THE EFFECTS OF ANTIBIOTIC TREATMENT ON FOOD AND WATER CONSUMPTION IN MICE SELECTIVELY BRED FOR HIGH VOLUNTARY WHEEL RUNNING

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Abstract

The trillions of microbes living in the mammalian gut play a role in many important host functions, such as maintaining homeostasis, regulating host inflammatory responses, and assisting in gastrointestinal development. Antibiotics that directly disrupt the gut microbiome can cause long-lasting physiological and neurobiological changes in mice. Here, we show that dysbiosis of the microbiome through antibiotic treatment can increase water consumption without an effect on food consumption in mice from lines selectively bred for high voluntary wheel-running behavior and their non-selected control lines. 100 female mice from an artificial selection experiment that consists of 4 replicate high runner (HR) lines and 4 non-selected control (C) lines were given broad-spectrum antibiotics (with Splenda to increase palatability) for 10 days to greatly reduce their microbiome composition. HR mice differ from C mice in several traits that likely involve the microbiome, such as 3-fold higher daily wheel-running distance and increased food consumption. Food and water consumption were measured by weighing food hoppers and water bottles over a 7-day period prior to antibiotic treatment and also at the start and end of antibiotic administration (10 days). With body mass as a covariate, HR mice consumed more food than C mice both before and during antibiotic treatment. Antibiotic treatment decreased food consumption of HR mice, but not of C mice. When the amount of wheel running (data from a companion study) was used as a covariate in statistical analyses, we found no differences between HR and C mice and no effect of antibiotics on food consumption. With body mass as a covariate, HR and C mice did not differ statistically in water consumption, but both linetypes drank more water when given antibiotics with Splenda, and the effect remained when the amount of wheel running was added as a covariate. The results of this study demonstrate some of the potential impact of antibiotics on host behavior and function.

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Introduction

The 10^{14} microbes coating an individual mammalian gastrointestinal tract are a collection of bacteria, archaea, and eukarya termed the "gut microbiota" (Thursby and Juge, 2017). The harmful effects that some microbes have on their host are well known, and with that, a newfound interest in the mutually beneficial relationship that hosts share with the microbes living inside of them is emerging. Thus far, researchers have found that the microbiome is crucial in maintaining homeostasis, resisting epithelial injury, regulating immunity through the balancing of Th1/Th2 responses, protection against pathogens, and more (Geuking et al., 2014; Rakoff-Nahoum et al., 2004a; Yamamoto et al., 2012; Zarrinpar et al., 2018; Zheng et al., 2020). Additionally, the microbiota play a large role in metabolism and digestion. For example, *Veillonella,* a genus of microbes that are abundant after exercise, utilize the lactate produced during exercise to make propionate, a chemical compound that can enhance exercise capacity (Scheiman et al., 2019). The microbiota in the GI tract is essential for managing lipid absorption, regulating the ability to store and extract energy from food, managing body weight, and fermenting complex carbohydrates (Thursby and Juge, 2017). Microbial assistance in digestion generates various microbial metabolites such as short-chain fatty acids that go on to regulate different cellular processes within the host. These processes include gene expression, chemotaxis, differentiation, proliferation, and apoptosis (Lazar et al., 2019; Martinez-Guryn et al., 2018; Thursby and Juge, 2017).

"Dysbiosis" is disturbance of the microbiome influenced by such factors as diet and antibiotic use. The use of antibiotics is becoming more prevalent as a means to eliminate pathogens and treat various infections (Ichinohe et al., 2011). As antibiotic use rises, so does antibiotic resistance, leading to switching to other antibiotics and thus creating a cycle (Rosa et

al., 2018). Furthermore, dysbiosis through antibiotic use can disrupt the important crosstalk between the host's functions and the microbes inside them. For example, one study using adult mice found that antibiotic-induced dysbiosis is associated with bacteria-derived metabolite depletion and changes in signaling molecules responsible for cognition (Fröhlich et al., 2016). Another study observed an upregulation of TLR4, AMP's, and pro-inflammatory cytokines, and changes in the mucosal barrier and sIgA secretion in mice that had undergone a 2 week antibiotic treatment (Aguilera et al., 2015). Host changes due to dysbiosis show that there is an adaptive response to microbial alterations, highlighting the existence of host-bacterial interactions.

