# Previous Repeated Exposure to Food Limitation Enables Rats to Spare Lipid Stores during Prolonged Starvation*

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## ABSTRACT

q1 The risk of food limitation and, ultimately, starvation dates back to the dawn of heterotrophy in animals, yet starvation remains a major factor in the regulation of modern animal populations. Researchers studying starvation more than a century ago suggested that animals subjected to sublethal periods of food limitation are somehow more tolerant of subsequent starvation events. This possibility has received little attention over the past decades, yet it is highly relevant to modern science for two reasons. First, animals in natural populations are likely to be exposed to bouts of food limitation once or more before they face prolonged starvation, during which the risk of mortality becomes imminent. Second, our current approach to studying starvation physiology in the laboratory focuses on nourished animals with no previous exposure to nutritional stress. We examined the relationship between previous exposure to food limitation and potentially adaptive physiological responses to starvation in adult rats and found several significant differences. On two occasions, rats were fasted until they lost 20% of their body mass maintained lower body temperatures, and had presumably lower energy requirements when subjected to prolonged starvation than their naive cohort that never experienced food limitation. These rats that were trained in starvation also had lower plasma glucose set -points and reduced their reliance on endogenous lipid oxidation. These findings underscore (1) the need for biologists to revisit the classic hypothesis that animals can become habituated to starvation, using a modern set of research tools; and (2) the need to design controlled experiments of starvation physiology that more closely resemble the dynamic nature of food availability.

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## Introduction

In his classic book on animal bioenergetics, *The Fire of Life*, Max Kleiber opens the second chapter by suggesting that animals can learn to starve. He tells the story of Oscar, a Scotch collie that on first exposure to starvation became moribund after 45 d but, during a second exposure to starvation, survived 117 d, ultimately losing 63% of his body mass ($m_b$; Kleiber 1975; reference to Howe et al. 1912). For more than a century, researchers have claimed that animals—including pigeons, rabbits, and dogs—that were previously starved had improved abilities (e.g., slower rates of mass loss and lower rates of nitrogen excretion) to survive subsequent bouts of starvation (reviewed in Howe 1910). Do animals really respond to starvation? If so, what are the physiological mechanisms underlying this phenomenon?

All animals are heterotrophic, and natural events—such as disease, fires, droughts, and habitat disturbance—can force animals to experience the physiological stress of food limitation (McCue 2012*a*). Modern researchers routinely study the physiological responses to single, isolated bouts of fasting and food limitation (reviewed in Castellini and Rea 1992; Wang et al. 2006; McCue 2010; Secor and Carey 2016), yet wild animals may experience many bouts of food limitation over the course of their lives. Unfortunately, we know little about how animals respond to these repeated bouts of food limitation.

It is well known that repeated exposure to physiological stressors other than food limitation can trigger a suite of adaptive responses in animals. For example, horses repeatedly exposed to submaximal training exhibit a range of subcellular to organ-level changes known as the training effect whereby whole-animal performance (e.g., speed) is improved (e.g., Guy and Snow 1977; Evans and Rose 1988; Yamano et al. 2002). Prior exposure to near-freezing temperatures is responsible for the cold hardening that is widely documented among insects (reviewed in Danks 1996; Clark and Worland 2008; Teets and Denlinger 2013). Exposure to hypoxia at high altitudes causes changes in the binding kinetics of hemoglobin that facilitates oxygen delivery in a variety of animals (e.g., Hopkins and Powell 2001; Bavis et al. 2007; Gilbert-Kawai et al. 2014). Female mammals often have second litters that are larger in number or size than their first litters (e.g., Ingram

et al. 1958; Sterning and Lundeheim 1995). Could exposure to the stress of food limitation induce similar potentially adaptive responses in animals?

Controlled studies of fasting and starvation have revealed that different animals exhibit a variety of responses to survive periods of food limitation (McCue 2012*a*; McCue et al. 2015*a*). The approaches used have broadly been categorized as supply-side or demand-side strategies (sensu McCue 2007). Adaptive demand-side strategies generally involve reductions in energy expenditure. For example, in addition to reducing total activity levels to conserve energy, ectothermic animals may select cooler microhabitats (reviewed in Bicego et al. 2007; Angilletta 2009), and endothermic animals may employ heterothermy (e.g., long term: hibernation or short-term daily torpor) and reduced metabolic demands (reviewed in McKechnie and Mzilikazi 2011; Geiser et al. 2014; Vuarin and Henry 2014; van Breukelen and Martin 2015). Supply-side strategies generally involve the regulated mobilization and subsequent oxidation of the different classes of metabolic fuels to meet animals' energy demands in a way that minimizes diminished functionality and performance (Caloin 2004; Speakman and Westerterp 2013; Higginson et al. 2014). For example, some starving animals preferentially protect some organs—including the heart, gonads, kidney, or particular skeletal muscles—from degradation (Navarro and Gutierrez 1995; McCue 2010; Bauchinger and McWilliams 2012).

We quantified the extent to which repeated exposure to food limitation affects both the supply-side and demand-side fasting strategies in laboratory rats (*Rattus norvegicus*). Our general hypothesis was that laboratory rats that have been previously exposed to food limitation will respond differently to prolonged fasting than naive rats that have never experienced food limitation. In particular, we predicted that rats with fasting experience will employ one or more of the following potentially adaptive responses: lower rates of $m_b$ loss, lower body temperatures, protracted protein-sparing response, lipid oxidation increases sooner after the onset of fasting, and greater total reliance on lipid oxidation. We further predicted that each of these fasting responses would be greater in rats that have previously experienced multiple bouts of food limitation than those exposed to only a single episode of food limitation.

