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Targeted ^{13}C enrichment of lipid and protein pools in the body reveals circadian changes in oxidative fuel mixture during prolonged fasting: A case study using Japanese quail

Marshall D. McCue [a,*], James A. Amaya [a], Alice S. Yang [a], Erik B. Erhardt [b], Blair O. Wolf [c], David T. Hanson [c]

[a] St. Mary's University, Department of Biological Sciences, San Antonio, TX 78228, USA
[b] University of New Mexico, Department of Mathematics and Statistics, Albuquerque, NM 87131, USA
[c] University of New Mexico, Department of Biology, Albuquerque, NM 87131, USA

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ABSTRACT

Many animals undergo extended periods of fasting. During these fasts, animals oxidize a ratio of macronutrients dependent on the nutritional, energetic, and hydric requirements of the fasting period. In this study, we use Japanese quail (*Coturnix coturnix japonica*), a bird with natural intermediate fasting periods, to examine macronutrient use during a 6 d fast. We raised groups of quail on isotopically labeled materials (^{13}C-1-leucine, ^{13}C-U-glucose, or ^{13}C-1-palmitic acid) with the intent of labeling specific macronutrient/tissue pools in each treatment, and then traced their use as fuels by measuring the δ^{13}C values of breath CO_2. Based on changes in δ^{13}C values during the fast, it appears that the carbohydrate label, ^{13}C-U-glucose, was largely incorporated into the lipid pool and thus breath samples ultimately reflected lipid use rather than carbohydrate use. In the lipid treatment, the ^{13}C-1-palmitic acid faithfully labeled the lipid pool and was reflected in the kinetics δ^{13}C values in breath CO_2 during the fast. Endogenous lipid oxidation peaked after 24 h of fasting and remained constantly elevated thereafter. The protein label, ^{13}C-1-leucine, showed clear diurnal periods of protein sparing and degradation, with maximal rates of protein oxidation occurring at night and the lowest rates occurring during the day time. This stable isotope tracer method provides a noninvasive approach to study the nutrient dynamics of fasting animals and should provide new insights into how different types of animals use specific nutrient pools during fasting and possibly other non-steady physiological states.

1. Introduction

Many vertebrate animals naturally endure prolonged periods of fasting during their annual cycle. Researchers have identified potentially adaptive physiological strategies used to survive starvation in several groups of animals including penguins (Robin et al., 1988; Castellini and Rea, 1992; Groscolas and Robin, 2001), marine mammals (Castellini and Costa, 1990; Champagne et al., 2005; Wheatley et al., 2008), migratory passerines (Lindstrom et al., 2000; Karasov et al., 2004; Jenni-Eiermann and Jenni, 2012), and ambush foraging reptiles (Secor and Diamond, 2000; McCue, 2007; McCue et al., 2012). In general these may include *supply-side strategies*, where large amounts of nutrients are stored in the body in anticipation of food limitation, or *demand-side strategies*, where animals reduce their energy requirements in the face of food limitation. Animals that may be considered 'fasting adapted' often employ both of these strategies to different degrees, but no animals can avoid having to oxidize their own body tissues when food is unavailable. Virtually all of the fasting animals studied to date apparently switch among oxidizing different metabolic substrates to meet energy demands. According to the current paradigm, they first oxidize their carbohydrate stores followed by their lipid stores (Castellini and Rea, 1992; Navarro and Gutierrez, 1995; Wang et al., 2006). As fasting continues and lipids become depleted, they increasingly catabolize and oxidize endogenous proteins, which eventually lead to organ-failure and death.

The time-course of these fasting-induced shifts in metabolic fuels can vary widely among species and can be difficult to identify using traditional physiological measures including changes in body mass, blood metabolites, nitrogen excretion, and respiratory exchange ratios (McCue, 2010). A new approach to track the changes in oxidative fuel mixture during fasting is tested, whereby different nutrient pools in the body (*i.e.*, carbohydrates, lipids, and proteins) that are artificially enriched with stable isotopes (*e.g.*, ^{13}C) was recently described (McCue, 2011, 2012). So far, only two studies have implemented this experimental technique. In one experiment, a population of house sparrows was given oral gavages of ^{13}C-glucose, ^{13}C-palmitic acid, or ^{13}C-leucine and subsequently fasted for 24 h during which their rates of $^{13}CO_2$ production were monitored (Khalilieh et al., 2012). The conclusion that sparrows may be unable to partition among different endogenous nutrient pools was likely confounded by the fact that the bolus of exogenous ^{13}C-tracers did not have sufficient time to become fully

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[*] Corresponding author. Tel.: +1 210 431 8005.
E-mail address: mmccue1@stmarytx.edu (M.D. McCue).

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integrated into the tissue pools. In the other experiment, three populations of mice were raised to adulthood on diets enriched with ^{13}C-glucose, ^{13}C-palmitic acid, or ^{13}C-leucine tracers and then fasted for 72 h (McCue and Pollock, 2013). That study revealed clear transitions in nutrient partitioning during starvation including a crash in carbohydrate oxidation followed by a coincidental spike in lipid oxidation and protein sparing. As fasting progressed, the mice exhibited a gradual transition toward increased reliance on protein catabolism and oxidation. These sequential changes in substrate oxidation were generally similar to those seen in animals able to tolerate comparatively long periods of fasting although they occurred over a much shorter time scale in mice. Given our existing data sets, it remains unclear whether this sequence in fuel switching is part of some universal starvation response.

Compared to birds that have been identified as being relatively well adapted to fasting (penguins) and birds that may succumb to starvation after one day (house sparrows), quail probably exhibit some intermediate ability to tolerate starvation. The goal of the present study was to examine fasting-induced changes in fuel oxidation over these intermediate periods (up to 6 d), in Japanese quail using ^{13}C-labeling of body tissues and analysis of exhaled breath $^{13}CO_2$. Japanese quail have become a popular model organism for studying the physiological effects of food limitation (Sartori et al., 1995), particularly in context of their ability to exhibit starvation-induced heterothermy to minimize energy expenditure (Hohtola et al., 1991; Ben-Hamo et al., 2010; Hohtola, 2012), yet little is known about fuel switching in these animals during fasting. Quail are known to tolerate starvation for as long as 21 d (Sartori et al., 1995), however most studies focus responses to more ecologically relevant periods lasting up to one week.