New studies have highlighted the detrimental impact of dysbiosis by way of antibiotics on animal behavior in murine models. For example, alteration of the microbiome has been shown to affect the central nervous system through alterations of gene expression in the infralimbic region of the brain, which is important for facilitating fear extinction learning. Mice that had undergone antibiotic treatment had a lower density of c-FOS expression in the infralimbic region, leading to deficits in fear extinction learning (Chu et al., 2019). Interestingly, mice with an absent or disrupted microbiome were also found to have microglia that exhibited an immature state, resembling that of a developing juvenile microglia (Chu et al., 2019). Antibiotic treatment has also been found to alter anxiety-like behavior, decrease sociability, social novelty, and social avoidance, increase aggression, and impair memory in murine models (Chu et al., 2019; Gareau et al., 2011; Lach et al., 2020; Leclercq et al., 2017). Furthermore, changes in animal behavior and function are often unable to restore to its prior self even after cessation of antibiotics. Failure of restoration shows that dysbiosis caused by antibiotic use leads to irreversible changes in the gut microbiota composition, leading to long-term ramifications in host function. For example, Chu and colleagues found that supplementing mice with symbiotic

microbes during adult life immediately following dysbiosis as adults did not reverse the detrimental effect that antibiotic use has on fear extinction learning (Chu et al., 2019).

Dysbiosis of microbes also causes disruption in host physiological functions, such as metabolic homeostasis and immune responses. Specifically, depletion of the microbiome deprives the colon of butyrate, a microbial metabolite, causing enterocytes to shift to glucose for metabolism, which leads to an increase in hepatic gluconeogenesis, and thus contributes to hyperglycemia in diabetes (Zarrinpar et al., 2018). Mice who lack a native gut microbiome have many immunological alterations. For example, germ-free (GF) mice show deficits in the development of gut-associated lymphoid tissue and defensins, defects in antibody production, smaller Peyer's patches and mesenteric lymph nodes, impaired development and maturation of isolated lymphoid follicles, altered patterns of microvilli formation, and decreased rates of cell turnover in intestinal epithelial cells (Round and Mazmanian, 2009). GF mice have defective T cell trafficking, are deficient in Th17 cells, and have reduced IL-17 and ATP levels in the colon (Round and Mazmanian, 2009).

Changes in dietary intake can also alter the community composition and diversity of the gut microbiome (Carmody et al., 2015; Denou et al., 2016; Martinez-Guryn et al., 2018). Mice fed a high-fat, high-sugar diet have a lower relative level of Bacteroides and a significantly higher level of Firmicutes and Verrucomicrobia compared to mice fed a low-fat, high-plant-polysaccharide diet (Carmody et al., 2015). Additionally, mice given a high-fat diet have a lower alpha diversity within the phylum Bacteroidetes compared to chow-fed mice (Denou et al., 2016). Changes in the microbiome can also have various metabolic effects. For example, GF mice inoculated with high-fat diet-induced microbiota still had an increased lipid absorption while fed a low-fat diet (Martinez-Guryn et al., 2018).

The present study is part of a larger one that examined the effects of antibiotic-induced dysbiosis on voluntary exercise in a unique mouse model consisting of artificially selected lines of mice specifically bred for high voluntary wheel-running behavior over many generations (McNamara et al., in preparation). This experiment began in 1993 (Swallow et al., 1998; Wallace and Garland, 2016). Each generation, young adult mice are individually housed with access to a wheel and daily wheel revolutions are counted for six days. In the High Runner (HR) lines, the highest-running male and female are chosen as breeders, whereas in the control (C) lines breeders are chosen without regard to their running behavior (Swallow et al., 1998). HR mice responded to the artificial selection and evolved to run about 3x more revolutions per day than C mice (Careau et al., 2013; Wallace and Garland, 2016). The amount of revolutions per day of HR mice plateaued at around generation 17 or later, indicating the existence of external and/or internal limiting factors that limit voluntary exercise (Careau et al., 2013). Also, when housed without wheels, HR mice have higher spontaneous physical activity in home cages than do C mice (Copes et al., 2015).

