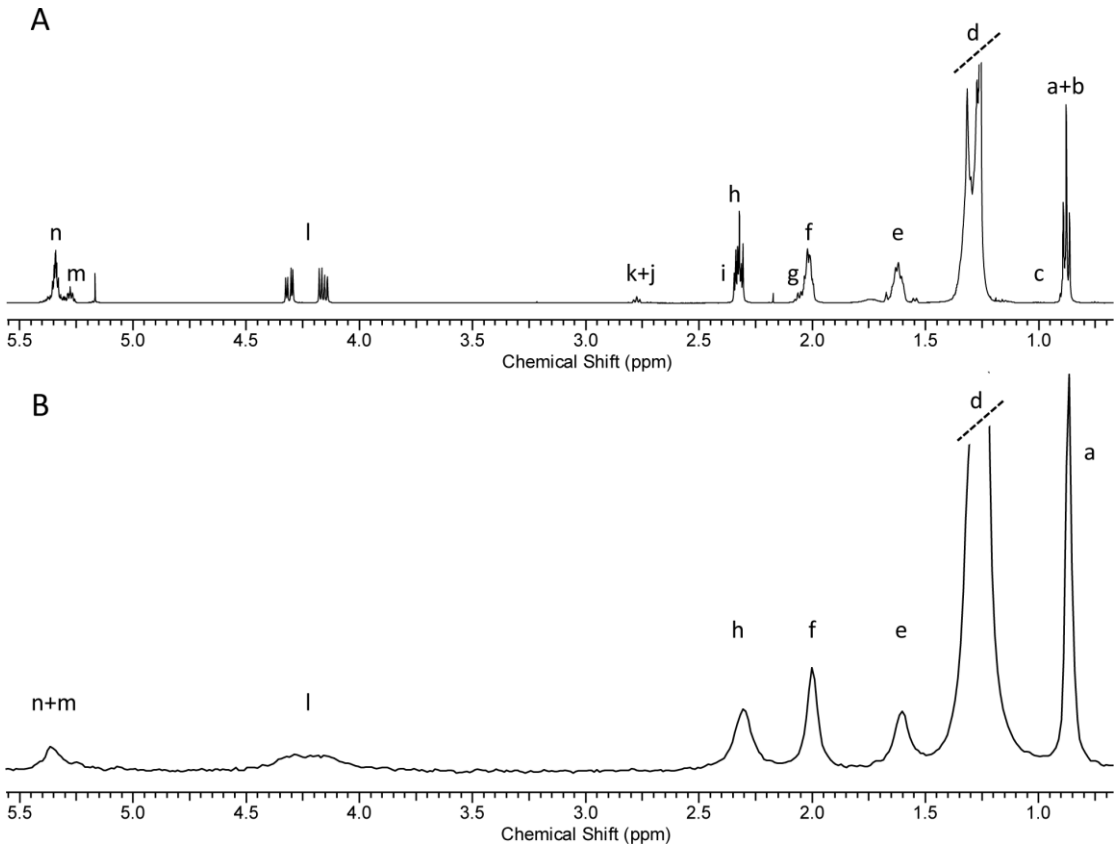

Figure 1. Representative ^{1}H (A) and ^{2}H (B) NMR spectra of subcutaneous fat triglycerides from black-tailed godwits submitted to two different dietary treatments in captivity and to $^{2}H_2O$ administration for 24 h. Assigned peaks as a: Non n-3 methyls; b: Partial n-6 methyls; c: n-3 methyls; d: Aliphatic chain methylenes; e: β methylenes; f: MU allylic hydrogens; g: PU 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 TG-bound glycerol; m: sn-2 of TG-bound glycerol; n: Olefinic hydrogens. Off the spectra: Chloroform (solvent; singlet at 7.25 ppm) and Pyrazine (internal standard; singlet at 8.50 ppm).

