Urinary Stable Nitrogen Isotopes Ratios Are Responsive to Controlled Short-Term Calorie Restriction

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Abstract

Purpose

Increased nitrogen losses observed during periods of energy deficiency are attributed to increased breakdown of endogenous proteins. Stable nitrogen isotope ratios ($\delta^{15}N$) measured in human hair have previously been shown to reflect prolonged periods of increased protein breakdown. The goal of this study was to determine whether stable nitrogen isotopes measured from urinary urea ($\delta^{15}N_{urea}$) represent a potential biomarker of more immediate changes in endogenous protein utilization.

Methods

We analyzed samples from recreationally active males (n = 6, 25.2 ± 1.0 years, BMI: 23.5 ± 0.6 kg/m^2) who took part in a repeated measures cross-over study during which they underwent 4 days of calorie restriction (~ 15 kcal/kg FFM) without and with exercise (CR-EX, CR + EX) and two control conditions in energy balance (CON-EX, CON + EX). $\delta^{15}N_{urea}$ was analyzed from urine samples. In addition, the trophic shift ($\Delta^{15}N$) was calculated as $\delta^{15}N_{urea}$ - $\delta^{15}N_{diet}$, with $\delta^{15}N_{diet}$ obtained from food logs.

Results

$\delta^{15}N_{urea}$ was significantly elevated in CR-EX (4.35 ± 0.16‰) when compared to CR + EX (3.84 ± 0.14‰; p = 0.050, *d* = 1.56), CON-EX (3.65 ± 0.09‰; p = 0.026, *d* = 1.63) and CON + EX (3.34 ± 0.13‰, p = 0.001, *d* = 3.52). $\delta^{15}N_{urea}$ was also significantly higher in CR + EX when compared to CON + EX (p = 0.050, *d* = 1.5). $\Delta^{15}N$ was positive in CR-EX (0.24 ± 0.15‰) and negative in all other conditions (CR + EX: -0.55 ± 0.46‰; CON-EX: -0.96 ± 0.19; CON + EX: -1.1 ± 0.15). CR-EX differed significantly from CON-EX (p = 0.005, *d* = 2.55) and CON + EX (p = 0.006, *d* = 2.3).

Conclusion

Results from this analysis suggest that $\delta^{15}N_{urea}$ as well as $\Delta^{15}N$ can serve as alternative tools to monitor changes in endogenous protein turnover during phases of calorie restriction.

Introduction

Nitrogen is an essential component of protein, amino acids and many other biomolecules. As the human body cannot synthesize nitrogen, it has to be taken up through the diet, which is primarily achieved in the form of dietary proteins (Tome, 2012). The largest compartment containing nitrogen within the human body are proteins, which are found as muscle, structural and secretory proteins. Depending on the metabolic and physiological demands of the body, nitrogen balance can vary (Tomé & Bos, 2000). During periods of anabolism (e.g., growth, pregnancy) nitrogen is retained in the human body, leading to a positive nitrogen balance. In contrast, a negative nitrogen balance is observed during periods of catabolism such as starvation or weight loss (Hambræus, 2014). However, assessment of nitrogen balance is complex, requiring accurate measurements of not only protein intake, but also nitrogen losses via urine, faeces and sweat (Tarnopolsky et al., 1988). Further, this approach reflects gross body protein turnover, but does not yield any information about the contribution of dietary and endogenous protein utilization.

Endogenous protein turnover becomes of particular interest during periods of catabolism, when nitrogen and essential amino acids are made available from skeletal muscle to serve as a substrate for essential functions and energy provision (Lecker et al., 2006). In addition to extreme states of muscle protein loss due to cachexia or sarcopenia, the loss of fat-free mass (FFM), which is largely made up of skeletal muscle, is a well-described side effect of weight loss (Weinheimer et al., 2010). Strategies to counteract loss of muscle during weight loss include incorporation of exercise and increased dietary protein intakes, which retain muscle mass through increasing muscle protein synthesis and attenuating muscle protein breakdown (Carbone et al., 2019). Outcomes of exercise and diet interventions aimed at preserving muscle are commonly assessed by measurement of muscle protein turnover. This however, requires

administration of labelled isotopes and/or muscle biopsies which are invasive methods and confined to tightly controlled laboratory conditions (Kim et al., 2016; Pasiakos & Carbone, 2014).

