



Three-dimensional diet regulation and the consequences of choice for weight and activity level of a marsupial carnivore

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Many animals consume foods that vary in all 3 macronutrients: carbohydrates, lipid, and protein. Yet most studies of diet regulation only consider pairs of nutrients (protein and carbohydrate or protein and lipid). Diet regulation also extends beyond nutrient and energy intake to include sources of energy expenditure, such as changes in activity level. We used a right-angled mixture triangle design to quantify the 3-dimensional intake target of fat-tailed dunnarts, *Sminthopsis crassicaudata*, and to test the consequences of free choice for energy intake, weight gain, and activity level relative to a standard maintenance diet. Dunnarts consistently preferred a relatively high-lipid, low-protein, and low-carbohydrate diet in 3 separate feeding experiments. Dunnarts also consumed a higher total energy intake during choice relative to no-choice periods. However, the weight of dunnarts was not consistently higher at the end of choice relative to no-choice periods, which is likely because dunnarts increased their activity level during periods of choice and decreased their activity when on no-choice diets. This shows that increases in the intake of lipid, which is an important component in the diet of dunnarts, does not necessarily lead to increases in weight gain because these animals can adjust energy expenditure to balance their energy budget. These results have important implications for the design of diets for animals in captivity and demonstrate that consideration of both energy intake and expenditure are needed for a more comprehensive and integrative understanding of diet regulation by animals.

Key words: carnivore, diet choice, dunnarts, marsupial, nutrition, *Sminthopsis*

The balance of nutrients consumed by animals can have large consequences for growth, survival, and reproduction (Sternler and Elser 2002; Simpson and Raubenheimer 2012). Food items vary in their nutrient composition and animals often have particular requirements for nutrients (Sternler and Elser 2002; Simpson and Raubenheimer 2012). Hence, many animals have evolved nutrient regulatory behaviors that allow them to balance food consumption to achieve a targeted intake of nutrients at which fitness or components of fitness are maximized (Simpson et al. 2004; Lee et al. 2008; Maklakov et al. 2008). For example, fruit flies, *Bactrocera tryoni*, and vinegar flies, *Drosophila*

melanogaster, will target foods with an intermediate balance of carbohydrates and protein that maximizes lifetime reproductive success by balancing the benefits of high-protein food for egg production and high-carbohydrate foods for lifespan (Lee et al. 2008; Fanson et al. 2009). Similarly, male and female crickets, *Teleogryllus commodus*, select slightly different ratios of macronutrients that allow them to maximize different components of their reproductive success: calling and egg production (Maklakov et al. 2008). The rules that animals use to regulate their diet will affect how they respond to diet imbalances and the consequences for animal health and fitness.

Diet regulation and nutrient-targeted foraging has been demonstrated unequivocally for herbivores but has also recently been shown to be important for carnivores (Mayntz et al. 2009; Hewson-Hughes et al. 2011; Simpson and Raubenheimer 2012; Hewson-Hughes et al. 2013). At trophic extremes, the study of pairs of nutrients has been effective for examining diet regulation behavior including carbohydrates and protein for herbivores versus lipid and protein for carnivores (Simpson and Raubenheimer 2012). For more omnivorous animals, balancing consumption of all 3 macronutrients may be important for diet regulation. Even for herbivores and carnivores where 2 nutrients are assumed to be the main drivers of diet regulation, the ability or extent to which animals balance all 3 macronutrients remains poorly understood but may be more important than previously assumed (Hewson-Hughes et al. 2011, 2013; Coogan et al. 2014; Erlenbach et al. 2014; Kohl et al. 2015). For example, cats, *Felis catus*, appear to have a “carbohydrate ceiling” or level of carbohydrate intake at which they will stop eating foods regardless of whether they have satisfied their total energy, lipid, or protein requirements (Hewson-Hughes et al. 2011, 2013). Relatively little is known about diet regulation and target intakes of other carnivores, especially for all 3 macronutrients: carbohydrates, lipids, and proteins. Yet, information on self-selected nutrient intake and the consequences of restricted diets versus free choice is important for understanding how animals cope with temporal and spatial variation in food abundance and nutrient content. In addition, data on self-selected nutrient intake can inform the design of diets to increase the well-being and reproductive output of animals in captivity. Much of the data used to formulate diets for captive animals comes from field observations of their diet intake, which may or may not represent an ideal or self-selected diet depending on the availability of foods in nature.