In order to ensure that their tissues became isotopically enriched, the quail in this study were raised from chicks on diets supplemented with one of three artificially ^{13}C-labeled molecules (*i.e.*, fatty acid, amino acid, or monosaccharide). The idea for this experimental approach was developed following studies where pigeons were subjected to a switch between C3- and C4-plant based diets and exhaled ^{13}C values were used to partition between endogenous and exogenous nutrient oxidation in pigeons (Hatch et al., 2002a, 2002b). The major difference is that this methodology avoids the 'scrambled egg premise' (*sensu* (Kelly and Martinez del Rio, 2010; Saris et al., 1993; Van Der Merwe, 1982)) where ^{13}C atoms are distributed roughly equally among classes of macronutrients, and it can thus be used to track the fates of individual classes of macronutrients. We hypothesized that changes in $^{13}CO_2$ excretion during fasting would enable us to identify changes in the types of endogenous substrates that quail oxidized as fasting progressed and that these changes would fit the typical pattern of fuel switching in animals. In particular, we predicted that $^{13}CO_2$ excretion in ^{13}C-glucose-raised quail would be elevated during the initial phases of starvation and would be followed by a clear period of elevated $^{13}CO_2$ excretion in the ^{13}C-palmitic-acid raised quail. We also predicted that $^{13}CO_2$ excretion would remain low in the ^{13}C-leucine-raised quail during most of the fasting period and then rise exponentially during the latter phases, although we did not have any clear predictions about the specific timing of each of these events. We also monitored changes in body mass (m_b), body temperature (T_b), and circulating metabolites during fasting to observe how changes in these variables overlapped with changes in substrate oxidation.

2. Materials and methods

2.1. Animals

Day-old, male Japanese quail, *Coturnix japonica* ($n = 60$) were purchased from Diamond H Ranch in Bandara, Texas in November 2012. The chicks were raised communally in the laboratory under a photoperiod of 14 h light and 10 h dark and relative humidity of 20–40% in the laboratory. Ambient temperature was held at 35 °C during the first week and then reduced to 33 °C during the second week. Nature Wise Chick Starter (Nutrena, Minneapolis, MN, USA) crumbles and water were provided *ad libitum*. At two weeks of age (approximately 60–70 g), each quail was uniquely marked with numbered plastic leg bands and relocated into 80 × 40 × 60 cm (L × W × H) stainless steel cages where they were maintained in smaller groups of 4 to 6. The ambient temperature was reduced to 30 °C but the photoperiod and relative humidity remained the same during the remainder of the study.

2.2. Nutrient oxidation and $\dot{V}CO_2$ trial

At two weeks of age, 36 quail chicks were randomly selected to participate in the isotope enrichment trial. This population was further divided into three experimental groups (*i.e.*, leucine, glucose, or palmitic acid) containing 10 quail each and a control group consisting of six quail. All of these quail continued to consume the chick crumble diet over the subsequent six weeks, but the diets of the quail belonging to the experimental groups were enriched with one of three isotope tracers, ^{13}C-1-leucine, ^{13}C-U-glucose, or ^{13}C-1-palmitic acid (99% pure; Cambridge Isotopes, Cambridge MA, USA), with the intent of specifically labeling the protein, carbohydrate and lipid pools, respectively. The ^{13}C-U-glucose was dissolved in tap water at a concentration of 250 mg/L and provided *ad libitum* to the glucose group. The ^{13}C-1-leucine was delivered by mixing 500 mg of crystalline leucine kg^{-1} of food available *ad libitum* to the leucine group. The hydrophobic ^{13}C-1-palmitic acid was dissolved in a minimal volume of ethanol and then atomized through a 30-gauge syringe needle over a thin layer of crumbles at 225 mg palmitic acid kg^{-1} of food. The ethanol was allowed to completely evaporate before the food was presented to the birds. ^{13}C-1-leucine and ^{13}C-1-palmitic acid were chosen as the amino acid and fatty acid tracers, respectively, because of minimal extent to which ^{13}C atoms become incorporated into different classes of nutrients once inside the body (McCue and Pollock, 2013); hereafter referred to as '*^{13}C-leakage*'. We recently measured negligible leakage of ^{13}C atoms derived from ^{13}C-1-leucine and ^{13}C-1-palmitic acid into the lipid and lean mass pools in the body, respectively, in chickens chronically exposed to similar, isotopically enriched diets (McCue et al., 2013).

Between eight and nine weeks of age, food was removed at 0800 h so the quail would become postabsorptive (McCue, 2006; Secor, 2008). At 1200 h the quail were placed into individual metabolic chambers (15 cm × 10.5 cm × 10.5 cm) (Lock & Lock, Hana Cobi, Korea) lined with a cardboard floor. Dry, CO_2-free air was constantly ventilated through each chamber (1000–1200 mL min^{-1}). Inlet and outlet ports were staggered on opposite sides of each chamber at quail eye-level to maximize gas mixing within the chambers (McNab, 2006). Ambient temperature was maintained at 30 °C within the thermal neutral zone of this species (Ben-Hamo et al., 2013; Burness et al., in press). Excurrent gas was serially subsampled (150 mL min^{-1}) from each chamber every 30 min using a programmable multiplexer (RM-8; Sable Systems International, Las Vegas, NV, USA) and diverted into a water vapor analyzer (RH-300; Sable Systems International) followed by a CO_2 analyzer (CD-3A; Applied Electrochemistry, Sunnyvale, CA, USA). $\dot{V}CO_2$ was calculated using standard equations (Lighton, 2008) and reported in STPD; $\dot{V}CO_2$ was not measured.

Every 4 h, during 6 d of fasting, we manually collected subsamples (~20 mL) of excurrent gas from the metabolic chambers of all of the birds in the experimental groups using a 50 mL gas-tight, glass syringe (Cadence Inc.; Staunton, VA, USA), and a 20-gauge stainless steel needle inserted into a resealable silicon injection port. The gas samples were injected into evacuated 12 mL Exetainers (Labco Limited, UK) until the contents of each vial were under positive pressure. Vials were stored at room temperature for up to 8 weeks until δ^{13}C-analysis. The fasting quail were allowed to drink for 15 min every 12 h after which their body mass (m_b) was measured to ±0.1 g and their metabolic chambers were cleaned. The quail were then returned to their respective metabolic chambers to continue fasting. After 144 h (6 d) of fasting the quail were euthanized *via* decapitation, except in one case where a subset of quail were fasted for seven days (see Discussion). Breath samples of the control birds were only collected at time zero and used to calculate AFE in Eq. (2) and were then euthanized.