There are no universally accepted terms to describe periods where animals consume inadequate amounts of food or do not eat at all (McCue 2012*b*). In the this study, we use the term "food limitation" to refer to a period of complete fasting (drinking water was permitted; sensu Kleiber 1975) that causes a biologically relevant amount of mass loss but does not overtly cause acute behavioral or physiological distress. Further, we defined the result of food limitation as a 20% reduction in the $m_b$ of otherwise healthy, nourished animals. We use the term "starvation" to refer to a prolonged period of complete fasting to a point where decrements in physiological performance occur and the risk of death is imminent. We selected 30% of $m_b$ loss as a critical threshold because we previously found that it was sufficient to elicit a sharp rise in endogenous protein oxidation (Guzman et al. 2015). While a 30% reduction in $m_b$ caused noticeable lethargy (M. D. McCue, personal observation), it was not lethal to ~300-g Sprague Dawley rats exposed to the same temperature and photoperiod (Guzman et al. 2015). It is noteworthy that while in previous studies rats were starved until they lost more than 40% of their initial $m_b$ (e.g., Kleiber 1975; Belkhou et al. 1991; Cherel et al. 1992), that level of inanition exceeded commonly accepted limits for acute mass loss in laboratory studies (National Research Council 2011).

## Material and Methods

### *Animals*

This project was done under the auspices of St. Mary's University Institutional Animal Care and Use Committee protocol 2015-4. Fifty-two 21-d-old, male Sprague Dawley rats (58 ± 5 g) were purchased from Harlan Laboratories (Indianapolis). We assigned equal numbers of rats to one of two diet groups (i.e., $^{13}$C lipid [$n = 26$] and $^{13}$C protein [$n = 26$]; fig. 1) and raised them in standard rat cages (480 mm × 265 mm × 210 mm; model 2154F, Techniplast, West Chester, PA) lined with ~2 cm of 3-mm corn cob bedding that was changed weekly. Rats were provided with ~12-cm segments of polyvinyl chloride pipe in which they could hide for behavioral enrichment (National Research Council 2011). Food and water were provided ad lib.

Temperature in the vivarium was maintained at 28° ± 1°C throughout the study. Because this temperature is within the thermal neutral zone for this species (Gordon 1990; Riek and Geiser 2013), we minimized the risk of confounding thermal stress with nutritional stress (Kleiber 1975). The photoperiod was 14L:10D, and for convenience of sampling procedures later in the experiment, it was shifted from natural conditions (i.e., photophase began at 0400 hours and scotophase began at 1800 hours). We weighed rats weekly during this growth period (fig. 2).

### *Isotope Tracers*

The $^{13}$C protein group was raised on standard rodent chow blocks (Teklad Global 2018; 18% protein, 6% fat) and drinking water supplemented with 500 mg $L^{-1}$ of $^{13}$C-1-L-leucine (>99%; Cambridge Isotope Laboratories, Tewksbury, MA) in order to isotopically label all of the proteins in their bodies over the course of their lives. We selected leucine because it is a common amino acid accounting for ~8% of the amino acid residues in the rat (McCue et al. 2005; Newsome et al. 2014). Moreover, because it is an essential amino acid, it is less likely to be oxidized as an energy source than nonessential amino acids on ingestion (McCue 2011; McCue et al. 2011). The location of the $^{13}$C carbon at the number 1 position also means that any degradation of the leucine (the first step of which is decarboxylation) generates a molecule of $^{13}CO_2$ that is unlikely to become incorporated into the lipids or carbohydrates in the body.

The $^{13}$C lipid group was raised on standard rodent chow blocks that were infused with 500 mg $kg^{-1}$ of $^{13}$C-1-palmitic acid (>99%; Cambridge Isotope Laboratories). In short, the

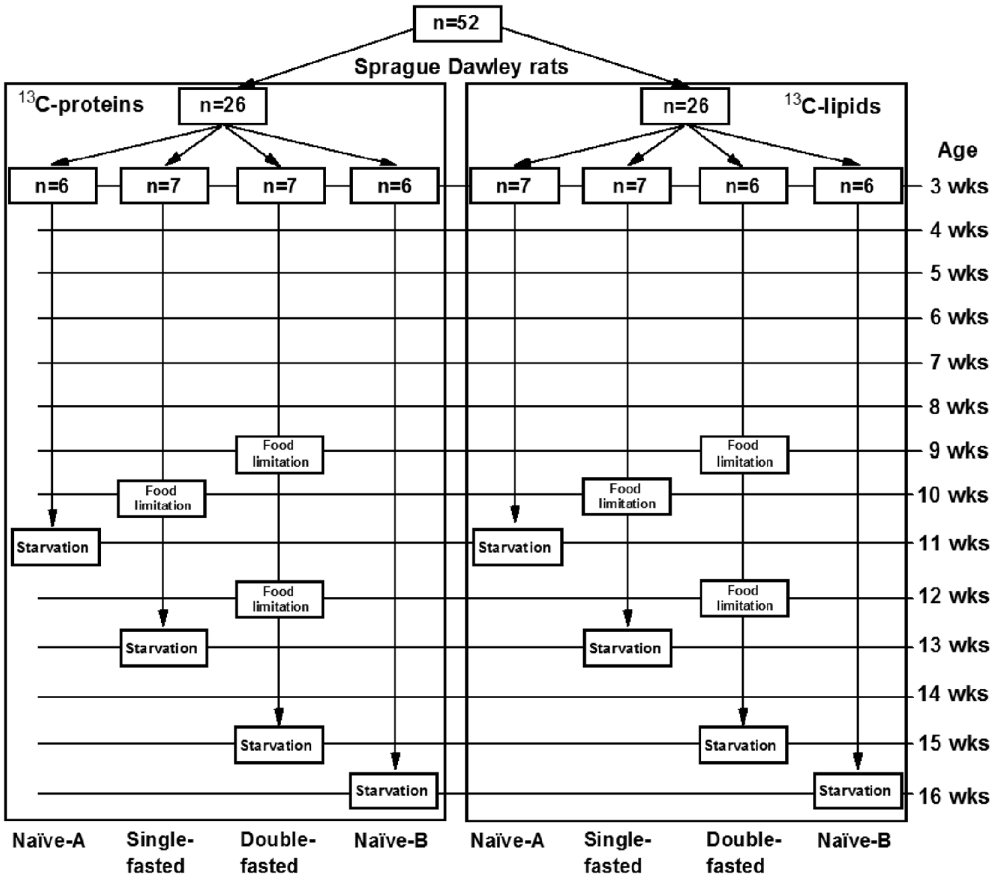


Figure 1. Experimental design and treatment groups. Juvenile rats were divided into one of two feeding groups, where they were raised on diets supplemented with either $^{13}$C leucine or $^{13}$C palmitic acid. These groups were further divided into one of four fasting treatments, where they were exposed to one (single fasted) or two (double fasted) periods of food limitation or were never exposed to food limitation (naive A or naive B) before starving.