Analysis of stable nitrogen isotope ratios ($\delta^{15}N$) could serve as a simple alternative to monitor changes in protein utilization in response to interventions in free-living subjects (O'Brien, 2015). Because the "lighter" isotope ^{14}N is preferentially metabolized and excreted, body protein is typically enriched in ^{15}N when compared to the diet (Schoeller, 1999). The difference between enrichment of body tissue ($\delta^{15}N_{tissue}$) and the diet ($\delta^{15}N_{diet}$) is termed the trophic shift (Δ) (Huelsemann et al., 2009).

In fact, stable nitrogen isotopes have already been successfully used to characterize dietary habits and the nutritional state in past and modern populations. In terms of dietary habits, it has been shown that $\delta^{15}N$ measured in human hair ($\delta^{15}N_{hair}$) is reflective of dietary protein source, with higher values observed in omnivores or lacto-ovo-vegetarians when compared to vegans (O'Connell & Hedges, 1999). However, $\delta^{15}N_{hair}$ was also found to be elevated during periods of extreme energy deficiency as observed during starvation or in patients with anorexia nervosa (Huelsemann et al., 2009, 2016; Mekota et al., 2006, 2009; Neuberger et al., 2013). Given that the body is enriched in ^{15}N, the elevation can be explained by the increased metabolization and subsequent excretion in states of elevated body protein breakdown (Huelsemann et al., 2016). In fact, results from animal studies show that $\delta^{15}N$ measured in various tissues increases during periods of energy deficiency (Barboza et al., 2020; Deschner et al., 2012; Gustine et al., 2014; Huneau et al., 2019).

In humans, preliminary data from our group suggests that measurement of $\delta^{15}N$ in urea ($\delta^{15}N_{urea}$), the end product of protein catabolism, is highly sensitive to short-term changes in whole body protein turnover and does not require large sample sizes (Hülsemann et al., 2017). While we have previously shown that $\delta^{15}N_{urea}$ is impacted by protein intake (Hülsemann et al., 2017), it has yet to be confirmed that short-term changes in $\delta^{15}N_{urea}$ can be reflective of changes in whole body protein turnover in response to controlled manipulation of energy status through caloric restriction and exercise. Therefore, the present study aimed to evaluate the value of $\delta^{15}N_{urea}$, as an indicator of whole-body protein metabolism in response to term caloric restriction and exercise. Our primary working hypotheses was that $\delta^{15}N_{urea}$ would increase in response to caloric restriction, indicating increased rates of body protein breakdown. We further anticipated that exercise, a known way to preserve muscle during caloric restriction, would attenuate elevations in $\delta^{15}N_{urea}$.

Material And Methods

Experimental Design

For this investigation, we analysed samples from a randomized controlled trial assessing the impact of calorie restriction and exercise on endocrine and metabolic markers (Koehler et al., 2016) as well as behavioural adaptations (Martin et al., 2021). In order to test the independent and combined effects of caloric restriction (CR) and exercise (EX), the study was conducted using a four-way crossover design during which participants underwent two 4-day conditions of CR and two 4-day control conditions in energy balance (CON) (Figure 1). During one CR and one CON condition, participants conducted aerobic exercise (CR+EX; CON+EX). During the other CR and CON conditions, no exercise was conducted (CR-EX; CON-EX). To match energy balance within CR and CON conditions, dietary energy intake was adjusted for the energy expended during exercise. The order of the experimental conditions was randomly assigned. After each condition, participants underwent wash out periods of *ad libitum* food intake. Wash-out periods were set to at least 4 days following CON and at least 10 days following CR conditions. The study was approved by the ethical review board of the German Sport University Cologne and was conducted in accordance with the Declaration of Helsinki.

Participants

Participants were recruited from the university community in accordance with the following inclusion criteria: male, 18-30 years of age, ≥3 h/week aerobic exercise, normal (body-mass index: 19-25 kg/m^2; body fat percentage below 15%) and stable body weight (±3 kg during the past 6 months). Exclusion criteria were: smoking, infectious disease within the past 4 weeks, intake of medication that could influence the study outcomes, cardiovascular disease or orthopaedic impairment interfering with conducting exercise, diabetes or history of a clinical eating disorder. All participants provided written informed consent prior to participation in the study.

Body Weight and Body Composition

At baseline and the beginning and end of each condition, body weight and body composition were assessed with a bioimpedance scale (Tanita BC 418 MA, Tanita, Amsterdam, The Netherlands). All measurements were carried out in the morning with participants in a fasted (≥10 hours) and well-hydrated state (Koehler et al., 2016).