In the aforementioned larger study, female mice from both HR and C lines were weaned and then housed individually for less than 1 week. At 7 weeks of age, all mice were allowed wheel access in individual cages. After 2 weeks of wheel access, mice were given a broad-spectrum antibiotic cocktail in their water to reduce the gut microbiome. Voluntary exercise was then measured as the total number of revolutions per day and was recorded continuously from 7 weeks of age (McNamara et al., in preparation).

In this part of the study, we examined the effect of antibiotic treatment on food and water consumption. HR and C mice have been shown to differ in their microbiome composition (McNamara et al., 2021). Thus, our overarching hypothesis, addressed in the companion study,

was that antibiotics would decrease wheel-running behavior in HR mice due to dysbiosis of the microbiome. We expected less of an adverse effect on running by C mice because they do not run enough to tax their physiological limits. Based on the energetic cost of wheel running and previous studies, we expected that the higher daily wheel running of HR mice prior to administration of antibiotics would be accompanied by greater food consumption as compared with C mice (Copes et al., 2015). We also expected to see an accompanying higher water consumption in HR mice as a direct result of increased food consumption, because fluid is required for digestion of food.

We hypothesized that because dysbiosis decreases energy availability, antibiotic treatment would decrease the amount of wheel running and thus decrease the amount of food consumed by HR mice during antibiotic treatment. In addition, antibiotics might also have a direct negative effect on food consumption due to illness and loss of appetite (Alcock et al., 2014; Cunha, 2001). With regard to water consumption, we hypothesized that HR mice would have a decrease in water consumption as a downstream effect of decreased wheel running and decreased food consumption. We also predicted a decrease in water consumption as a direct result of decreased wheel running because wheel running is a predictor for water consumption (Thompson et al., 2017).

Methods

All procedures were approved by the Institutional Animal Care and Use Committee of the University of California, Riverside.

Experimental design

Mice were sampled from generation 89 of an ongoing experiment in which 4 replicate High Runner (HR) lines are selectively bred for voluntary wheel-running behavior and compared with 4 replicate non-selected Control (C) lines. Briefly, the selection experiment began in 1993 with 224 outbred, genetically variable Hsd:ICR strain mice (Swallow et al., 1998). Mice were randomly mated for 2 generations and then randomly assigned into 4 replicate HR lines bred for high wheel running and 4 replicate C lines. Home cages are attached to 1.12m circumference wheels (Lafayette Instruments, Lafayette, IN). Each generation, the highest-running HR female and male from each of 10 families are chosen as breeders, based on the average revolutions on days 5 and 6 of a 6-day period of wheel access (Swallow et al., 1998). Breeders in the C lines are chosen without regard to wheel running. Mice are paired outside their family but within their line, and sibling mating is never allowed. Photoperiod is 12:12 L:D and temperature is ~22°C. All mice receive Standard Laboratory Rodent Diet (SD) from Harlan Teklad (W-8604), which contains 24.3% kJ from protein, 4% kJ from fat, and 40.2% from carbohydrate. Pregnant dams are given Harlan Teklad Lab mouse breeder diet [S-2335] 7004 through weaning.

For the present experiment, 12 females from each of the 4 replicate HR and 4 C lines were weaned at 21 days of age, plus an additional 4 from HR line 6, which is polymorphic for the mini-muscle phenotype (see Garland, Jr. et al., 2002). Mice were housed individually for <1 week, then randomly cohoused in groups of 4 in an attempt to homogenize the gut microbiome,

as mice are coprophagic (Laukens et al., 2016). Each week, mice were again randomized into clean cages, and this occurred three times (Figure 1).