palmitic acid was dissolved in a minimal amount of 95% ethanol, and the chow blocks were repeatedly coated with aliquots (~10 mL kg$^{-1}$) of the solution and allowed to dry in an incubator at 60°C between each application. Food coloring was added to the solution to ensure thorough distribution of the isotopic tracer. We selected palmitic acid because it is one of the most common fatty acids in the body and is regularly found in both the neutral (e.g., triacylglycerols) and polar (e.g., phospholipids) lipid fractions (Abbott et al. 2010; Ben-Hamo et al. 2013; Price et al. 2013; Pierce and McWilliams 2014; Louis et al. 2015). Given that $\beta$ oxidation of fatty acids yields $^{13}$C acetyl–coenzyme A that is immediately consumed in the tricarboxylic acid cycle, the $^{13}$C from the palmitic acid tracer is unlikely to become incorporated into proteins or carbohydrates (McCue and Welch 2016; Welch et al. 2016).

### *Body Temperature*

Between 40 and 50 d of age, the rats were lightly anesthetized using Isoflurane (CAS 26675-46-7; IsoSol, Vedco, St. Joseph, MO) and subcutaneously injected with sterile heat-sensitive microchips (Bio-Thermo, Destron Fearing, South St. Paul, MN) into the right inguinal skin fold. In short, these are glass-encapsulated passive integrated transponders approximately the size of a grain of rice. The temperatures of the implanted chip (hereafter body temperature, $T_b$) and unique identification numbers of each rat were read using a hand-held radio-frequency chip reader (Pocket Reader model RE6016, Destron Fearing). According to the manufacturer, the chips are accurate to $\pm 0.5$°C over a range of 25°–40°C.

During the starvation trials, $T_b$ was measured three times per day (i.e., early day, 0800 hours; late day, 1600 hours; and middle night, 2400 hours), noninvasively through the floor of the cages before other measurements were made (e.g., $m_b$, breath collections, and plasma glucose). Following the experiment, the microchips were removed from the carcasses and placed in a water bath at 37°C to determine any temperature offsets. We corrected the measured $T_b$ by as much as 0.3°C to account for slight differences among the individual microchips (sensu Langer and Fietz 2014).

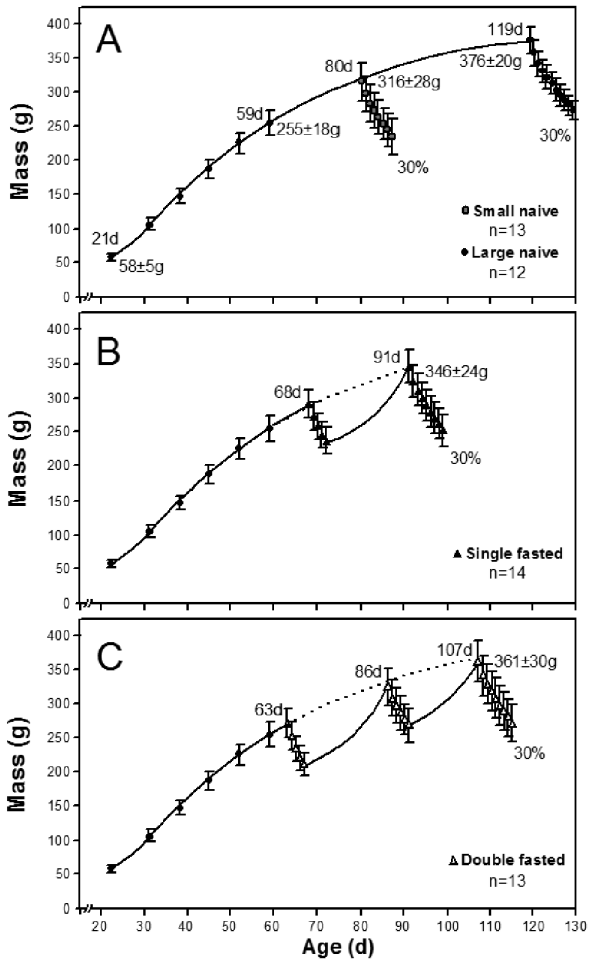


Figure 2. Body masses and ages of rats over the course of this study. *A*, Masses of rats ($n = 56$) between days 21–59, during which their body lipids or proteins were becoming enriched in $^{13}C$. Gray circles represent rats starved at age 80 d (naive A; $n = 13$). Black circles represent rats starved at age 119 d (naive B; $n = 12$) during starvation. Solid line is an interpolation of the body masses of naive rats and is repeated in the other panels to facilitate visual comparisons. *B*, Body masses of rats (single fasted; $n = 14$) exposed to one bout of food limitation at age 68 d and starved at age 91 d. Dotted line represents the curve generated from the solid line in *A* and illustrates the similarity in $m_b$ with the naive rats in *A*. *C*, Body masses of rats (double fasted; $n = 13$) exposed to two bouts of food limitation at age 63 and 86 d and later starved at age 107 d. Dotted line represents the curve generated from the solid line in *A* and illustrates the similarity in $m_b$ with the naive rats in *A*. Error bars are $\pm 1$ SD.

### *Food Limitation*

At 59 d of age, the two diet groups (i.e., $^{13}C$ lipid and $^{13}C$ protein) were assigned to one of four fasting treatments in a factorial design (fig. 1). Because we did not yet know the effect that age and nutritional history would have on $m_b$ and the other physiological responses, we established two control groups that were never deprived of food (i.e., naive A [$n = 13$] and naive B [$n = 12$]; fig. 1). At 80 and 119 d of age, respectively, those groups were starved until their initial $m_b$ was reduced by 30% (fig. 2*A*).