Diet Prescription

Caloric restriction was operationally achieved by reducing energy availability, defined as dietary energy intake minus exercise energy expenditure, to 15 kcal/kg FFM. Energy balance was assumed at an energy availability of 40 kcal/kg FFM (Loucks & Thuma, 2003). Detailed meal plans were provided to ensure energy intake was in accordance with the respective conditions. Macronutrient distribution was set to recommendations of the German Nutrition Association (50-55% carbohydrates, 30-35% fat, 10-15% protein (Deutsche Gesellschaft für Ernährung, 2012). During all conditions, participants weighed all foods consumed as well as leftovers with a food scale and reported their intake daily. Analysis of food logs occurred on a daily basis (EBIS pro version 7.0, University of Hohenheim, Stuttgart, Germany, 2005) and meal plans were adjusted if reported and prescribed intake differed by ≥50 kcal.

Exercise prescription

During exercise conditions, participants performed supervised exercise on the bicycle ergometer at 60% of their VO_{2peak} until an exercise energy expenditure of 15 kcal/kg FFM was achieved. Outside of the intervention, participants were instructed to abstain from any additional exercise, which was monitored with an activity tracker (SenseWear Pro3 armband, Bodymedia, Pittsburgh, USA).

Stable Nitrogen Isotope Ratio Analysis and Calculations

For analysis of $\delta^{15}N_{urea}$, participants were asked to collect three urine samples per day. Samples were collected in the morning in a fasted state as well as in the afternoon and at bedtime. Participants were instructed to record the time of sample collection and the total urine volume. A 50-mL aliquot of each sample was stored in the participants' refrigerators until delivery to the laboratory, which occurred within 24-48 hours. Urinary urea was isolated using the xanthydrol method (Hülsemann et al., 2017), and $\delta^{15}N$ of urea was assessed using elemental analysis-isotope ratio mass spectrometry. Isotope ratios are expressed relative to atmospheric nitrogen (AIR). The elemental analyser (Eurovektor EA 3000, Hekatech, Wegberg, Germany) was coupled to a Delta C continuous-flow isotope ratio mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The working standard gas (N2, purity 5.0, Linde, Munich, Germany) and the working standard (creatine-monohydrate, AlzChem, Trostberg, Germany) were scale calibrated using IAEA-N-1 (+0.4 ‰) and IAEA-N-2 (+20.3 ‰) for $\delta^{15}N$ values (IAEA, Vienna, Austria). All measurements were carried out in triplicates and the standard deviation for triplicate measure of the working standard was ±0.2‰. Instrument stability and analytical performance were checked by regular analysis of the working standard and zero blanks. For the present analysis, data from the three daily samples was aggregated into a daily average of $\delta^{15}N_{urea}$, and $\delta^{15}N_{urea}$ was further averaged across each of the 4-day study condition. The trophic shift ($\Delta^{15}N$) was calculated as the difference between $\delta^{15}N_{urea}$ and dietary nitrogen isotope ratio ($\delta^{15}N_{diet}$), which was obtained from food logs as previously described (Huelsemann et al., 2013).

Statistical Analysis

Statistical analyses were performed with R (version 4.1.1). If not stated otherwise, data are reported as mean ± standard error of the mean (SEM). Normality of the data was assessed with Shapiro-Wilk-test. Linear mixed model analyses were used to identify differences in outcomes over time and between study conditions, using the subject identifier to account for repeated measures design. If the linear models revealed a trend for time or condition effects ($p<0.1$) post-hoc analyses were performed. Based on normality of the data, one-sided paired t-tests were conducted. Significance was set at $p\leq0.05$. In case of multiple comparisons, p-values were adjusted for multiple testing using the Holm-correction. Effect sizes were calculated from the difference of means and standard deviation of those differences, with d=0.2 considered as small, d=0.5 as medium and d>0.8 as large effect (Cohen, 1992).

In order to evaluate to the impact of potential confounders on $\Delta^{15}N$ is, multiple regression analyses were performed using $\Delta^{15}N$ as dependent and protein intake (g), calorie restriction (kcal/kg FFM) and an interaction term as independent variables. Subject identifiers were included in all models and adjusted R^2 was interpreted. The standardized coefficient (β) was obtained from multiple linear regression with Z-transformed variables.

Results

Study Participants

Participants were 25.2±1.0 years old, had a BMI of 23.5 ± 0.6 kg/m^2 and physically trained according to their VO_{2peak} (49.3±2.4 ml/kg/min). Participants completed all conditions, adherence to the prescribed diet was high (97%-106% of the prescribed energy intake), and attended all supervised exercise sessions (Table 1).