2.3. ^{13}C analyses

A subsample (~5 mL) from each Exetainer was removed using a gas-tight syringe and injected into a tunable diode laser absorbance spectrometer (TDL, TGA100, Campbell Scientific, Logan, UT, USA) in a manner similar to that of Engel et al., 2009. Briefly, the TDL injection port and sample cell were maintained under light vacuum (~20 mbar) and continuously sampling a CO_2-free air stream at a rate of 100 mL min^{-1}. Samples were injected into the CO_2-free air stream and the absolute concentration of $^{13}CO_2$ and $^{12}CO_2$ in the sample was recorded at a frequency of 10 Hz (see Barbour et al., 2007 for a description of the TDL system). Every 20 min the TDL was programmed to change inlets and sample two calibration gas cylinders containing known isotopic compositions ($\delta^{13}C$) and CO_2 concentrations. The $\delta^{13}C$ of the exhaled CO_2 samples were calculated using an R package (Erhardt and Hanson, 2013). In this package, the periodic measurements of the calibration gasses (high and low) were interpolated using a cubic smoothing spline to account for slow drifts throughout the measurement period; the sample concentrations were calibrated using a gain and offset determined from the mean interpolated tank values. Finally, the $^{13}C/^{12}C$ ratios were converted to $\delta^{13}C_{VPDB}$ units using the following equation (Craig, 1957):

$$\delta^{13}C_{VPDB} = \left(\frac{\left(^{13}C/^{12}C\right)_{sample} - \left(^{13}C/^{12}C\right)_{std}}{\left(^{13}C/^{12}C\right)_{std}}\right) \cdot 10^3 \tag{1}$$

where $(^{13}C/^{12}C)_{sample}$ is the ratio of ^{13}C to ^{12}C atoms in a sample and $(^{13}C/^{12}C)_{std}$ is the ratio of the ^{13}C to ^{12}C atoms in an international standard (VPDB; 0.011237).

We calculated the ^{13}C atom fraction excess (AFE) in the exhaled breath at each time point for quail raised on the ^{13}C-tracers. The AFE values are unitless and were calculated by subtracting isotope values measured in quail from the ^{13}C treatment groups ($\delta^{13}C_{enriched}$) from the mean values measured in quail raised only on the base diet ($\delta^{13}C_{control}$) and calculated using the following equation:

$$AFE = \left[\frac{\left(^{13}C/^{12}C\right)_{std} \cdot \left[\left(\frac{\delta^{13}C_{enriched}}{1000}\right) + 1\right]}{1 + \left[\left(^{13}C/^{12}C\right)_{std} \cdot \left[\left(\frac{\delta^{13}C_{enriched}}{1000}\right) + 1\right]\right]}\right] - \left[\frac{\left(^{13}C/^{12}C\right)_{std} \cdot \left[\left(\frac{\delta^{13}C_{control}}{1000}\right) + 1\right]}{1 + \left[\left(^{13}C/^{12}C\right)_{std} \cdot \left[\left(\frac{\delta^{13}C_{control}}{1000}\right) + 1\right]\right]}\right]. \tag{2}$$

The changes in the isotopic enrichment of exhaled carbon dioxide, alone, provide no direct information about the actual rates of $^{13}CO_2$ production. We therefore calculated the fasting-induced changes in the rates that the ^{13}C-labeled tracers were oxidized (T; mol min^{-1}) using the following equation modified from McCue et al. (2011):

$$T = \left[\frac{AFE \times VCO_2}{k \times m \times \theta}\right] \tag{3}$$

where VCO_2 is the carbon dioxide production (mL CO_2 min^{-1}), k is the volume of CO_2 (mL) produced per gram of tracer oxidized (Romijn et al., 1992; Welch et al., 2008; McCue et al., 2010), m is the molar mass of each tracer, and θ is the number of isotopically enriched atoms per tracer molecule (note: $\theta = 6$ for ^{13}C-U-glucose).

2.4. Temperature and plasma metabolites trial

The remaining 24 quail chicks were used in the plasma metabolites and body temperature trial and were raised on chick crumbles (with no artificial ^{13}C supplementation) for the subsequent six weeks. At 4 weeks of age, each bird had a temperature-sensitive radio-frequency identification (RFID) microchip (Life Chip; Destron Fearing) injected subcutaneously into the lower abdominal region using a sterile syringe provided by the manufacturer. The birds were allowed to recover for two weeks after the tag injection.

At eight weeks of age food and water were removed from these birds at 0800 h, and at 1200 h they were individually placed into translucent metabolic chambers (as described in Section 2.2) under the same environmental conditions in order to minimize variation between this trial and the isotope trial; gas exchange was not recorded in this trial. The subcutaneous skin temperatures (hereafter: *body temperatures*: T_bs) were measured every 12 h through the walls of the chambers using a handheld RFID reader. Every 12 h 100 μL of blood was collected from seven quail into a non-heparinized capillary tube by puncturing the brachial vein with a sterile, 26-gauge hypodermic needle. Plasma glucose and β-hydroxybutyrate were measured using a Precision Xtra meter (Abbott Laboratories, Abbott Park, IL, USA) and triacylglycerol (TAG) levels were measured using a CardioChek Monitor (Polymer Technology Systems, Inc., Indianapolis, IN, USA) (Khalilieh et al., 2012). The birds were rotated for blood sampling and repeated sampling events on individuals were separated by a minimum of 36 h. These quail were then allowed to drink for 15 min and body mass (m_b) was measured to ±0.1 g while their metabolic chambers were cleaned. After 144 h (6 d) of fasting these quail were euthanized *via* decapitation.

2.5. Statistical methods

The daytime T_bs taken at the same time point each day (*i.e.*, 1200 h) were compared using repeated measures ANOVA. Fasting T_bs were also compared to postabsorptive, prefasting values at each time point using Holm–Sidak multiple comparisons tests. Plasma metabolites were compared across 12-h intervals using Kruskal–Wallis ANOVA on ranks. The $\delta^{13}C$ of exhaled CO_2 were compared across 4-h intervals within treatment groups using Kruskal–Wallis ANOVA on ranks. Dunn's Q tests were used to compare the postabsorptive, prefasting $\delta^{13}C$ values with the fasting values at each time point. The $\delta^{13}C$ values collected from a subset of the quail between 144 and 168 h were not used for statistical analyses although they are presented graphically (see Discussion for explanation). Means are presented ±1 standard deviation (s.d.) and $\alpha = 0.05$ was chosen as the level of significance. All statistical analyses were done with SigmaPlot 12.0 (Systat Software, San Jose, CA, USA).