Foraging and regulation of diet intake have featured prominently in studies of nutritional ecology. However, nutritional homeostasis or regulation of body mass and composition in an animal is a consequence of multiple interacting factors affecting the intake, storage, and expenditure of energy and nutrients. Animals have the ability to manipulate some or all of these factors to regulate their body mass including adjusting food intake, digestion efficiency, heat production, and activity level. Increases in energy intake that are not associated with concomitant increases in energy expenditure can result in an increase in body mass through fat storage. In the wild, seasonal increases in body mass or fat content can be important to prepare for seasonal changes or unpredictability in food supply (e.g., bears, *Ursus arctos*—Coogan et al. 2014) and are often balanced by low-food-availability periods that result in loss of gained fat. But, under stable conditions in captivity, fat gain is often not balanced by periods of fat loss and can lead to obesity and undesirable health outcomes (West and York 1998). Understanding the consequences of diet for both energy intake and usage is critical for providing a more holistic view of the regulation of nutritional homeostasis or body mass by animals.

We used a Right-angled Mixture Triangle framework (Raubenheimer 2011) to quantify 3-dimensional diet regulation

by fat-tailed dunnarts, *Sminthopsis crassicaudata* (Gould, 1844), and the consequences of free diet choice for energy intake, weight gain, and activity level. Fat-tailed dunnarts were chosen because they can be maintained in captivity in the laboratory and may provide a model system for beginning to understand diet regulation by marsupial carnivores, many of which are endangered. Specifically, we quantified the target intake of carbohydrate, lipid, and protein eaten by dunnarts in 3 diet experiments in which different combinations of macronutrient-biased food dishes were presented. We conducted experiments in 2 sets that used different sources of protein: experiments 1 and 2 used soy protein powder and experiment 3 used lean ground beef. We then compared the total calorie intake, body weight, and activity level of animals between periods when they were restricted to a maintenance diet versus given free choice of ad libitum nutrients. We predicted that dunnarts would prefer intakes with relatively low protein, high lipid, and low carbohydrate content, consistent with recent nutritional studies of some placental carnivores (Mayntz et al. 2009; Hewson-Hughes et al. 2011). We also predicted that differences in total energy intake between choice and no-choice periods would be associated with changes in activity level as dunnarts attempted to balance energy intake with energy expenditure.

MATERIALS AND METHODS

Study animals.—Experiments 1 and 2 were conducted in July 2013 and used 12 adult fat-tailed dunnarts (*S. crassicaudata*), aged from 11 to 12 months and with body mass 14.34 ± 0.97 g (mean ± 1 SE), captive-bred at the University of Sydney. These animals consisted of 6 pairs of siblings (1 female and 1 male of each sibling pair). The use of siblings allowed us to control for family effects in statistical analyses. Experiment 3 was conducted in September 2014 and used 12 different adult fat-tailed dunnarts aged from 11 to 12 months and with body mass 16.03 ± 1.97 g, also captive-bred at the University of Sydney. They were related to the animals used in experiments 1 and 2; many were genetic siblings but born in later litters. These animals also consisted of 6 pairs of siblings (1 female and 1 male of each sibling pair).

Throughout the study, dunnarts were housed individually in cages (internal dimension $20 \times 20 \times 30$ cm) with nest boxes (2 clean cardboard rolls that were sealed at one end), and under natural photoperiod with ambient temperature of $20 \pm 2^\circ\text{C}$. For each animal, a 12-cm-diameter wheel with bicycle pedometer (Cateye CC-VL820) was provided to record activity. Pedometers collected data on distance travelled (km) and time spent moving each day. We only present results for distance traveled because these 2 measures of activity were highly correlated (all $R^2 > 0.79$). Animals were fed daily and had access to water ad libitum. For the first 2 experiments, the eye surface temperature of each dunnart was measured to the nearest 0.1°C in the morning (07:30–08:30) of the beginning and the end of every experimental period (2 washout periods and 2 diet-choice periods) by placing an infrared digital thermometer (SE-100, Sein Electronics Co., Ltd., Gyeonggi, South Korea)

about 2 cm away from the surface of the eye. The temperature of the eye has been demonstrated to be a good proxy for body temperature in a closely related species, *S. macroura* (Song and Geiser 1997). All animals were normothermic when measured during the study. The study was approved by the Animal Ethics Committee of University of Sydney (K25/5-2013/3/6000) and conformed to guidelines approved by the American Society of Mammalogists (Sikes et al. 2016).