Table 1. Energy and protein intake during each of the 4 study conditions.

Condition	Energy Intake (kcal/kg FFM/day)	Exercise Energy Expenditure (kcal/kg FFM/day)	Energy Availability (kcal/kg FFM/day)	Protein Intake (g/kg/day)
CR-EX	15.9 ± 0.4	0	15.9 ± 0.4	0.8 ± 0.1
CR+EX	29.7 ± 1.27	15	14.9 ± 1.0	1.4 ± 0.2
CON-EX	39.9 ± 0.6	0	39.9 ± 0.6	1.5 ± 0.1
CON+EX	52.0 ± 2.4	15	37.1 ± 2.3	1.8 ± 0.1

CR-EX: calorie restriction without exercise, CR+EX: calorie restriction with exercise, CON-EX: control without exercise, CON+EX: control with exercise

Changes in Body Weight and Composition

Significant reductions in body weight occurred in both CR conditions (Table 2). In CR-EX, losses were primarily attributed to reductions in fat-free mass (67%), whereas in CR+EX, fat mass and fat-free mass losses contributed almost equally.

Table 2. Changes in body weight and body composition over the course of each of the 4 study conditions

	Body Weight (kg)			Fat Mass (kg)			Fat-Free Mass (kg)		
Condition	Pre	Post	Change						
CR-EX	79.6±3.3	77.2±3.1***	-2.4±0.3^{ab}	7.9±1.4	7.2±1.2	-0.7±0.6	71.7±2.3	70.1±2.1*	-1.6±0.8
CR+EX	80.4±3.0	78.6±3.0**	-1.8±0.4^{a}	8.0±1.3	7.2±1.3	-0.8±0.5^{a}	72.5±2.5	71.5±2.3	-1.0±0.8
CON-EX	79.5±3.1	79.1±3.1	-0.5±0.3	7.8±1.4	8.2±1.6	0.4±0.3	71.9±2.0	71.0±2.0	-0.9±0.5
CON+EX	79.2±3.3	79.2±3.2	-0.1±0.5	7.6±1.1	7.9±1.3	0.3±0.2	71.7±2.4	71.3±2.3	-0.4±0.6

CR-EX: calorie restriction without exercise, CR+EX: calorie restriction with exercise, CON-EX: control without exercise, CON+EX: control with exercise; *,**,***: significantly different from pre ($p<0.05$, $p<0.01$, $p<0.001$; $^{a:}$ significantly different from CON ($p<0.05$)

Stable Nitrogen Isotope Ratio Analysis

One participant failed to provide urine samples on days 1-3 of the CR+EX condition, so $\delta^{15}N_{urea}$ and $\Delta^{15}N$ data are from n=5. $\delta^{15}N_{urea}$ in CR-EX was significantly higher (4.35±0.16 ‰) when compared to CR+EX (3.84±0.14‰; p=0.050, *d*=1.56), CON-EX (3.65±0.09‰; p=0.026, *d*=1.63) and CON+EX (3.34±0.13‰, p=0.001, *d*=3.52). $\delta^{15}N_{urea}$ was also significantly higher in CR+EX when compared to CON+EX (3.84±0.14‰ vs. 3.34±0.13‰; p=0.050, *d*=1.51) (Figure 2). Although no significant differences were observed for $\delta^{15}N_{diet}$, changes in $\delta^{15}N_{urea}$ closely mirrored $\delta^{15}N_{diet}$ over the course of each condition (Figure 3).

$\Delta^{15}N$ was positive in CR-EX (0.24±0.15‰) but negative in all other conditions (CR+EX: -0.55±0.46‰; CON-EX: -0.96±0.19; CON+EX: -1.1±0.15). When compared among each other, CR-EX differed significantly from CON-EX

(p=0.005, *d*=2.55) and CON+EX (p=0.006, *d*=2.31). The differences in $\Delta^{15}N$ between CR+EX and CON+EX (p=0.244) and between CR-EX and CR+EX (p=0.244) were not significant (Figure 2, C).