At ~7 weeks of age, mice were weighed and placed into individual cages with wheels (same as used in the routine selective breeding protocol) for 2 weeks to allow daily running distances to reach a stable plateau (Copes et al., 2015; Thompson et al., 2017). After 2 weeks, mice were weighed, a fecal sample was collected, and all of the mice were given a broad-spectrum antibiotic cocktail in the drinking water to greatly reduce the gut microbiome. The antibiotic cocktail contained 1 g/L ampicillin, 1 g/L neomycin, 125 mg/L vancomycin, and 2.5 g/L Splenda (similar to Ichinohe et al., 2011b; Rakoff-Nahoum et al., 2004b; Reikvam et al., 2011). The antibiotic treatment lasted for 10 days, during which body mass, antibiotic water consumption, and food consumption were measured. After completion of the antibiotic treatment, sample size was reduced to ~76 for logistical reasons, another fecal sample was collected, and the mice were given sterile water for 3 days to wash out the antibiotics. Mice were then provided with tap water for 7 days to allow the gut microbiome to naturally "recover" (either from environmental sources or through bacteria present in the gut).

Food and Water Consumption

Average daily water consumption during the initial wheel access (7 days) and during antibiotic treatment (ten days) was calculated by weighing the water bottle at the start and end of the period. Average daily food consumption was calculated in a similar fashion, by weighing food hoppers with due allowance for wastage (Koteja et al., 2003).

Fecal Sampling and Plating

To confirm reduction of the gut microbiome, we determined the number of colony-forming units for a subset of mice from a baseline sample collected after 2 weeks of wheel access and a post-antibiotic treatment fecal sample from the same subset of mice. Mice were placed into clean individual cages until defecation. Fecal pellet mass was determined by weighing sterile tubes before and after collection of the pellet. Pellets were suspended in 500 µL sterile phosphate-buffered saline (PBS) using a BeadBeater for 30 seconds at 1,400 rpm. We then cultured a serial dilution of fecal samples for 47 of the mice. 5 µL of the fecal suspension was plated on Luria Bertani media in a serial dilution to 10^{-6} (2 per mouse) and incubated both aerobically and anaerobically at 37°C. After a 24-h incubation, the colonies were counted and the colony-forming units calculated by dividing the number of colonies per mL plated by the total dilution factor.

Statistical Analyses

We used linear mixed models to determine the effects of genetic background (High Runner versus Control lines), antibiotic treatment (pre-antibiotic versus during-antibiotic), and their interaction on food and water consumption. In the companion study, we also analyzed wheel running, spontaneous physical activity in home cages, and body mass (McNamara et al., in preparation). Following numerous previous studies of these mice, the effect of linetype was tested relative to the variance among replicate lines, which are a nested random effect within linetype, with 1 and 6 d.f. (SAS Procedure Mixed with repeated measures, SAS Institute, Cary, NC, USA) (e.g., Acosta et al., 2015; Swallow et al., 1998). The effect of antibiotic and the

interaction with linetype were also tested with 1 and 6 d.f. Covariates of body mass and the amount of wheel running (refs/day) were included in the models where appropriate.

Outliers were removed and we accepted statistical significance at $P<0.05$. Data are presented as least squares means and standard errors from SAS Procedure Mixed.

Results

Depletion of the Gut Microbiome

Prior to antibiotic treatment, plates averaged 4.18 x 10^6 aerobic colony-forming units, with no statistical difference between High Runner and Control mice (ANOVA, $F_{1,6}$=0.32, p=0.5905). Ten days of antibiotic treatment reduced the colonies to a non-detectible amount for both linetypes. Anaerobic plates were not usable due to technical issues.