3. Results

3.1. Body mass and $\dot{V}CO_2$

Quail lost 20.7 ± 1.6% of their initial body mass during the 6 d of fasting (Fig. 1A). The mean $\dot{V}CO_2$ decreased by approximately 20% during the first 4 h and continued to decrease through the first night (Fig. 2A). In the morning the $\dot{V}CO_2$ increased above nighttime values, but the magnitude of this pattern diminished as fasting progressed and the $\dot{V}CO_2$ during the final 48 h of the experiment showed no recognizable circadian pattern. Mass-specific $\dot{V}CO_2$ fell sharply during the first 4 h, but thereafter remained relatively constant over the remaining 6 d (Fig. 2B).

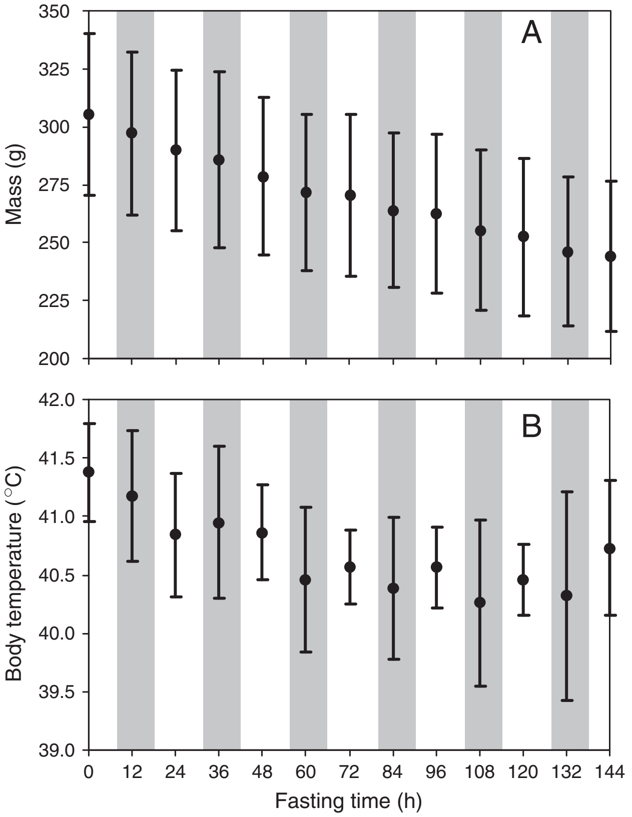

Fig. 1. A) Body masses (mean ± s.d.) of adult quail during 6 d of complete fasting ($n = 60$). B) Subcutaneous body temperatures (mean ± s.d.) of adult quail during 6 d of complete fasting ($n = 21$) measured using implantable RFID tags. Note that food was removed from the birds at 0800 to allow them to become postabsorptive and fasting time 0 h refers to 1200 on day 1.

3.2. *Nutrient oxidation and* $\dot{V}CO_2$ *trial*

Five of these birds were removed from the isotope enrichment trial after experiencing lacerations from aggressive cage-mates; the final sample sizes for three the experimental groups were: leucine, $n = 8$; palmitic acid $n = 10$; and glucose $n = 7$. The $\delta^{13}C$ in the exhaled CO_2 of the postabsorptive, prefasting quail raised on the ^{13}C-labeled glucose was $-15.0 \pm 2.3‰$ (Fig. 3A). Fasting had a significant effect on $\delta^{13}C$ of exhaled CO_2 (ANOVA on ranks, $df = 36$, $H = 96.881$, $p < 0.001$) and $\delta^{13}C$ values from the glucose quail were significantly more enriched at all fasting time points than postabsorptive, prefasting values (Dunn's, $Q = 2.785–4.767$, $p < 0.05$ in all cases).

The $\delta^{13}C$ in the exhaled CO_2 of quail raised on the ^{13}C-labeled palmitic acid had a postabsorptive, prefasting $\delta^{13}C$ value of $-14.3 \pm 0.9‰$ (Fig. 3A) that also changed as a result of fasting (ANOVA on ranks, $df = 36$, $H = 128.654$, $p < 0.001$). Similar to the glucose group, the CO_2 of the birds raised on the palmitic acid tracer became isotopically enriched in ^{13}C at all fasting time points (Dunn's, $Q = 3.083–5.402$, $p < 0.05$ in all cases), suggesting a general increase in the rate of endogenous lipid oxidation (see Discussion).

The $\delta^{13}C$ of the exhaled CO_2 of the postabsorptive, prefasting quail raised on the ^{13}C-leucine tracer was $-10.4 \pm 2.1‰$ (Fig. 3B) and significantly changed as a result of fasting (ANOVA on ranks, $df = 36$, $H = 59.200$, $p = 0.009$). Specifically, the $\delta^{13}C$ in the exhaled CO_2 of fasting quail became more depleted in ^{13}C than postabsorptive, prefasting values (Dunn's, $Q = 3.038–4.590$, $p < 0.05$ in all cases), suggesting a general reduction in the rate of endogenous protein oxidation (see Discussion).

The mean rate of $^{13}CO_2$ excretion in the quail raised on the ^{13}C-palmitic acid tracer peaked at approximately 20 h during which the rate of ^{13}C-palmitic acid oxidation was nearly 1 nMol min^{-1} greater than the postabsorptive, prefasting rates (Fig. 4A). Between 24 and 144 h of fasting the rates of ^{13}C-palmitic acid oxidation gradually decreased but always remained more than 0.5 nMol min^{-1} greater than the postabsorptive, prefasting rates. The apparent rates of ^{13}C-glucose oxidation were not calculated because of the peculiar similarities between the $\delta^{13}C$ profiles in the breath of quail belonging to the glucose and palmitic acid treatment groups (see Discussion for explanation).

The mean rate of ^{13}C-leucine oxidation decreased sharply during the first 8 h of fasting, exhibiting rates that were at least 1 nMol min^{-1} lower than the postabsorptive, prefasting rates (Fig. 4A). During the first night of fasting this rate of oxidation further diminished, but then increased the next day. Thereafter, a distinct circadian pattern was observed over the subsequent 5 d of fasting whereby the daily maximal rate of ^{13}C-leucine oxidation occurred in the middle of the night and the daily minimum rate of ^{13}C-leucine oxidation occurred in the morning.

3.3. *Body temperature and plasma metabolites*

The daytime T_b differed significantly as a result of fasting (RM-ANOVA; $df = 6$, $F = 10.822$, $p < 0.001$) and the daytime T_b during fasting was always lower than in the postabsorptive, prefasting birds (Holm–Sidak, $t = 3.907–6.946$, $p < 0.001$ in all cases; Fig. 1B).