The two other treatment groups were exposed to either one or two periods of food limitation until they lost 20% of their initial $m_b$ (i.e., single fasted [$n = 14$] or double fasted [$n = 13$]; fig. 1). The single-fasted group was fasted at age 68 d (for ~5 d) and later starved at age 91 d (fig. 2*B*). The double-fasted group was fasted at age 63 d (for ~5 d) and again at age 86 d before undergoing starvation at age 107 d (fig. 2*C*).

During the food limitation and starvation trials, the rats were maintained individually in their home cages, but their bedding was removed to prevent them from eating it. The behavioral enrichment tubes remained inside the cages. Drinking water (containing no $^{13}C$ tracer) was provided ad lib. We weighed the animals once each day during the food limitation treatments. When at least half of the rats in a treatment group lost at least 20% of their $m_b$, food and bedding were returned to their cages.

During the starvation trials, we weighed the rats three time each day but only after recording their $T_b$ and then collected breath samples. These measurements continued until each rat lost at least 30% of its initial $m_b$, determined at the late-day measurement period (i.e., at 1400 hours, 10 h into the 14-h photophase). At that time, animals were killed in their home cages by loosely sealing the cage and introducing pure $CO_2$ at a rate of ~5 L $min^{-1}$. We used cervical dislocation to ensure death (AVMA 2013) before freezing the carcasses.

### *Isotope Analyses*

Breath samples were collected three times each day. In short, rats were placed inside 2,130-mL resealable plastic food containers for 10 min to allow $CO_2$ levels to accumulate to ~2%, the minimum concentration required by our isotope analyzer. We used a 20-mL glass syringe (Cadence, Staunton, VA) to collect 15-mL subsamples of the gas within the containers through a 1-mm hole in the lid. These samples were injected into 12-mL evacuated Exetainer vials (Labco, Lampeter), and determinations of $^{13}C$ content were made within 3 d of collection.

We measured the $\delta^{13}C$ in the gas samples using a nondispersive infrared spectrometer (HeliFan Plus, Fischer ANalysen Instrumente, Leipzig) interfaced with a FanAS autosampler (Fischer Analysen Instrumente). Vials containing 2.5% $CO_2$ calibration gas (Mesa Gas, Santa Ana, CA) with $\delta^{13}C$ (previously verified at the University of Arkansas Stable Isotope Laboratory) were analyzed in parallel with the unknown samples to detect and correct for analytical drift (as described in McCue et al. 2015*b*). All $^{13}C$ values are presented in units of $\delta^{13}C_{VPDB}$ (IAEA 2000).

### *Plasma Glucose*

We measured plasma glucose concentration (mg $dL^{-1}$) during the late-day measurements using blood collected from a tail vein. On any given day, these measurements were made on

Table 1: One-way ANOVA showing that body mass at the start of starvation in rats differed among the four treatment groups

| Source of variation | df | SS | MS | *F* | *P* |
|---|---|---|---|---|---|
| Treatment | 3 | 25,355.135 | 8,451.712 | 12.339 | <.001 |
| Residual | 48 | 32,877.788 | 684.954 | | |
| Total | 51 | 58,232.923 | | | |

half of the rats and alternated each day to minimize handling stress and possible stress associated with blood loss (Khalilieh et al. 2012). Determinations were made using a MediSense glucose analyzer (Abbott Laboratories, Alameda, CA; McCue and Pollock 2013).

### *Statistics*

We used general linear mixed models in SigmaPlot 13.0 (San Jose, CA) to calculate degrees of freedom, critical values, and *P* values. An $\alpha$ of 0.05 was used to determine significance. Mean values are reported followed by standard deviations.

Prestarvation $m_b$ and rates of $m_b$ loss during starvation were compared among the four treatments using one-way ANOVAs. We used ANCOVAs to compare the relationships between $m_b$ and starvation time using age as a covariate. Two-way ANOVAs were used to compare $T_b$, plasma glucose, and $\delta^{13}C$ of breath. Treatment and time were main factors; therefore, we also examined the treatment × time interactions. Because we expected $T_b$ to follow a circadian pattern, we analyzed the three daily time points separately. We used one-sample *t*-tests to compare the $\delta^{13}C$ of breath with that of the base diet.

When ANOVA revealed significant differences, we used Holm-Sidak pairwise comparisons to further examine the patterns. Data that were not normally distributed were examined using Kruskal-Wallis one-way ANOVA on ranks or a Mann-Whitney rank sum test when appropriate. We used Pearson product-moment correlation tests to examine the relationship between initial $m_b$ and reductions in $m_b$.

## Results

### *Masses and Mass Loss*

While the juvenile rats were integrating the $^{13}C$ tracers into their body tissues, they increased their $m_b$ more than fourfold from 58 ± 5 g at age 21 d to 255 ± 18 g at age 59 d (fig. 2*A*). During their exposure to food limitation, the single-fasted group decreased its $m_b$ by 19% from 291 ± 21 g at age 68 d to 237 ± 19 g during ~5 d (fig. 2*B*). The double-fasted group at age 63 d decreased from 272 ± 22 to 212 ± 18 g during ~4 d (fig. 2*C*) of food limitation. At age 86 d, this group was fasted again, and $m_b$ decreased from 325 ± 27 to 269 ± 25 g during ~5 d. Following both bouts of food limitation, the $m_b$ of the double-fasted rats increased to match those of the unfasted rats.

The naive A group (316 ± 28 g) was starved at age 80 d followed by the single-fasted group (346 ± 24 g) at age 91 d. Thereafter, the double-fasted rats (361 ± 30 g) at age 107 d were fasted immediately followed by the naive B rats (376 ± 20 g) at age 119 d. The prestarvation $m_b$ of the four groups differed significantly (table 1). Using pairwise comparisons, we found that the $m_b$ of the naive A rats was significantly less than the single-fasted rats (Holm-Sidak, $t = 2.953$, $P = 0.015$). While the $m_b$ of the single-fasted rats did not differ from those of the double-fasted rats ($t = 1.541$, $P = 0.243$), they were less than the $m_b$ of the naive B rats ($t = 2.987$, $P = 0.018$). These differences in $m_b$ preclude comprehensive comparisons among the treatment groups. However, because of the compensatory growth in the double-fasted group, their $m_b$ did not differ significantly from those of the naive B group.