Diets.—We experimentally manipulated the macronutrient content of foods to provide animals with diets biased in carbohydrate, lipid, and protein. All diets started with a base of commercial canned cat food, “Whiskas,” jellymeat variety with 7% protein and 5.5% lipid by wet mass. To this base cat food, we added mixtures of concentrated nutrients depending on the type of diet we were making. Carbohydrate content was manipulated by adding a 1:1:1 mixture of sugar, wheat flour, and rice flour. Lipid content was manipulated by adding cold pressed extra virgin olive oil. Protein content was manipulated by adding soy protein powder (experiments 1 and 2) or lean beef mince (experiment 3). One package of lean beef mince was frozen at -20°C for 1 month before being used in experiments. Separate portions of 100 or 300 g were thawed in a refrigerator for 24–48 h before being microwaved for 1 min, which was more than sufficient to fully cook the beef. A small amount of water was added to some diets to standardize moisture and we formulated diets to ensure that the total energy density of diets was similar. The diets in experiment 3 did not have as high lipid contents as experiments 1 and 2 because adding too much oil to the beef-based diets would have changed the texture too much. Diets were mixed into a fine paste to avoid selective feeding within a food dish. Macronutrient calculations were based on manufacturer specifications for each ingredient, as these have been shown to be accurate (Stannard et al. 2014). Macronutrient content was quantified as a percentage of the total metabolizable energy derived from each macronutrient (Table 1).

General procedure and animals.—Our experiment involved subjecting animals to alternating periods of 5–7 days on either a no-choice diet similar to their normal maintenance diet (~12 g food/animal/day, composition: protein 32%, lipid

51%, carbohydrate 17%; Table 1) and choice periods during which animals were provided with 3 food dishes: high-protein, high-lipid, and high-carbohydrate foods (~12 g food/dish/animal/day; for composition, see Table 1). We ran 3 separate diet-choice trials in 2 blocks. In the 1st set of experiments (experiments 1 and 2), we used soy protein powder to manipulate diet protein content. The 1st set of experiments involved dunnarts being presented sequentially with a 5-day pretreatment exposure to a no-choice diet during which we did not collect data, a 5-day free-choice period for experiment 1, a 5-day no-choice period, and a 7-day free-choice period for experiment 2. Experiment 1 (high protein, high lipid, high carbohydrate) and experiment 2 (high protein, high lipid, similar nutrients) used different sets of diets (Table 1). The 2nd set of experiments used lean ground beef to manipulate diet protein content. The 2nd set of experiments involved animals being presented sequentially with a 5-day no-choice period, a 7-day free-choice period, and a 5-day no-choice period.

We weighed the food presented to dunnarts and the food remaining after 24 h. During all experiments, 2 dishes for each diet were used to calculate the dehydration rate every day and consumption rates were adjusted for dehydration. Throughout the study, animals had ad libitum access to food and drinking water and were provided with vitamins and minerals mixed in the food on Wednesdays and calcium powder mixed into the food on Sundays. The body mass and tail width of dunnarts were measured at the beginning and the end of each no-choice period or diet-choice period.

Statistical analysis.—We tested if animals fed randomly from their 3 food dishes during the diet-choice trials by comparing the mass of food removed from each dish with the predicted mass of food that would have been removed if dunnarts had fed randomly. Predictions for random food consumption from dishes were determined by adding the mass consumed from all 3 dishes and dividing by 3 (Hawley et al. 2016). Multivariate analysis of variance (MANOVA) was used to compare the mass of food consumed from each of the 3 nutrient dishes with predictions from random feeding. We then converted mass consumed into macronutrient consumed by each animal and used analysis of variance (ANOVA) to compare intake of each macronutrient with expectations from random feeding. Predictions for random nutrient intake for each macronutrient were determined by taking the average of the nutrients available in the diet (Table 1). Analysis and presentation of data on macronutrient intake allowed for more direct comparisons to studies of other species.

To test the effects of choice, total energy intake and mass of dunnarts was compared between no-choice and choice periods using ANOVA. Distance traveled per day was compared between no-choice and choice periods first using repeated measures ANOVA. When significant time \times treatment interactions were observed, we calculated the change in distance traveled per dunnart per period (final – initial daily distance traveled) and compared this between no-choice and choice periods using ANOVA. Tukey post hoc tests were used to determine individual differences among treatments for all ANOVAs.