Multiple linear regression analysis

Multiple linear regression analyses showed that the induction of CR as the primary intervention, explained 39% of variation in $\Delta^{15}N$ (p=0.026). Exercise as the secondary intervention did not predict $\Delta^{15}N$ and was subsequently excluded from the analysis. Dietary protein intake improved the prediction of $\Delta^{15}N$ to 64% (p=0.001), and the interaction between CR and dietary protein intake further improved the prediction of $\Delta^{15}N$ to 72% ($R_{adjusted}$=0.72, p<0.001, Table 3). Altogether, the model showed that an increase in protein intake by 1 unit (g) translates into a reduction in $\Delta^{15}N$ by 0.026‰. A decrease in the magnitude of calorie restriction by 1 unit (kcal/kg FFM) reduced $\Delta^{15}N$ by 0.076‰.

Table 3. Multiple Linear Regression Analysis Results

Coefficient	Estimate	Error	β	t-value	p
Intercept	2.30	0.73	-0.48	3.17	0.007
Caloric Restriction	-0.076	0.03	-0.25	-2.53	0.024
Protein Intake	-0.026	0.007	-0.55	-3.76	0.002
Caloric Restriction x Protein Intake	5.5×10^{-4}	2.5×10^{-4}	0.33	2.219	0.044

Discussion

Results from this study indicate that measurement of $\delta^{15}N$ in urea can serve as a non-invasive tool to track short-term changes in protein metabolism during CR. Further, we were able to demonstrate that protein metabolism reflected by $\Delta^{15}N$ is impacted not only by dietary protein intake but also energy and to a lesser degree exercise status.

To date, findings on the relation between $\delta^{15}N$ and dietary intakes in humans are primarily based on analyses of $\delta^{15}N_{hair}$, where dietary changes become detectable in $\delta^{15}N$ only within a matter of days or weeks (Huelsemann et al., 2009; Petzke & Lemke, 2009). However, protein metabolism responds almost immediately to changes in dietary intake and exercise stimuli, making $\delta^{15}N_{hair}$ inadequate to evaluate short-term changes. Contrary, $\delta^{15}N$ measured in urinary urea, which is the end product of protein catabolism, has already been shown to reflect changes in protein metabolism more immediately under conditions during which habitual diet and physical activity levels are maintained (Hülsemann et al., 2017). In fact, we have previously linked changes in $\delta^{15}N_{urea}$ to dietary protein intake (Hülsemann et al., 2017).

Data from the present study shows that $\delta^{15}N_{urea}$ is not only impacted by protein intake but also energy status. The importance of energy status in prediction of $\delta^{15}N_{urea}$ levels seems plausible, as protein demands during periods of CR are primarily met through breakdown of bodily proteins (Friedlander et al., 2005; Lecker et al., 2006). Based on that knowledge, we hypothesized that an increased metabolization and excretion of body nitrogen would become noticeable in a shift in $\delta^{15}N_{urea}$. Similar results were already reported for $\delta^{15}N_{hair}$ measured over the course of a 25 day dessert crossing (Huelsemann et al., 2016). More precisely, $\delta^{15}N_{hair}$ was found to increase as a consequence of low energy and protein intake, which did not match the high energy expenditure of the expedition. Anecdotally, $\delta^{15}N_{hair}$ was highest when the energy deficit was greatest (Huelsemann et al., 2016; Koehler et al., 2011). In line with those findings, our data also show elevated $\delta^{15}N_{urea}$ levels in both CR conditions at 15 kcal/kg FFM.

Incorporation of exercise during periods of weight loss is a well described strategy to counteract the loss of FFM (Carbone et al., 2019; Weinheimer et al., 2010). Indeed, a protective effect of exercise on preservation of FFM was also observed in this study. While both CR conditions lead to a significant weight loss, 67% of weight loss during the CR-EX condition could be attributed to loss of FFM, while only ~50% were attributed to FFM in the CR+EX condition. The difference of approximately 17% falls in line with estimations from Weinheimer et al. 2010 who reported that exercise can decrease losses of lean mass from ~24% up to ~11% during caloric restriction (Weinheimer et al., 2010). Based on its capacity to preserve lean mass, we also anticipated exercise to impact $\delta^{15}N_{urea}$

during calorie restriction. However, we failed to detect such an effect in the present study. It is possible that this discrepancy is due to the fact that participants only conducted endurance training, while the muscle sparing effects during CR is most prominent with resistance exercise (Carbone et al., 2019).