During starvation, $m_b$ decreased in all rats (fig. 2); however, it did so at different rates during the first week (table 2). For example, by 176 h of starvation, the $m_b$ decreased by 27.2% ± 2.7% in the naive A group, 25.3% ± 2.0% in the single-fasted group, 23.2% ± 1.8% in the double-fasted group, and 22.2% ± 1.0% in the naive B group (fig. 3). Using pairwise comparisons, we found that the decrease in $m_b$ of the naive A rats was greater than the single-fasted rats (Holm-Sidak, $t = 2.481$, $P = 0.033$). While the decrease in $m_b$ of the single-fasted rats was also lower than that of the double-fasted rats ($t = 2.706$, $P = 0.028$), there was no difference in $m_b$ loss between the double-fasted and the naive B rats ($t = 1.306$, $P = 0.198$).

An ANCOVA using age as a covariate showed that changes in $m_b$ did not differ among the four treatment groups ($P = 0.894$; table S1; tables S1–S5, fig. S1 available online); however, correlation analysis showed that starvation-induced $m_b$ loss was negatively related to initial $m_b$ (coefficient = −0.989, $P = 0.001$; fig. 3, inset). Because $m_b$ of the naive A rats was lower than that of the single-fasted rats, direct comparisons between these groups could be misleading. However, since neither prestarvation $m_b$ nor the rates of starvation-induced $m_b$ loss differed between the double-fasted and the naive B groups, we used these two groups as the focus for comparisons of the physiological responses to starvation.

### *Body Temperature*

Temperatures followed a daily cycle where $T_b$ tended to be approximately 1°C higher during the scotophase (fig. S1). As starvation progressed, both daytime and nighttime $T_b$ gradually

Table 2: One-way ANOVA showing that the treatment groups of rats had different percent changes in body mass after 172 h of starvation

| Source of variation | df | SS | MS | *F* | *P* |
|---|---|---|---|---|---|
| Treatment | 3 | 187.348 | 62.449 | 15.922 | <.001 |
| Residual | 48 | 188.268 | 3.922 | | |
| Total | 51 | 375.616 | | | |

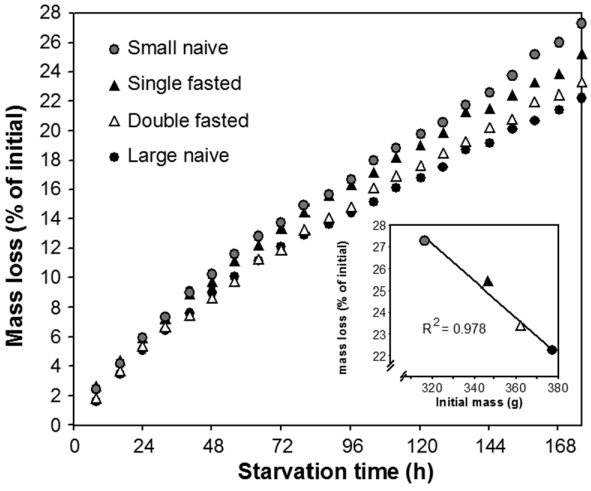


Figure 3. Reductions in body mass during the first 176 h of starvation in rats subjected to four treatments. The single- or double-fasted rats had previously experienced food limitation, whereas the two groups of naive rats had never experienced food limitation. Error bars are not shown for clarity. *Inset*, relationship between initial body mass and mass loss during the first 176 h of starvation.

decreased in all treatment groups. We compared $T_b$ measured in the early day, late day, and middle night and found that there were significant differences by treatment (table 3).

As starvation progressed, $T_b$ tended to decrease in all treatment groups. As such, treatment × time interactions were also significant at each point in time (table 3). Pairwise comparisons of $T_b$ of naive B versus double-fasted rats had significant differences at nine points in time (fig. 4); at eight of these time points, the $T_b$ was significantly higher in the naive B rats (table S3). We interpret these differences as evidence that the repeated exposure to food limitation resulted in a decrease in the $T_b$ set points of the double-fasted rats, particularly during the latter half of the starvation period.

### *Plasma Glucose*

Plasma glucose averaged 111 ± 15 mg $dL^{-1}$ at 8 h and fell to an average of 73 ± 14 mg $dL^{-1}$ between 32 and 200 h (fig. 5). Plasma glucose among the four treatments did not differ ($P = 0.497$; table S2); however, there was a significant effect of time ($P < 0.001$; table S3). A second two-way ANOVA comparing the naive B versus the double-fasted rats indicated a significant treatment effect but also a treatment × time interaction (table 4). Pairwise comparisons between these two treatments revealed that overall the plasma glucose was lower in the double-fasted rats (Holm-Sidak, $t = 3.280$, $P = 0.001$). This difference was also apparent at several specific points in time (i.e., 32, 152, 224, and 248; fig. 6; table S4). We interpret these differences as evidence that the repeated exposure to food limitation reduced the plasma glucose set point of the double-fasted rats.

### *Protein and Lipid Oxidation*

The $\delta^{13}C$ of the base diet (i.e., before the addition of any $^{13}C$ tracer) was −21‰. Therefore, any breath $\delta^{13}C$ values greater than this indicate that the $^{13}C$ leucine in the body proteins or $^{13}C$ palmitic acid in the body lipids were being oxidized. The initial $\delta^{13}C$ of breath from the $^{13}C$ protein rats did not differ among the four treatment groups (ANOVA on ranks, df = 3, $H = 4.971$, $P = 0.174$) and fell sharply during the first 8 h (Mann-Whitney ranks, $U = 29.5$, $t = 997.5$, $P < 0.001$; fig. 7*A*). We attribute the elevated values at 0 h to the fact that the rats were not fully postabsorptive and were processing recent ingested nutrients from their previous meal (McCue 2006; Secor 2009).