Plasma glucose concentrations significantly changed during fasting (ANOVA on ranks; $df = 12$; $H = 21.924$; $p = 0.038$; Fig. 5A). *Post hoc* analyses indicated that fasting values were significantly lower than postabsorptive, prefasting values at 12 h (Dunn's $Q = 3.504$, $p < 0.05$) and 24 h (Dunn's $Q = 2.912$, $p < 0.05$). Levels of plasma ketone bodies significantly changed during fasting (ANOVA on ranks; $df = 12$, $H = 44.988$, $p < 0.0001$; Fig. 5B). All fasting values were significantly higher than the postabsorptive, prefasting values (Dunn's Q, $p < 0.05$ in all cases). Unlike the concentrations of glucose and ketone bodies the plasma TAG values were not normally distributed (Shapiro–Wilk, $p < 0.05$) and did not vary significantly over time (ANOVA on ranks; $df = 12$, $H = 15.460$, $p = 0.217$; Fig. 5C). No transformations or further analyses were done on these TAG data (see Discussion for explanation).

4. Discussion

4.1. *Changes in body mass, body temperature, and* $\dot{V}CO_2$

With a few exceptions, nearly all animals showed reduce body mass during fasting. The rate of mass loss over 6 d of fasting in this study (~21%) was similar to those previously reported for quail fasting for 3 d (~13%; Laurila et al., 2005) or 4 d (~13%; Ben-Hamo et al., 2010), lower than reported for larger species including yellow-legged gulls (~15% over 8 d (Alonso-Alverez and Ferrer, 2001)) and greater than snow geese (~44% over 34 d (Boismenu et al., 1992)). It is noteworthy that we did not see evidence of differential changes in mass loss (Fig. 1A) that could be used to delimit phase transitions as has been reported for penguins (Le Maho et al., 1988); rather, the general pattern of mass loss in quail followed a curvilinear response similar to that previously documented in fasting geese (Boismenu et al., 1992).

One of the best documented responses to fasting in Japanese quail is a progressive reduction in nighttime core body temperature that functions to minimize energy expenditure when resources are limited (Hohtola et al., 1991; Underwood et al., 1999; Laurila et al., 2005; Ben-Hamo et al., 2010). In some cases the T_b of fasting quail can decrease by several degrees during the scotophase, although the magnitude of this heterothermy is highly sensitive to ambient temperature (Hohtola et al., 1991; Hohtola, 2012). The observed changes in T_b reported in this study (Fig. 1B) were not as dramatic as those previously reported in fasting quail maintained within their thermoneutral zone (Ben-Hamo et al., 2010; Ben-Hamo et al., 2011), an outcome that is likely

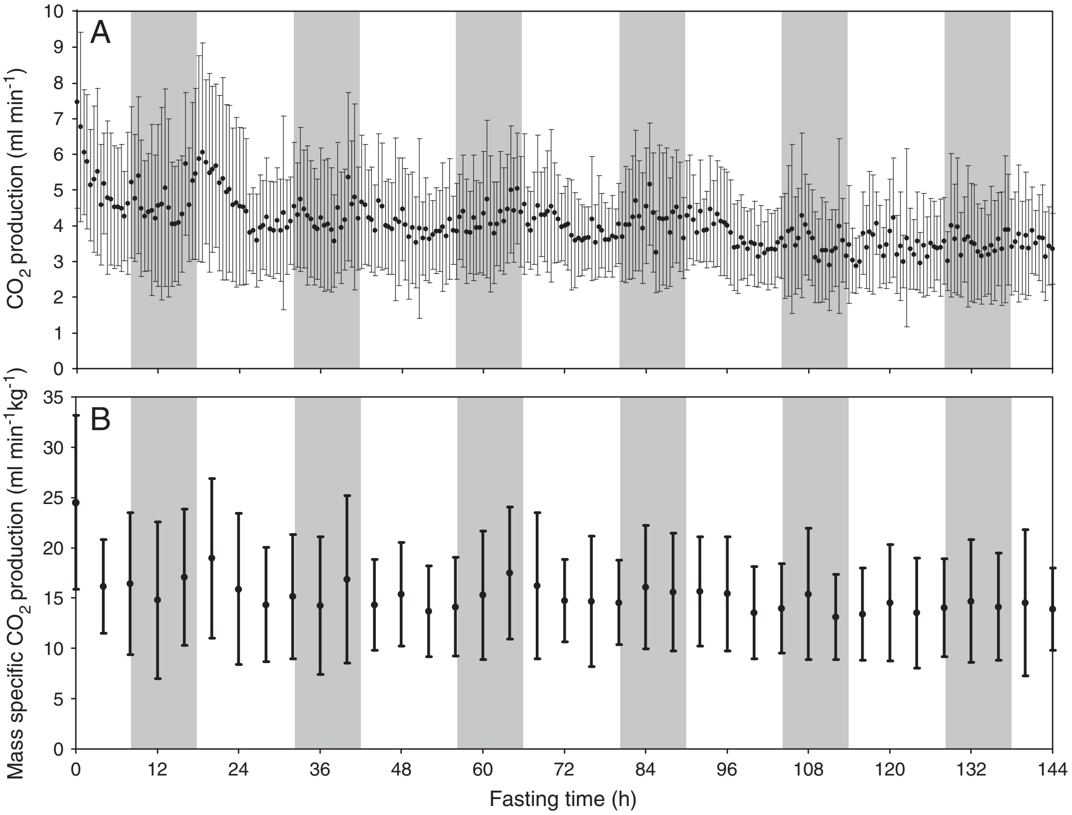

Fig. 2. Carbon dioxide excretion in fasting adult quail (n = 25). A) $\dot{V}CO_2$ (mean ± s.d.) averaged across 30-min intervals (n = 25). B) Mass-specific rates of $\dot{V}CO_2$ (mean ± s.d.) calculated at 4-h intervals (n = 25). Note that food was removed from the birds at 0800 to allow them to become postabsorptive and fasting time 0 h refers to 1200 on day 1.

related to 1) the 12-h sampling intervals (*i.e.*, 1200 and 2400) which did not include time points in the middle of the scotophase and 2) the fact that we were measuring subcutaneous temperatures rather than core body temperatures.