Table 1.—Macronutrient composition of the diets used in the experiments as a percentage of the metabolizable energy of the diets. Values are based on manufacturer’s analysis of nutrient content.

Diet	Percent of total macronutrients by energy		
	Protein	Lipid	Carbohydrate
Cat food	32	51	17
Diets with soy protein (experiments 1 and 2)			
High protein	53	37	10
High lipid	22	68	10
High carbohydrate	22	34	44
Similar nutrients	36	35	29
Diets with lean beef (experiment 3)			
High protein	50	40	10
High lipid	29	62	9
Similar nutrients	32	34	34

RESULTS

Intake target during choice.—In all 3 experiments, dunnarts fed nonrandomly from their food dishes (MANOVAs: experiment 1: Wilks' $\lambda = 0.041$, $F_{2,10} = 118.2$, $P < 0.001$; experiment 2: Wilks' $\lambda = 0.050$, $F_{2,9} = 86.2$, $P < 0.001$; experiment 3: Wilks' $\lambda = 0.087$, $F_{2,10} = 52.5$, $P < 0.001$). In experiments 1 and 3, dunnarts consumed less protein (experiment 1: $F_{1,11} = 958.1$, $P < 0.001$; experiment 3: $F_{1,11} = 217.9$, $P < 0.001$) and more lipid (experiment 1: $F_{1,11} = 31.0$, $P < 0.001$; experiment 3: $F_{1,11} = 4.6$, $P = 0.056$) than would be expected from random feeding but carbohydrate intake was not significantly different from random (experiment 1: $F_{1,11} = 1.6$, $P = 0.23$; experiment 3: $F_{1,11} = 0.9$, $P = 0.37$). In experiment 2, dunnarts consumed less protein ($F_{1,10} = 258.9$, $P < 0.001$), less carbohydrate ($F_{1,10} = 200.5$, $P < 0.001$), and more lipid ($F_{1,10} = 75.6$, $P < 0.001$) than would be expected from random feeding. The exact values of the intake target (i.e., the self-selected balance of nutrients) differed among experiments (Wilks' $\lambda = 0.068$, $F_{4,40} = 28.3$, $P < 0.001$). However, the triangles of available nutrient space were not completely overlapping, such that it was not possible for animals in experiment 2 to consume the same mixture of nutrients as the nutrient target for experiment 1 and the same for animals in experiment 3 compared to the intake targets of experiments 1 and 2. Yet, animals were consistently in the upper left corner of the triangle (i.e., low protein, low carbohydrate, and high lipid) relative to random (i.e., the geometric center of each triangle; Fig. 1A). The regulation of a low protein (23–32%), high lipid (49–61%), and low carbohydrate (13–19%) intake can also be seen in comparisons of average intake relative to the range of available nutrients (Fig. 1B).

Choice versus no choice.—**Energy intake:** Daily energy intake was significantly higher during the choice than during the no-choice periods. In the 1st set of experiments, energy intake during choice experiment 1 was 32% higher and choice experiment 2 was 28% higher than the no-choice periods ($F_{2,21} = 28.6$, $P < 0.001$). In the 2nd set using meat-based diets, energy intake during choice experiment 3 was 9% higher than the 1st and 20% higher than the 2nd no-choice period ($F_{2,22} = 19.5$, $P < 0.001$).

Body mass: There were significant differences but no consistent patterns in comparisons of dunnart body mass between choice and no-choice periods. Comparisons revealed higher body mass of dunnarts during the no-choice period for experiment 1, no difference between choice versus no-choice in experiment 2, and higher body mass of dunnarts during the choice period for experiment 3 (Fig. 2A: $F_{2,20} = 19.7$, $P < 0.001$; Fig. 2B: $F_{2,22} = 12.5$, $P < 0.001$).

Daily activity: The average daily distance traveled by dunnarts ranged from 1.7 to 4.5 km/day. In repeated measures analyses of the activity data, there were no significant effects of choice versus no-choice in experiments 1 and 2 ($F_{2,32} = 0.76$, $P = 0.48$) and experiment 3 ($F_{1,28} = 0.14$, $P = 0.71$). However, there were strong temporal patterns in the data (Fig. 3; time \times treatment: experiments 1 and 2: $F_{8,128} = 6.75$, $P < 0.001$; experiment 3: $F_{4,112} = 11.96$, $P < 0.001$), which we explored by calculating the change in daily distance traveled from the first to the last day of each experimental period. In experiments 1 and 2, dunnarts increased their average distance traveled by between 0.43 and 0.52 km/day over the course of the choice periods but decreased