Food Consumption

With body mass as a covariate, antibiotics had no effect on food consumption for C mice, but HR mice consumed less food during antibiotic treatment (days 15-24) than pre-antibiotics (days 8-14) (repeated measures interaction, $F_{1,6}$ = 6.24, p=0.0467; Figure 2A). The High Runner mice also consumed significantly more food than C mice during both the pre-antibiotic and antibiotic periods (repeated measures linetype $F_{1,6}$ = 9.15, p=0.0232; Figure 2A).

When the amount of wheel running (revs/day) was added as a covariate, it was a highly significant predictor of food consumption (Figure 3A, p<0.0001) and the effects of linetype (p=0.9365) as well as the linetype * antibiotic interaction became non-significant (p=0.2854).

Water Consumption

Accounting for body mass (p<0.0001), both linetypes drank significantly more of the antibiotic water during experimental days 15-24 compared to regular water during days 8-14 (repeated measures $F_{1,6}$ = 24.76, p=0.0025; Figure 2B).

With wheel running as an additional covariate (Figure 3B, p=0.0009), the effect of antibiotics was still highly significant (p=0.0012)

Discussion

Overview

The main purpose of this study was to examine the effect of microbial dysbiosis caused by antibiotic treatment on food and water consumption in a mouse model that includes 4 replicate lines that have been selectively bred for voluntary wheel-running behavior and 4 non-selected controls. This was done by administration of a broad-spectrum antibiotic cocktail for 10 days, which reduced gut microbiota to a non-detectable amount in aerobic plating tests (McNamara et al., in preparation). Food hoppers and water bottles were weighed pre- and after antibiotic treatment (Fig. 1).

Food Consumption

Based on numerous previous studies, HR mice have increased wheel-running activity compared to C mice (Careau et al., 2013; Copes et al., 2015; Wallace and Garland, 2016). As a result, when housed with access to a wheel and controlling statistically for variation in body mass, HR mice consume more food than C mice (e.g., see Copes et al., 2015; Hiramatsu and Garland, 2018 and references therein). In the present study, HR mice consumed more food than C mice, both before and during antibiotic administration (Fig. 2A).

Antibiotics have been shown to disrupt bacterial-gut function and decrease energy availability in studies of human adults (Burstein et al., 1993; Hernández et al., 2013). In one study, adult human patients who had undergone a 14-day antibiotic treatment showed carbohydrate maldigestion and an altered energy balance in the gastrointestinal tract similar to that in obese patients when compared to non-treated patients and lean patients (Hernández et al.,

2013). If mice on antibiotics experience reduced energy availability, then it might decrease their ability to exercise. We hypothesized that an antibiotic-induced decrease in wheel running would also reduce food consumption because of reduced daily energy expenditure. Additionally, antibiotics might directly decrease food consumption because dysbiosis has been found to dysregulate appetite (Alcock et al., 2014).

In the companion paper (McNamara et al., in preparation), we found that HR mice decreased wheel running while on antibiotics, but C mice did not, perhaps because C mice do not run enough to tax their physiological limits. Consistent with this effect, HR mice had decreased food consumption while on antibiotics, but C mice did not (Fig. 2A).

As the microbiome is crucial for helping the host immune system and aiding in digestion, another possible explanation for a decrease in food consumption in HR mice could be that dysbiosis of an established microbiome caused them to become ill, thus decreasing their appetite. However, this explanation is unlikely, as mice showed no significant difference in body mass and food consumption pre- versus during antibiotic treatment, indicating no apparent illness caused by antibiotics (McNamara et al., in preparation).

When taking into account the amount of wheel running (revs/day) before and during antibiotic treatment by analysis of covariance, there was no apparent change in food consumption of HR mice pre- and during antibiotic treatment and no difference between the HR and C lines (Fig. 3A). Additionally, C mice still had no change in food consumption when taking wheel running into account. Together, these results suggest that both the higher food consumption of HR mice in general and their decreased food consumption during antibiotic treatment are a function of their wheel-running behavior (compare Fig. 2A with 3A).