Between 24 and 72 h of starvation, the $\delta^{13}C$ in breath of the protein and lipid rats remained relatively low (−17.6‰ ± 1.9‰) but was significantly higher than baseline isotope levels (one-sample *t*-test, df = 181, $t = 24.241$, $P < 0.001$), indicating that protein oxidation had not completely ceased. Thereafter, the trajectories were unique to each treatment group (fig. 6*A*). The $\delta^{13}C$ and thus the protein oxidation increased first in the naive A rats and then followed by the single-fasted rats. However, because we previously determined that these two treatment groups had lower $m_b$ than the other groups, we did not examine these differences further. Comparisons of the $\delta^{13}C$ in the $^{13}C$ protein rats belonging to the naive B versus double-

Table 3: Two-way ANOVA comparing body temperatures of starving naive B versus double-fasted rats in time

| Source of variation | df | SS | MS | *F* | *P* |
|---|---|---|---|---|---|
| Early day: | | | | | |
| Treatment | 1 | 2.594 | 2.594 | 12.258 | <.001 |
| Time | 11 | 50.333 | 4.576 | 21.622 | <.001 |
| Treatment × time | 11 | 5.623 | .511 | 2.416 | .007 |
| Residual | 262 | 55.445 | .212 | | |
| Total | 285 | 113.753 | .399 | | |
| Late day: | | | | | |
| Treatment | 1 | .926 | .926 | 3.896 | .049 |
| Time | 11 | 32.232 | 2.93 | 12.322 | <.001 |
| Treatment × time | 11 | 9.648 | .877 | 3.688 | <.001 |
| Residual | 262 | 62.303 | .238 | | |
| Total | 285 | 106.545 | .374 | | |
| Middle night: | | | | | |
| Treatment | 1 | .8598 | .859 | 4.413 | .037 |
| Time | 10 | 33.088 | 3.309 | 16.992 | <.001 |
| Treatment × time | 10 | 7.494 | .749 | 3.848 | <.001 |
| Residual | 239 | 46.539 | .195 | | |
| Total | 260 | 87.511 | .337 | | |

Note. Analyses were done separately for each of the three times of day when body temperature was measured. Pairwise comparisons at times can be found in table S1, available online..

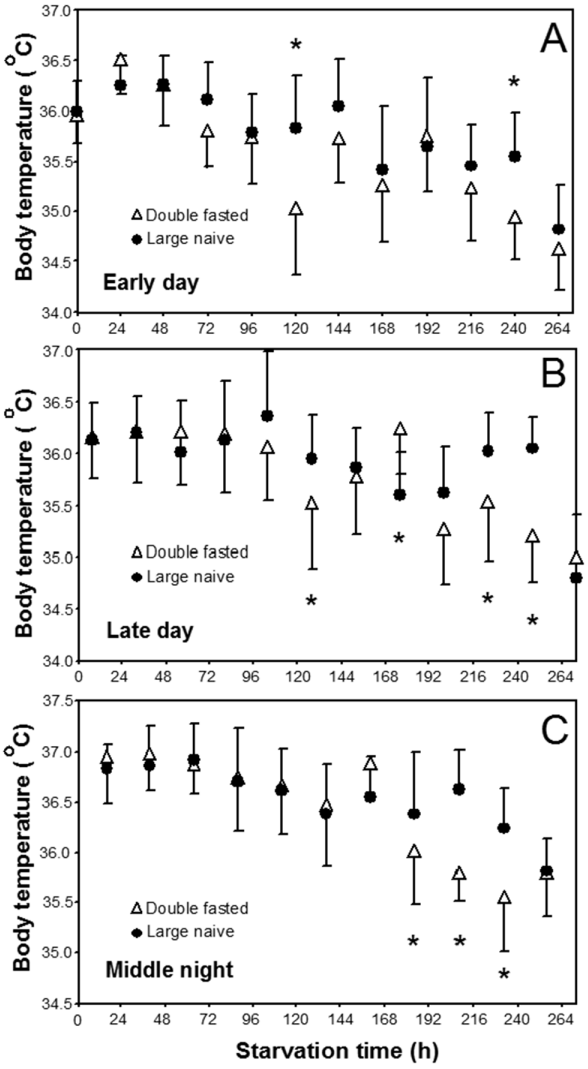


Figure 4. Body temperatures of naive B and double-fasted rats during starvation. The double-fasted rats had previously experienced food limitation, whereas the naive rats had never experienced food limitation. Asterisks indicate times at which body temperatures were significantly different (see table S1, available online). Error bars are ±1 SD.

fasted groups did not indicate a significant effect of treatment but did indicate a significant effect of time (table 5).

The initial $\delta^{13}C$ of breath (i.e., at the start of starvation) from the $^{13}C$ lipid rats differed among the four treatment groups (ANOVA, df = 3, $F = 14.725$, $P < 0.001$), but the $\delta^{13}C$ of breath did not differ between the naive B versus the double-fasted groups ($t$-test, df = 10, $P = 0.083$). During the first 24 h of fasting, the $\delta^{13}C$ of breath increased sharply in all groups (fig. 6*B*). Between 72 and 144 h, the two previously fasted treatment groups tended to have lower lipid oxidation; however, because of the differences in $m_b$, we did not explore this statistically. A two-way ANOVA comparing the naive B versus the double-fasted rats did indicate a significant treatment effect (table 5). Pairwise comparisons between those two treatments revealed that the $\delta^{13}C$ of breath was generally higher in the naive B rats (Holm-Sidak, $t = 33.878$, $P < 0.001$). Pairwise comparisons between these groups showed significant differences at all fasting times (i.e., >0 h; table S5). We interpret these differences as evidence that the repeated exposure to food limitation reduced the rates at which the double-fasted rats oxidized endogenous lipids.

## Discussion

We predicted that rats that had been previously exposed to food limitation would exhibit potentially adaptive supply-side (e.g., adjustments in metabolic fuel type) and demand-side (e.g., adjustments in energy expenditure) responses when subjected to starvation. While some of our specific predictions were supported by the data, others were not.