Fasting quail also showed marked changes in resting $\dot{V}CO_2$ (Fig. 2A). Fasting-induced reductions in $\dot{V}CO_2$ can be generally attributed to one or more of the following phenomena. 1) Fasting animals lose body mass; and smaller animals have lower metabolic rates — Dahnel's phenomenon (Calder, 1987; McNab, 1999). 2) Individuals that maintain lower body temperatures have lower metabolic rates (Geiser, 2004; McCue, 2004). 3) Energetically intense activities (*e.g.*, protein turnover (Bauchinger and McWilliams, 2012; Houlihan, 1991; Linares et al., 1992)) and highly active tissues (*e.g.*, those lining the digestive tract (Secor and Diamond, 1997; Starck, 1999)) are down-regulated. 4) The ratio of $\dot{V}CO_2$ to energy expenditure (in Watts) tends to decrease during fasting (Castellini and Rea, 1992; Walsberg and Wolf, 1995; Wang et al., 2006). In this study the greatest reduction in resting $\dot{V}CO_2$ occurred during the first few hours presumably as the quail were becoming habituated to the metabolic chambers. Transient, albeit diminishing, sequential peaks in mean $\dot{V}CO_2$ occurred on the second, third, and fourth mornings (Fig. 2A).

Metabolic rates scale allometrically (McNab, 2002) and smaller bodies tend to have higher mass specific metabolic rates, but in this study the mass-specific $\dot{V}CO_2$ of quail remained relatively constant after the first 12 h of fasting (Fig. 2B). This response coupled with the general continual decrease in body mass and the reduction in T_b after 12 h of fasting raises the possibility that mass specific $\dot{V}CO_2$ corrected for a constant T_b and body mass actually increased during the experiment. Such increases are not uncommon occurrences during prolonged fasting, and can usually be explained in part by changes in body composition — particularly the decrease in fractional lipid content in the body (Caloin, 2004; Price and Valencak, 2012).

4.2. Endogenous substrate oxidation

The artificially ^{13}C-enriched tracers in each of the experimental diets were effective at enriching the body tissues of the quail and measurements of the δ^{13}C of exhaled CO_2 and provided clear evidence that quail altered the rates at which they mobilized and subsequently oxidized different endogenous substrates during prolonged fasting. We expected to observe a rapid increase in δ^{13}C of exhaled CO_2 of ^{13}C-glucose quail during the initial portion of the fasting experiment as those birds metabolized their ^{13}C-labeled glycogen stores. That peak was expected to be followed by a rapid drop in the δ^{13}C of exhaled CO_2 as they shifted to metabolize unlabeled lipids, proteins, and carbohydrates generated from gluconeogenesis. As expected, we found a rapid increase in the δ^{13}C of exhaled CO_2, but we then found no subsequent drop in the δ^{13}C of exhaled CO_2. Although we did not examine the δ^{13}C of lipid tissues in the body we have two lines of evidence to conclude that a large proportion of the ^{13}C-atoms from the exogenous ^{13}C-glucose tracer became incorporated into the lipid pool of the body and did not accurately reflect the oxidative kinetics of endogenous carbohydrates: 1) the strong similarities between the kinetics of $^{13}CO_2$ excretion in the glucose and palmitic acid treatment groups and 2) the unlikely scenario that fasting quail were oxidizing significant amounts of endogenous carbohydrate after several days of fasting (Gannes et al., 2001). Consequently, we do not use the raw δ^{13}C measurements from the glucose quail for any further modeling purposes. We believe the transfer of glucose-derived ^{13}C into different nutrient pools in the body might be minimized in future studies by providing animals with a dose of ^{13}C-glucose tracer only one or two days prior to fasting (*e.g.*, (Gay et al., 1994; Tanis et al., 2003)).

The δ^{13}C-values of the quail raised on ^{13}C-leucine decreased by approximately 3‰ during the first 24 h of fasting (Fig. 3B), and over the next day they further decreased by approximately 1‰. Beginning