Moreover, another well-described strategy to maintain FFM is the consumption of high protein diets. Overall, intakes above the recommended daily allowance of 0.8 g/kg in the range of 1.6 up to ≥2.4 g/kg/BM are reported as beneficial (Hector & Phillips, 2018; Helms et al., 2014), although the exact threshold at which protein intake maximizes FFM retention during CR is still to be determined (Carbone et al., 2019; Hector & Phillips, 2018). Unfortunately, the current study design did not allow us to discern an effect of different protein intakes on $\delta^{15}N_{urea}$. Protein intakes were more or less comparable among the CR+EX and CON-EX, CON+EX conditions (1.4 ± 0.2 g/kg, 1.5 ± 0.1 g/kg, 1.8 ± 0.1 g/kg respectively), while participants consumed less protein during CR-EX (0.8 g ± 0.1 g/kg). Given that dietary protein intake impacts $\delta^{15}N_{urea}$, the different intakes certainly influenced the study outcomes driving higher $\delta^{15}N_{urea}$ levels in CR-EX. Therefore, future studies with matched protein intakes need to confirm that $\delta^{15}N_{urea}$ may be used as an indicator of increased endogenous protein breakdown during CR.

However, in situations in which food logs are available, calculation of $\Delta^{15}N$ as the difference of $\delta^{15}N_{urea}$ and $\delta^{15}N_{diet}$ may serve as another predictor accounting for dietary intakes. Given that body proteins are commonly enriched in ^{15}N, we hypothesized that $\Delta^{15}N$ will become more positive as a result of increased body protein breakdown (Hülsemann et al., 2017; Schoeller, 1999). Indeed, our findings support this hypothesis as both CR conditions resulted in the highest $\Delta^{15}N$ values. Ultimately, our analysis demonstrated, that in addition to dietary protein intake, energy status also impacts $\Delta^{15}N$, further emphasizing its potential as biomarker.

Conclusion

Results from this investigation confirm previous findings, which showed that changes in $\delta^{15}N$ levels can reflect short-term changes in nutritional status. Yet, we are the first to report that an increase in $\delta^{15}N_{urea}$ and $\Delta^{15}N$ might be reflective of elevated utilization of body protein as a result of CR. Future studies with controlled dietary protein intake are needed to verify $\delta^{15}N_{urea}$ and $\Delta^{15}N$ as a biomarker of increased body protein breakdown during CR.

Abbreviations

CR	Calorie Restriction
FFM	Fat-Free Mass
VO_{2peak}	Peak oxygen uptake

Declarations

Competing Interests: There are no conflicts of interest to disclose.

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Author contribution statement

KK designed the study. KK and FH conceived the methodology. Formal analysis was performed by PW and KK. PW, KK and FH interpreted the data. PW drafted the original manuscript. PW, KK and FH reviewed and edited the manuscript. All authors read and approved to the final version of the manuscript.

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Figures

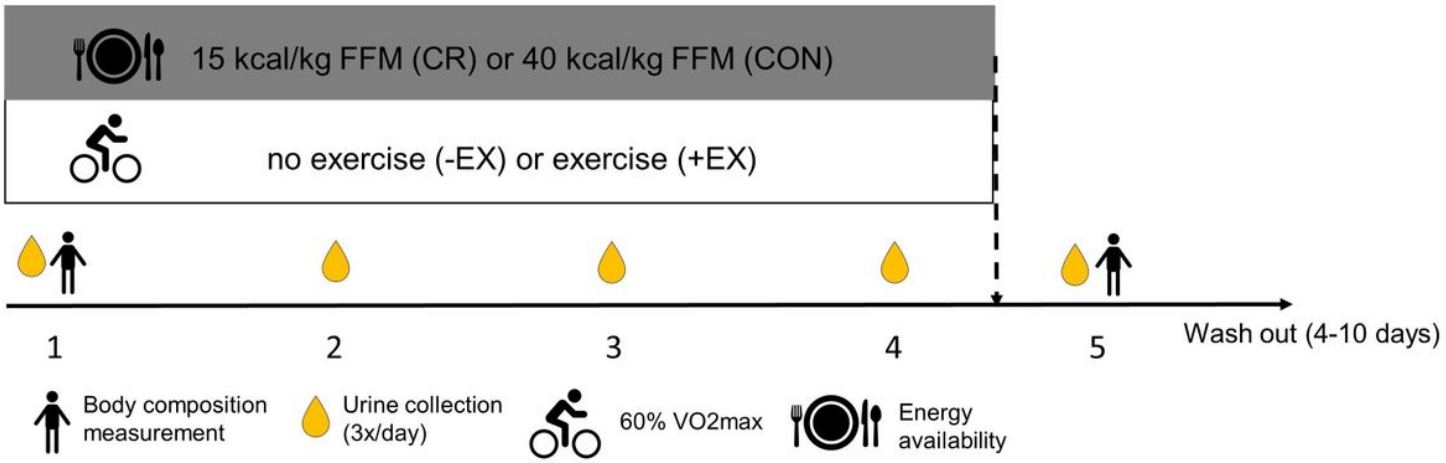

Figure 1

Overview of study flow.

