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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

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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 $\delta^{13}C$ values of breath CO_2. Based on changes in $\delta^{13}C$ 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 $\delta^{13}C$ 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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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