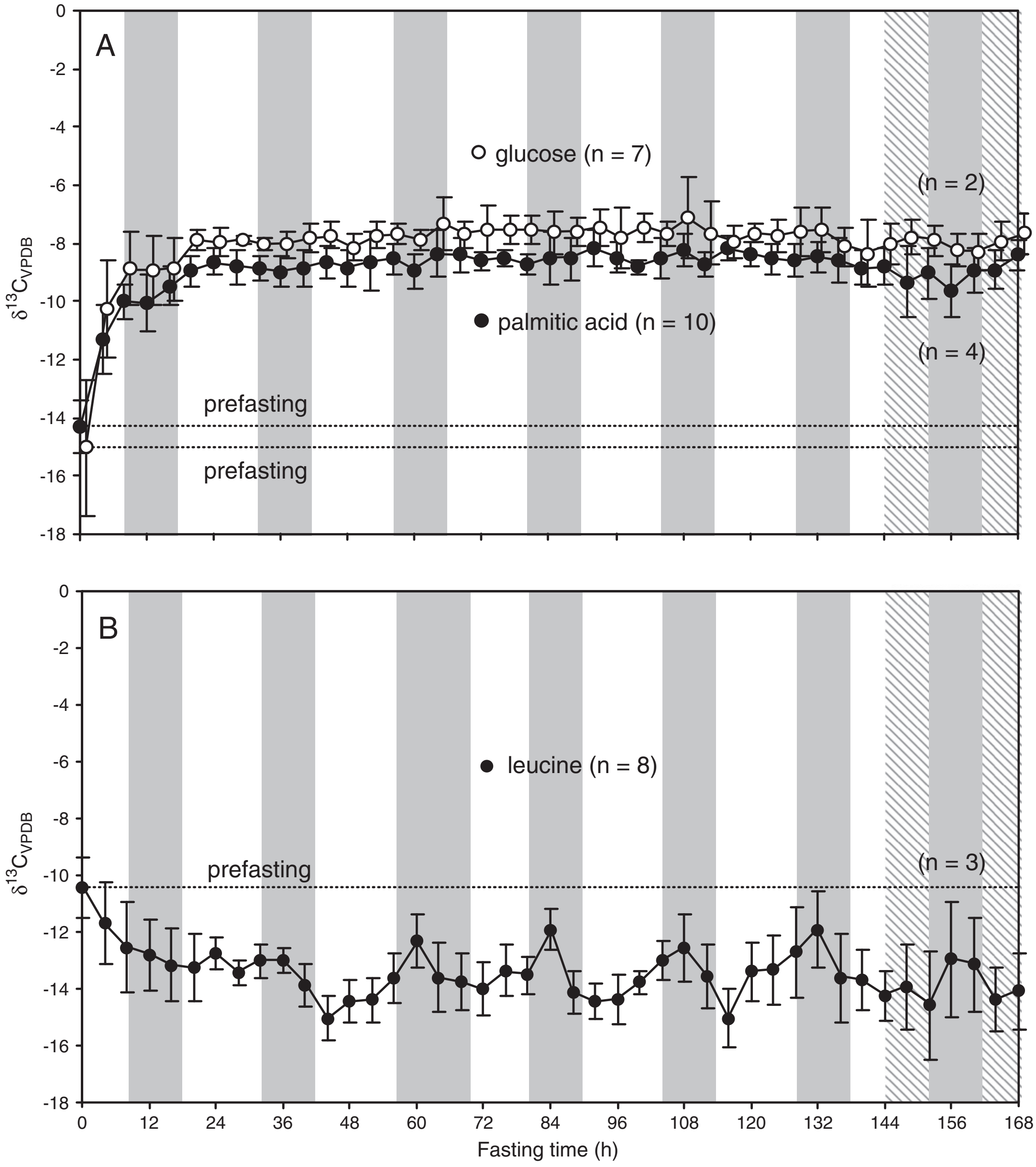

Fig. 3. The isotopic values of carbon in exhaled carbon dioxide of quail fasting for 6 d. A) Closed circles refer to birds raised on a diet supplemented with ^{13}C-palmitic acid tracer ($n = 10$). Open circles refer to quail raised on a diet supplemented with ^{13}C-glucose tracer ($n = 7$). The dashed lines represent the mean $\delta^{13}C$-values of postabsorptive, prefasting birds. B) $\delta^{13}C$ values of exhaled carbon dioxide quail raised on a diet supplemented with ^{13}C-leucine ($n = 8$). The dashed lines represent the mean $\delta^{13}C$-value of postabsorptive, prefasting birds. Food was removed from the birds at 0800 h to allow them to become postabsorptive and fasting time 0 h refers to 1200 h on day 1. Note: the cross-hatched region indicates responses measured in a subset of experimental quail during an additional seventh day of fasting.

on the second night of fasting, these $\delta^{13}C$-values began to exhibit a distinct cycle of ^{13}C enrichment during the scotophase and ^{13}C depletion during the photophase over the remaining 6 d. In the final round of fasting and breath collection we decided to extend the window of $^{13}CO_2$ measurements by 24 h (using $n = 8$ quail) to determine if some unusual physiological change might be occurring immediately after the 144-h time point. The $\delta^{13}C$ values from those quail are presented in Fig. 3A and B, but with no hint at any deviation from the pattern seen in the preceding days. If we assume that the rate of leucine oxidation is proportional to that of total endogenous oxidation (McCue et al., 2012) and that the amount of endogenous carbohydrate oxidation is negligible after 24 h (Gannes et al., 2001), then this periodic pattern of reduced protein oxidation between peak values could function to reduce total protein oxidation by as much as 33% (Fig. 4B).

Compared to mammals, birds are particularly effective at meeting their routine energy requirements through lipid oxidation (Jenni and Jenni-Eiermann, 1998; McWilliams et al., 2004). A review of fuel use in migratory birds suggested that during prolonged fasting, rates of endogenous lipid mobilization and oxidation in birds are likely to be maximized and thus the extent of protein oxidation should be proportional to the birds' metabolic rate (Jenni and Jenni-Eiermann, 1998). The results of this study failed to support this conclusion. During prolonged fasting the quail maintained relatively constant metabolic rates and constantly elevated rates of lipid oxidation. Because the rate of protein oxidation was highly variable over the course of this study, it suggests to us that the extent of protein oxidation may not be tightly coupled to the overall metabolic demands.

4.3. Blood metabolites

The plasma metabolites were not as useful as ^{13}C excretion measurements in tracking sequential changes in substrate oxidation during

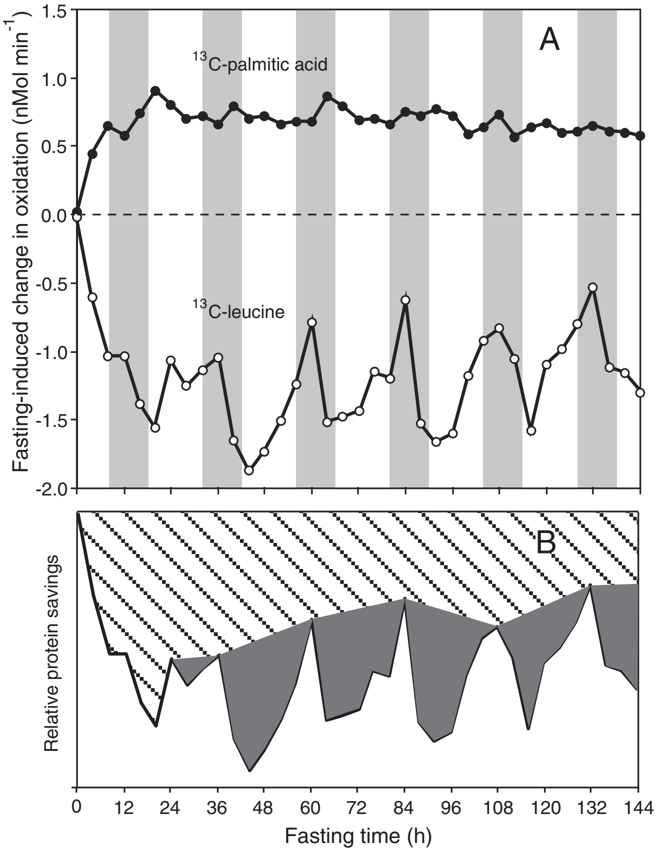

Fig. 4. A) Fasting-induced changes in instantaneous rates of endogenous ^{13}C-palmitic acid (solid circles) or ^{13}C-leucine (open circles). The changes in the rates of the fatty acid and amino acid oxidation are representative of the changes in net rates of mobilization and oxidation of the endogenous pools of lipids and proteins, respectively. Note that food was removed from the birds at 0800 to allow them to become postabsorptive and fasting time 0 h refers to 1200 h on day 1. All fasting values were statistically different from the postabsorptive, prefasting values; see Materials and methods section for calculations. B) The area of the cross hatched region represents the relative reduction in protein oxidation (*i.e.*, the rate of protein oxidation below postabsorptive, prefasting values) in a hypothetical fasting quail that does not exhibit the circadian reductions in protein oxidation seen in this study. The area of the gray region represents the additional relative reduction in protein oxidation exhibited by the actual quail in this study. Note that the extent of protein oxidation is reduced in both cases and that the area of the gray region accounts for 33% of the combined shaded and cross-hatched regions.