Because we did not know the magnitude of the compensatory growth responses in either the single- and the double-fasted rats, we used two naive treatment groups (i.e., A and B) to increase the chances that we would have matched—albeit not perfectly age matched—groups for statistical comparisons. The naive A group would have provided an appropriate match for $m_b$ if the food limitation treatments caused a significant decrement in growth. Indeed, the younger naive A rats and the slightly older single-fasted rats had similar $m_b$, but the double-fasted rats were significantly larger than the naive A rats, thereby precluding rigorous comparisons among those three groups. Ultimately, the single- and double-fasted rats exhibited near perfect compensatory growth, an observation that will permit the use of age-matched treatment groups in future studies. Because the single-fasted rats were significantly smaller than the double-fasted and the naive B rats, we focused our comparisons on these latter two treatment groups since they

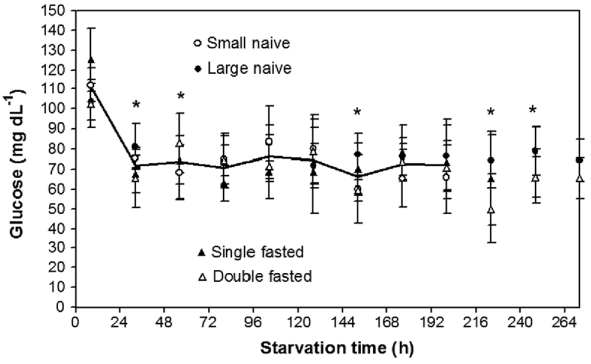


Figure 5. Plasma glucose levels in starving rats. Solid line illustrates the mean values for the four treatment groups between 0 and 200 h. The single- and double-fasted rats had previously experienced food limitation, whereas the two groups of naive rats had never experienced food limitation. Asterisks indicate times when the glucose values were significantly different between the double-fasted rats and the naive B rats (see table S2, available online). Error bars are ± 1 SD.

Table 4: Two-way ANOVA on plasma glucose values of starving naive B versus double-fasted rats through time

| Source of variation | df | SS | MS | *F* | *P* |
|---|---|---|---|---|---|
| Treatment | 1 | 1,692.441 | 1,692.441 | 10.759 | .001 |
| Time | 11 | 13,436.724 | 1,221.52 | 7.765 | <.001 |
| Treatment × time | 11 | 4,117.81 | 374.346 | 2.38 | .01 |
| | | 20.44 | | | |
| Residual | 130 | 9.424 | 157.303 | | |
| Total | 153 | 39,410.727 | 257.586 | | |

Note. Two-way ANOVA of plasma glucose values for all treatment groups can be found in figure S2, available online. Pairwise comparisons of naive B versus double-fasted rats at specific times can be found in table S2, available online.

had similar $m_b$ yet had the most divergent feeding histories. Recall that the naive B rats had never been exposed to food limitation, whereas the double-fasted rats experienced two bouts of food limitation before being starved.

### *Body Mass and Temperature*

One study of Siberian hamsters reported that prior exposure to 32 h of fasting had greater rates of $m_b$ loss during a subsequent 56-h period of starvation than naive hamsters (i.e., 11.6% vs. 8.8%; Wood and Bartness 1996); however, another study found that Siberian hamsters that were previously fasted for 32 h lost less $m_b$ (13%) when starved for 56 h than naive hamsters who lost 15% of $m_b$ over an identical period of time (Day et al. 1999). We initially predicted that previously fasted rats would also lose $m_b$ slower than naive rats; however, we found no evidence to support this prediction. Note that starvation-induced changes in $m_b$ alone should be interpreted with caution, primarily because the energy densities (in terms of wet mass) of lipids and nonlipids can vary by fivefold or more (Allen 1976; Gerson and Guglielmo 2011). Moreover, even small changes in the water content of the body can obscure accurate interpretations of changes in $m_b$ (see hypothetical example in McCue 2010).

Fasting rats are known to gradually reduce their $T_b$ (Closa et al. 1992; Challet et al. 1997; Nagashima et al. 2003; Garami et al. 2010), but none of the measurement periods reported exceeded 5 d. We predicted that $T_b$ in rats with fasting experience would be reduced to a greater extent during starvation than in naive rats. The $T_b$ of the naive B rats and the double-fasted rats decreased, but the double-fasted rats consistently maintained lower $T_b$, particularly during the second half of the starvation period. Although we did not quantify activity levels, we did not notice any differences in behavior among the treatment groups. We interpret these differences in $T_b$ as evidence that the double-fasted rats also maintained lower metabolic rates, though it is possible that differences in conductance may contribute to these differences

Several studies have shown that starving rats gradually reduce their resting metabolic rates (Montemurro and Stevenson 1960; Westerterp 1977; Ma and Foster 1986; Munch et al. 1993; Fuglei and Oritsland 1999; Nagashima et al. 2003; Garami et al. 2010). Indeed, we previously identified strong positive correlations between metabolic rate and $T_b$ in starving mice (McCue et al. 2016) and quail (Ben-Hamo et al. 2010), supporting our contention that the reductions in $T_b$ we measured in the rats likely reflect an energy-sparing response to starvation (see also Langer and Fietz 2014; van der Vinne et al. 2015). We did not measure the metabolic rates of these rats, but assuming a typical $Q_{10}$ value of 2.5 (Geiser 2004), a 1°C reduction would equate to an ~8% drop in metabolic rate.