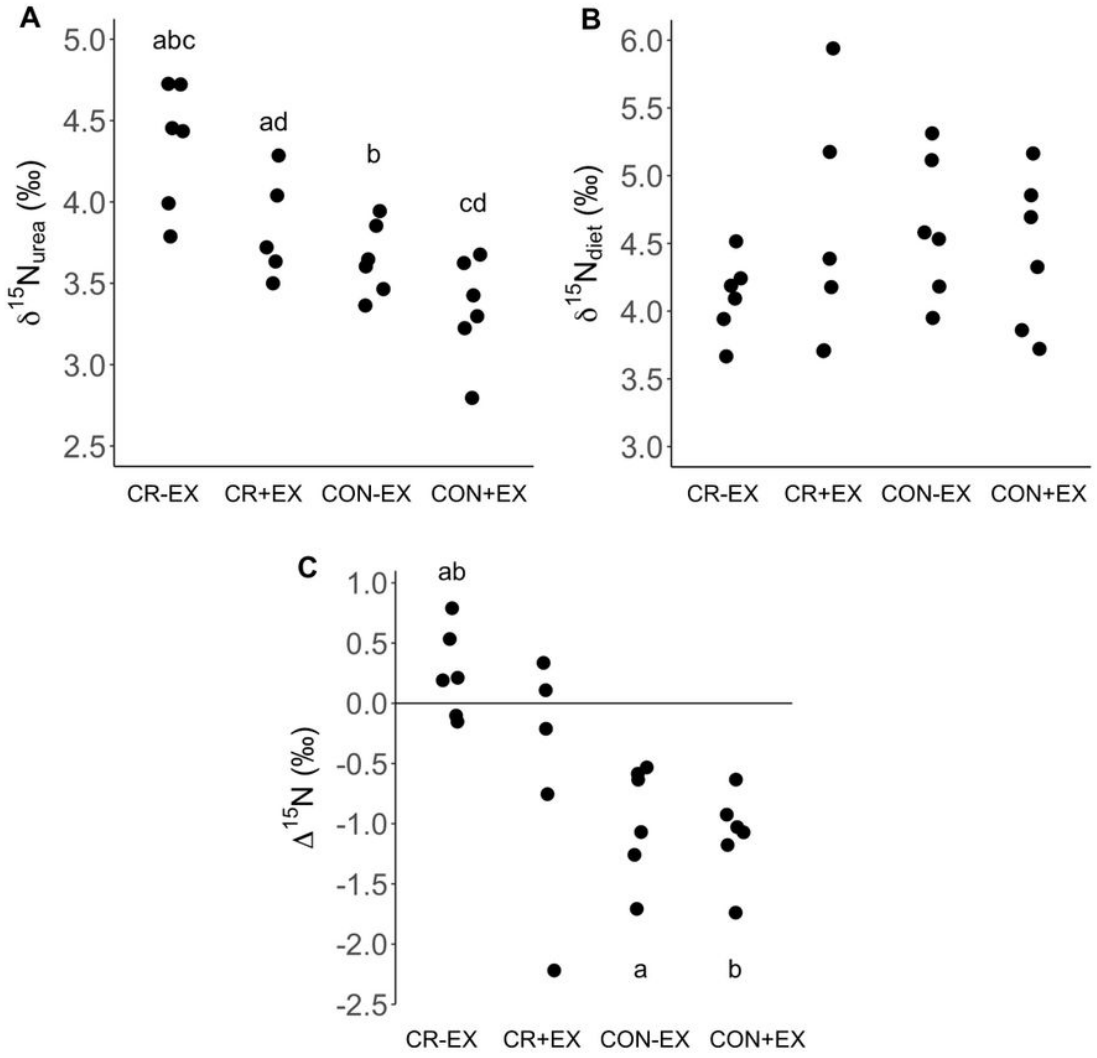

Figure 2

Mean $^{15}N/^{14}N$ isotope ratios in urinary urea ($\delta^{15}N_{urea}$; A) and in the diet ($\delta^{15}N_{diet}$;B) and the difference between urea and dietary $\delta^{15}N$ ($D^{15}N$; C) during 4 days of energy deficit with (+EX) and without exercise (-EX) and energy balance with (+EX) and without exercise (-EX). Similar letters denote statistical significance between groups ($p<0.05$).