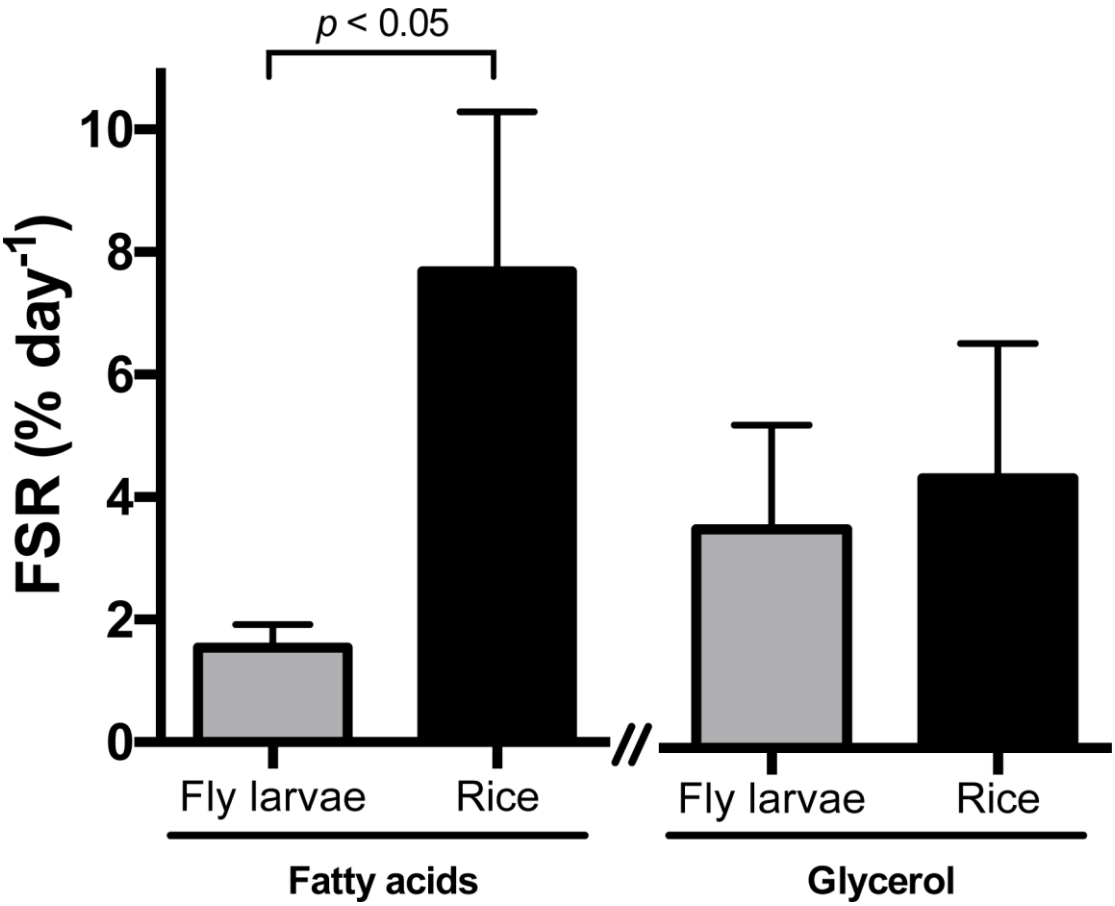

Figure 2. Triglyceride-bound fatty acid and glycerol fractional synthetic rates (FSR) expressed as percent per day of subcutaneous fat triglycerides from black-tailed godwits submitted to two different dietary treatments in captivity and to $^{2}H_2O$ administration for 24 h. Mean values ± SEM are presented (n=6). Differences between dietary treatments are indicated (t-test, $p<0.05$).

Table 1. Proximate composition and lipid species as determined from ^{1}H NMR spectra of the two experimental diets provided to black-tailed godwits in captivity.

Proximate composition	Fly larvae [1]	Rice [2]
Dry matter (DM; %)	30.8	88.7
Ash (%)	3.9	3.6
Gross energy (kJ g^{-1} dry weight)	22.6	17.1
Crude protein (% of DM)	52.3	7.5
Crude fat (% of DM)	20.8	2.0
Crude fiber (% of DM)	6.6	9.6
Starch (% of 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	nd

[1] Live fly larvae from *Protophormia terraenovae* - Comercial Las Grullas, Badajoz, Spain

[2] Unprocessed rice seeds harvested in Extremadura, Spain

nd: not detected.

Table 2. Somatic and blood parameters from black-tailed godwits submitted to two different dietary treatments in captivity. Mean values ± SEM are presented (n=6). No differences between dietary treatments were found for any of the parameters (t-test, $p>0.05$).

	Fly larvae	Rice
Initial weight (g)	250.0±19.7	253.8±14.6
Final weight (g)	265.8±18.4	258.0±16.1
Blood glucose (mg/dL)	223.3±27.2	205.0±12.9
Triglycerides (mg/dL)	69.1±17.4	63.6±7.6
Weight fat sample (mg)	12.9±2.6	13.1±1.8
Body water ^{2}H-enrichment (%)	7.5±0.5	8.8±0.3

Table 3. Lipid species and chemical structure of esterified fatty acids as determined from ^{1}H NMR spectra of subcutaneous fat triglycerides from black-tailed godwits submitted to two different dietary treatments in captivity. Mean values ± SEM are presented (n=6). Differences between dietary treatments are indicated by asterisks (t-test, $^{*}p<0.05$, $^{**}p<0.01$; $^{***}p<0.001$; ns: not significant; nd: not detected). Chemical structure: FA were considered polymers of olefinic (HC=CH) and methylenic (CH_2) subunits, [i.e. $^{-}OOC–(CH_2)_x–(HC=CH)_y–CH_3$], calculated as in Viegas et al. 2016.

Lipid species	Fly larvae	Rice
% SFA	37.7±3.6	36.8±0.6 ^{ns}
% UFA	62.3±3.6	63.2±0.6 ^{ns}
% PUFA	15.3±3.0	6.8±0.5 *
% MUFA	47.0±2.1	56.4±0.3 **
% n-3	0.4±0.2	nd
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 ^{ns}
Methylenic units (CH_2)	12.6±0.1	13.9±0.1 *
FA/Glycerol	3.1±0.1	3.0±0.0 ^{ns}