### *Plasma Glucose*

We predicted that the plasma glucose levels in the rats with prior exposure to fasting would either decrease less or decrease more slowly during starvation than in naive rats; this was apparently not the case. Contrary to our prediction, the double-fasted rats maintained lower plasma glucose levels than the naive B rats. This difference usually ranged between ~10 and 20 mg $dL^{-1}$ and was most pronounced during the last stages of starvation. Previous studies—wherein rats were fasted for 3 d—reported that liver glycogen stores fell to near 0 and did not recover (Bjorntorp et al. 1983; Cherel et al. 1992). Therefore, by 6–8 d of starvation, it is reasonable to expect that any

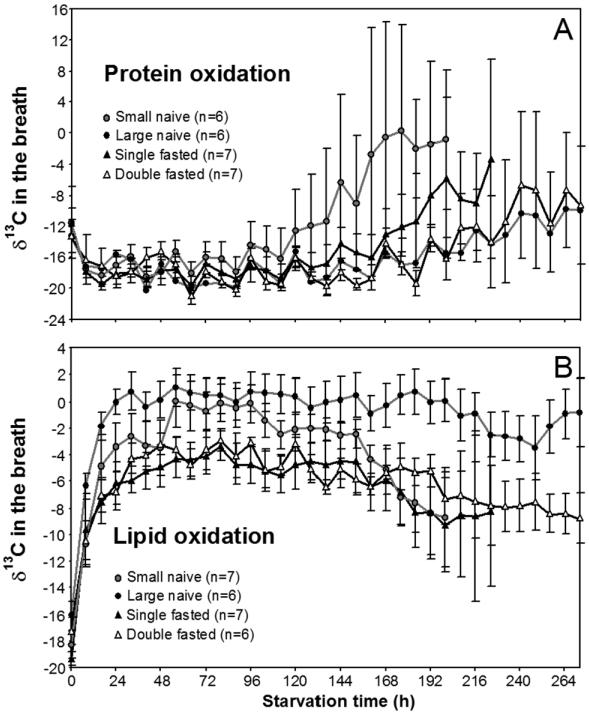


Figure 6. $^{13}C$ content in breath of starving rats as a measure of endogenous protein (*A*) and lipid oxidation (*B*). Error bars are ± 1 SD.

Table 5: Two-way ANOVA on values of $^{13}C$ in breath of starving naive B versus double-fasted rats through time

| Tracer and source of variation | df | SS | MS | *F* | *P* |
|---|---|---|---|---|---|
| Protein: | | | | | |
| Treatment | 1 | 25.094 | 25.094 | 2.344 | .127 |
| Time | 34 | 4,481.911 | 131.821 | 12.312 | <.001 |
| Treatment × time | 34 | 314.862 | 9.261 | .865 | .688 |
| Residual | 373 | 3,993.714 | 10.707 | | |
| Total | 442 | 8,893.056 | 20.12 | | |
| Lipid: | | | | | |
| Treatment | 1 | 2,500.864 | 2,500.864 | 1,147.708 | <.001 |
| Time | 34 | 3,096.496 | 91.073 | 41.796 | <.001 |
| Treatment × time | 34 | 170.695 | 5.02 | 2.304 | <.001 |
| Residual | 326 | 710.356 | 2.179 | | |
| Total | 395 | 6,368.407 | 16.123 | | |

Note. Analyses were done separately for each of the $^{13}C$-labeled pools (i.e., proteins and lipids). Pairwise comparisons of lipid oxidation at specific times can be found in table S3, available online.

glucose in the body (i.e., in circulation or found in hepatic or muscle glycogen stores) was derived from gluconeogenesis (e.g., from amino acids or glycerol precursors).

The demand for glucose during starvation is partially offset in most tissues by increased oxidation of fatty acids and lipid-derived ketone bodies (McGarry et al. 1970; de Bruijne and van den Brom 1986; Belkhou et al. 1991). However, the central nervous system cannot oxidize fatty acids for energy (Masoro 1968; Robinson and Williamson 1980), and therefore plasma glucose levels (and possibly ketone bodies; Andrews et al. 2009) need to be maintained at some minimal level. Interestingly, in this study, the minimum set point for plasma glucose in the double-fasted rats appears to have decreased. Although the response was quite clear, future research is needed to identify the mechanisms responsible for this change in glucose regulation. In particular, it would be useful to determine whether the reductions in glucose levels are being driven by supply-side responses (e.g., decreased rates of gluconeogenesis from protein substrates) or demand-side responses (e.g., increased preference for lipid oxidation by peripheral tissues or increased uptake of ketone bodies by the brain). It would also be informative to determine how prior exposure to food limitation affects insulin sensitivity in these animals.

### *Protein and Lipid Oxidation*

We predicted that rats with fasting experience would differ from rats with no experience. The naive A rats evidently exhausted their lipid stores sooner (fig. 6*B*) and increased their reliance on protein catabolism earlier than the naive B rats (fig. 6*A*); however, the aim of this study was not to examine the effects of $m_b$ (or age) on starvation. Comparisons of the double-fasted rats with the naive B rats did not reveal differences in the duration of their protein-sparing response. Moreover, the double-fasted rats did not oxidize more lipid than naive B rats. In fact, the reliance on lipid oxidation was consistently higher in the naive B rats at all times.

Bjorntorp and Yang (1982) fasted rats until their $m_b$ decreased by 25% and then showed that the compensatory growth during 8 d of refeeding was sufficient to restore their $m_b$ to the levels of the control rats. Although the $m_b$ of the control and refed rats in that study were identical, the carcasses of the refed rats contained higher protein content and lower lipid content than control rats. Reduced adiposity of refed rats was also reported by Cherel and Le Maho (1991). In the extreme case of obese rats, Cherel et al. (1992) determined that lipid oxidation accounted for a greater proportion of energy production than lean rats. We think that it would be useful to examine the relationship between repeated exposures to food limitation and body composition on otherwise normal rats to help identify the mechanisms responsible for the differences in fuel selection we observed here.

### *Conclusions*

We conclude that repeated exposure to food limitation causes a suite of physiological changes in rats. Interestingly, each of these changes became most apparent during the lattermost stages of starvation. In particular, the rats in this study reduced their $T_b$, presumably as a mechanism to reduce energy expenditure. They also regulated circulating glucose concentrations at lower levels, possibly as a mechanism to minimize gluconeogenic requirements. Finally, rats reduced lipid oxidation possibly because of a concomitant reduction in metabolic rate. None of these physiological changes are necessarily mutually exclusive, but they are sufficiently distinct to suggest that they are part of an integrated response involving several regulatory systems.

This study may not prove that animals can learn to starve (sensu Kleiber 1975), but it does provide clear evidence that

previous exposure to food limitation will affect how animals respond to future bouts of starvation. It is not yet clear whether these observations can be generalized to other species, but they do challenge us to question the taxonomic reach of these responses as well as the limitations of controlled studies of starvation in naive animals.

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