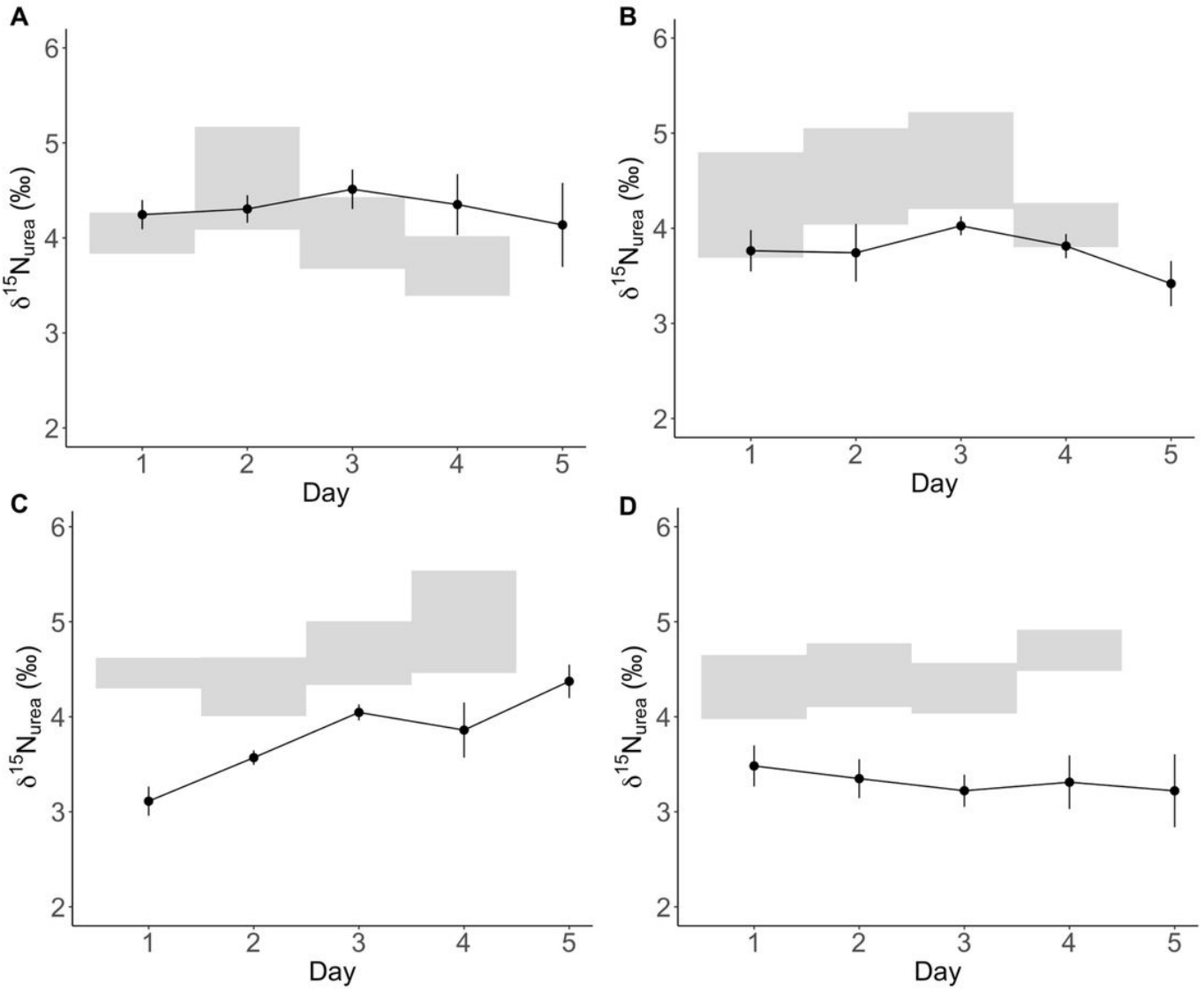

Figure 3

Changes in the $^{15}N/^{14}N$ isotope ration in urinary urea ($\delta^{15}N_{urea}$) over the course of each intervention. Grey shaded areas denote the $^{15}N/^{14}N$ isotope ration in the diet ($\delta^{15}N_{diet}$). A) calorie restriction without exercise; B) calorie restriction with exercise; C) control without exercise; D) control with exercise.