Water Consumption

Although we did not explicitly test the impact of food consumption on water consumption, current understanding of mammalian physiology makes it logical to assume that an increase in food consumption leads to increased water consumption to aid in the digestion and break-down of food. Wheel running is also a strong predictor of fluid consumption in mice (Thompson et al., 2017). Thus, we predicted that HR mice would have decreased water consumption during antibiotic treatment as a result of decreased wheel running and decreased food consumption. Contrary to our hypothesis, with body mass as a covariate, both HR and C mice had a significant increase in water consumption during antibiotic treatment when compared to pre-antibiotic values (Fig. 2B). Adding the number of revolutions run per day as a covariate did not change this pattern (Fig. 3B).

One possible explanation for an increase in water consumption during antibiotic treatment is that antibiotics can lead to kidney injury, causing the mice to consume more water (Sinha Ray et al., 2016; Yang et al., 2019). For example, vancomycin can cause acute kidney injury (AKI) in 40% of human cases due to its cytotoxicity (Yang et al., 2019). In rats, the pathogenesis of nephrotoxicity is caused by superoxide radicals generated from vancomycin use, leading to inflammation in and around proximal tubule cells and thus injury of the glomerulus (Nishino et al., 2003).

Another possible explanation could be that the mice liked the taste of the Splenda in the antibiotic cocktail, leading them to consume more of the antibiotic cocktail as compared with ordinary tap water. One study using the same artificially selected mouse model found that both

HR and C mice always drank more water with Splenda (and with other sweeteners) as compared with plain tap water (Thompson et al., 2017).

Conclusions

Antibiotic treatment and the resulting dysbiosis of the gut microbiome was associated with reduced food consumption in selectively bred HR lines of mice (Fig. 2A), but our analyses that incorporated the reduction in wheel running (Fig. 2B) suggest that dysbiosis per se did not have a direct effect on food consumption. With respect to water consumption, any effects of antibiotic-caused dysbiosis, changes in wheel running, and/or changes in food consumption appear to have been masked by large positive effects of mice liking to drink solutions that contain Splenda.

Figures

Figure 1. Experimental timeline.