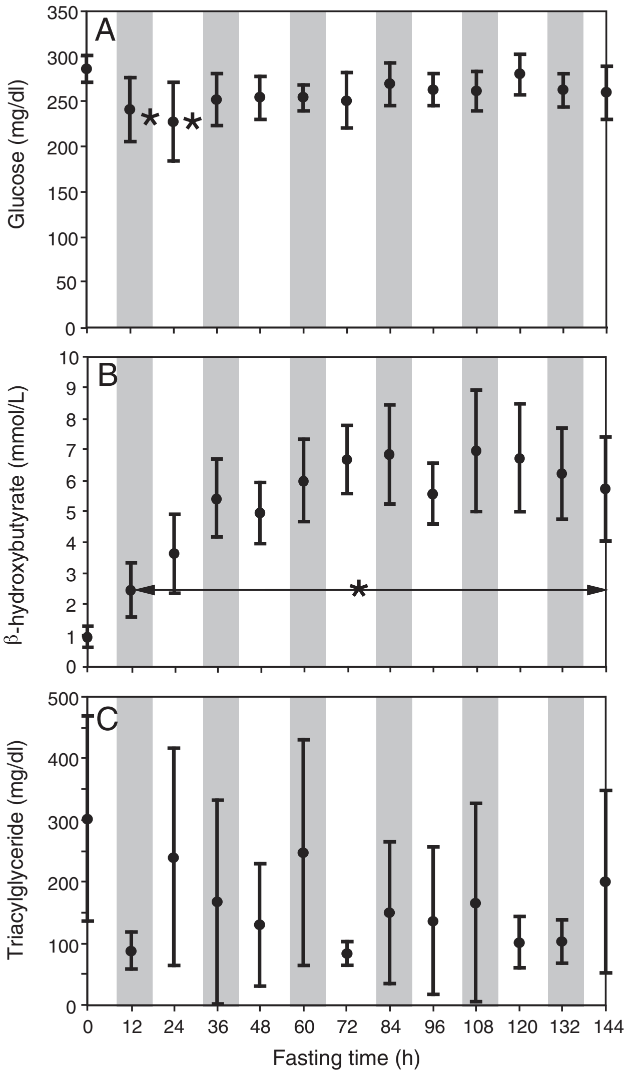

Fig. 5. Plasma metabolite concentrations (mean ± s.d.) in quail fasting for 6 d (n = 7 at each time point). A) Glucose. B) Ketone bodies (β-hydroxybutyrate). C) Triglyceride. Asterisks denote time points where values are statistically different from postabsorptive, prefasting values. Note that food was removed from the birds at 0800 h to allow them to become postabsorptive and fasting time 0 h refers to 1200 h on day 1.

fasting. This result could be related to several factors including: 1) the infrequent sampling intervals [12 h for metabolites *vs.* 4 h for breath samples]; 2) the types of metabolites measured [although newly developed metabolomic approaches would offer a wider range of potential biomarkers]; 3) plasma metabolites do not provide information about turnover rates of molecules (Robin et al., 1987; Thouzeau et al., 1999; Gannes et al., 2001). Nevertheless we were able to identify some significant trends that might be used to circumstantially corroborate some of the fasting-induced changes in nutrient oxidation using ^{13}C-breath testing.

Plasma glucose levels changed as a result of fasting. It is noteworthy that glucose concentrations reported here were consistently higher than those reported in fasting quail by one study (Sartori et al., 1995) and consistently lower than a second study (Sartori et al., 1996), yet similar to those reported in a third, more recent study (Laurila et al., 2005). We have no explanation for these inconsistencies within the literature. Nevertheless, in each of the aforementioned studies the general pattern of plasma glucose regulation was similar with minimal values occurring around 24 h and a complete recovery in plasma glucose levels thereafter (Fig. 5A). It remains unclear whether the transient dip in glucose levels were an effect of decreased carbohydrate availability and/or were a trigger for increased lipid oxidation. Nevertheless, Sartori et al. (1995) reported a sustained, greater than two-fold increase in gluconeogenesis rates in fasting quail between days 2 and 5 that is likely responsible for the recovery in plasma glucose. The ability to tightly regulate blood glucose levels during prolonged fasting appears to be an ability that is unique to birds and some reptiles (Veiga et al., 1982; Castellini and Rea, 1992; Jenni-Eiermann and Jenni, 1998; McCue et al., 2012).

The concentration of ketone bodies in circulation increased dramatically over the course of fasting and reached peak values that were over five-fold greater than postabsorptive, prefasting values after 5 d (Fig. 5B). The peak concentrations of ketone bodies in these quail were nearly twice as high as values reported for chickens fasting for a similar duration (Brady et al., 1978) and pigeons fasting for 48 h (Gannes et al., 2001), but were within the ranges reported for several species of passerines fasting for only 1 or 2 h (Jenni-Eiermann and Jenni, 1994, 1997). Gannes et al., 2001 found a direct correlation between the concentration of ketone bodies and free fatty acids in pigeons but the authors conceded that because ketone bodies can also be produced from degradation of ketogenic amino acids the concentration of ketone bodies may not accurately reflect lipid turnover rates. Future experiments using ^{13}C-labeled ketone bodies would be useful to investigate this relationship.

The plasma TAG levels were highly variable (Fig. 5C) and did not show the clear decreases previously reported in fasting quail by Sartori et al. (1995) and Lamsova et al. (2004). A study of pigeons fasting for 48 h found no clear changes in TAG (Gannes et al., 2001) although the variance among measurements in that study was much lower than the present study. We used the same model of instrument that Khalilieh et al. (2012) used to measure TAG in fasting house sparrows and more recently TAG in fasting mice (McCue and Pollock, 2013). But, given the unusually high variances (*e.g.*, coefficients of variation 0.23–0.99) we cannot be certain that the values are reliable. As such, we did not conduct statistical comparisons between postabsorptive, prefasting and fasting values.

4.4. Conclusion

We demonstrated that targeted, dietary isotopic enrichment was an effective way to differentially enrich lipid and protein pools of the body. Moreover, measurements of the kinetics of $^{13}CO_2$ excretion during fasting enabled us to identify heretofore undocumented changes in substrate oxidation in quail. While the sharp decrease in the rate of endogenous protein oxidation and the increase in rate of lipid oxidation during the early period of fasting are responses exhibited by many animals that are adapted to fasting, the pattern of continually elevated lipid oxidation coupled with strong circadian cycle of protein oxidation during prolonged fasting has not, to the best of our knowledge, been documented in a fasting animal. Future comparative studies will be useful to identify the extent to which this physiological strategy is unique to quail or employed by other types of animals. The physiological strategies that different animals use to partition among endogenous metabolic fuels are undoubtedly products of the evolutionary history of each species (Jenni and Jenni-Eiermann, 1998; Wang et al., 2006; McCue, 2007; Lignot and LeMaho, 2012) and we conclude that this novel approach can be an effective tool to complement more traditional methods to explore starvation physiology, and possibly other physiological situations involving non-steady physiological states.

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