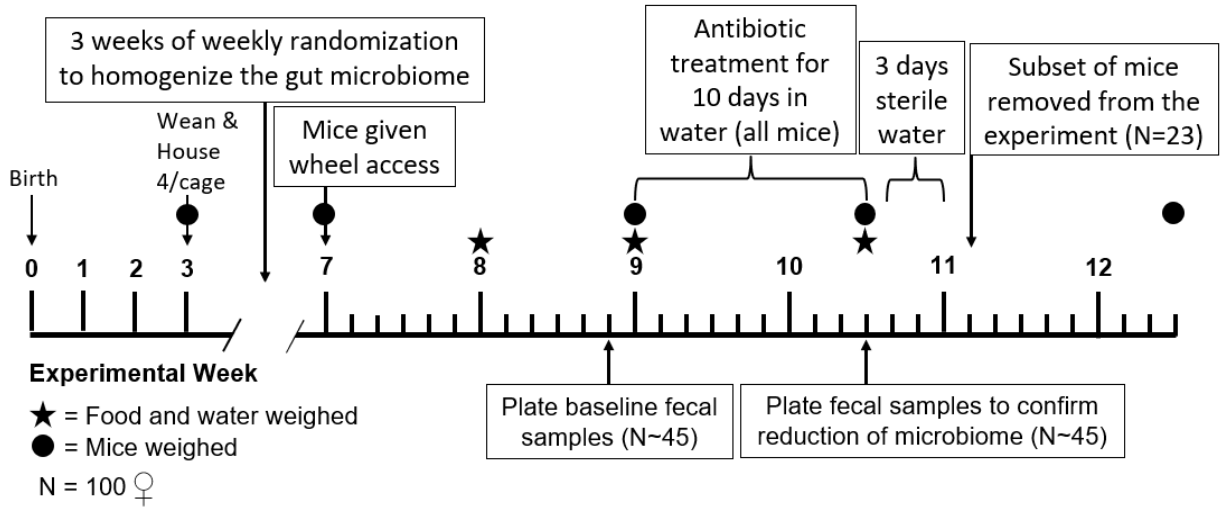
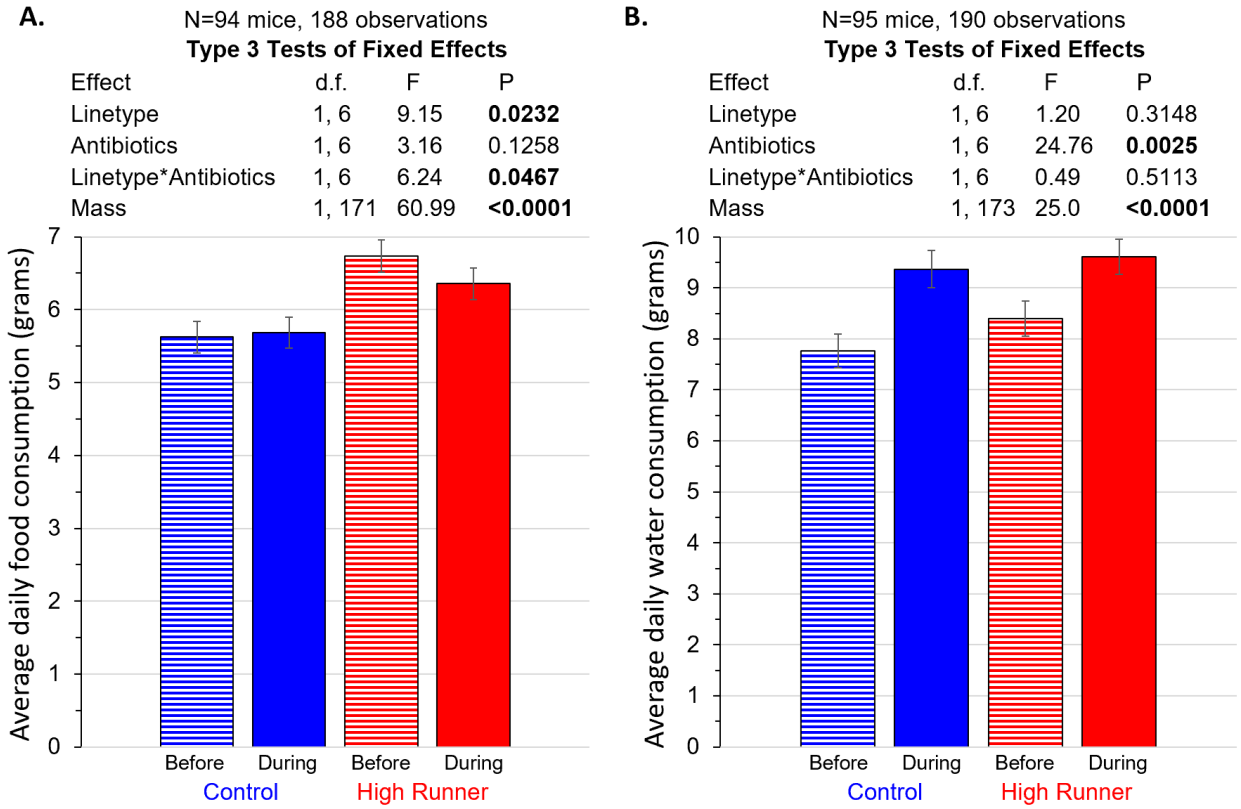
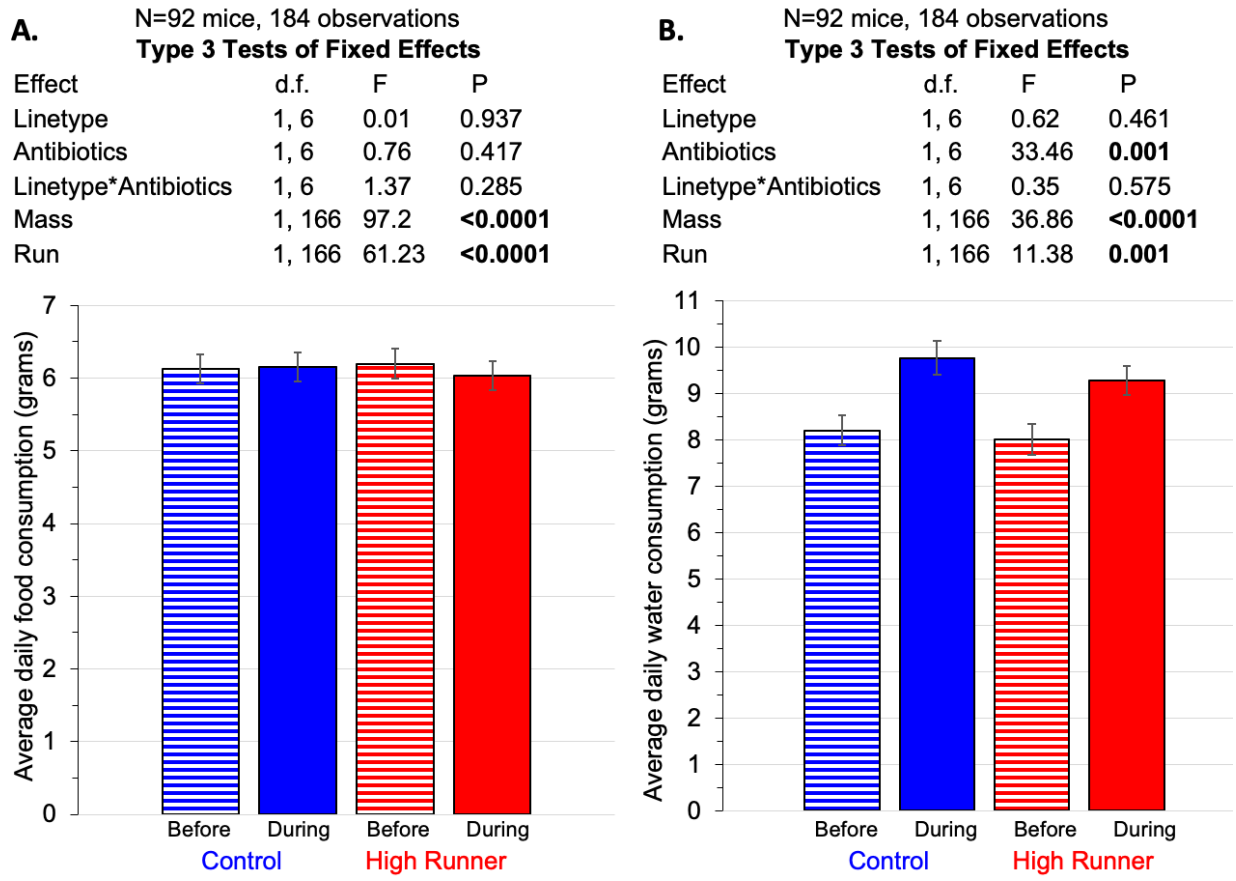


Figure 2. The effects of antibiotics on food and water consumption.



Repeated-measures analysis of average daily (A) food and (B) water consumption during the baseline period (days 8-14) compared to during the antibiotic treatment period (days 15-24). Data are presented as untransformed least squares mean plus or minus s.e.m.

Figure 3. The effects of antibiotics on food and water consumption with wheel running as a covariate.



Repeated-measures analysis of average daily (A) food and (B) water consumption during the baseline period (days 8-14) compared to during the antibiotic treatment period (days 15-24), as in Figure 2, but with wheel running as a covariate. Data are presented as untransformed least squares mean plus or minus s.e.m.

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