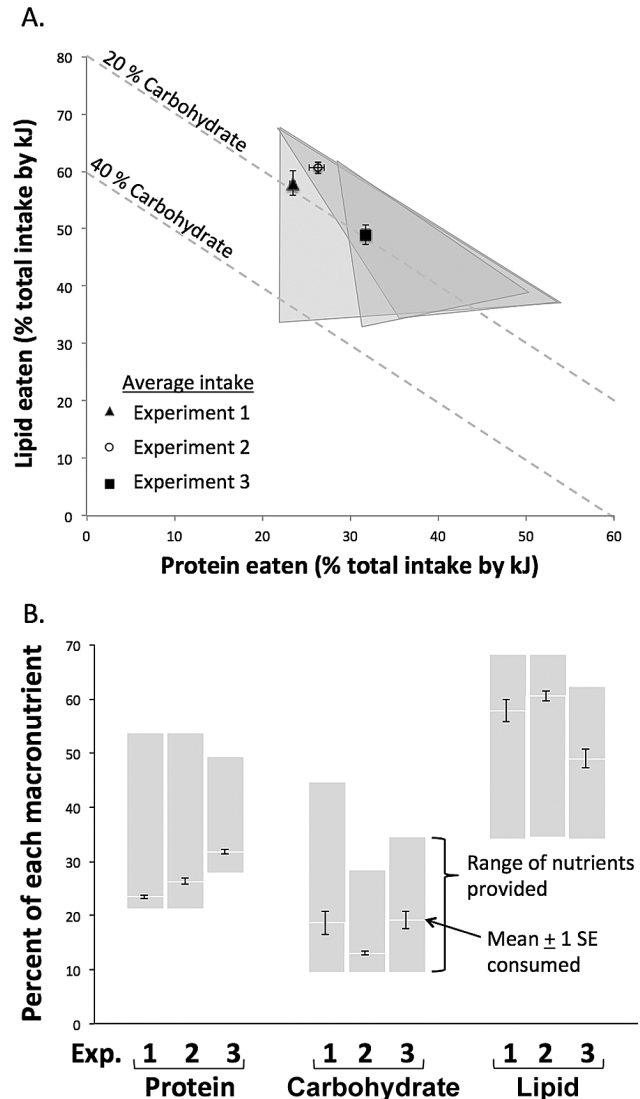


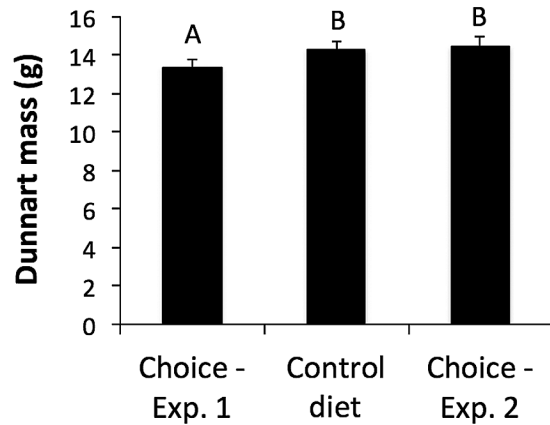
Fig. 1.—Nutrient intake of dunnarts when presented with free choice from protein-, carbohydrate-, and lipid-biased food dishes. A) Mixture triangles indicate the mean diet balance selected by dunnarts (points ± 1 SE) within the available nutrient space within which dunnarts could have regulated their diets (gray shaded triangles) for each experiment. Each animal was provided with 3 diet dishes and each of these dishes corresponded to one of the 3 corners of each triangle. B) Mean ± 1 SE intake of each macronutrient relative to the range of availabilities of each nutrient in diet dishes.

their activity by 1.63 km/day over the course of the no-choice period (Fig. 3A; $F_{2,21} = 14.39$, $P < 0.001$). Similarly, for experiment 3, animals increased their activity by 1.65 km/day over the course of the choice period but decreased their activity by 1.27 and 1.86 km/day over the course of the 1st and 2nd no-choice periods, respectively (Fig. 3B; $F_{2,18} = 20.89$, $P < 0.001$).

DISCUSSION

Fat-tailed dunnarts regulated their consumption of all 3 macronutrients by selecting intakes that had relatively low protein (23–32%), high lipid (49–61%), and low carbohydrate (13–19%) content. This result is contrary to conventional

A. Experiments 1 and 2



B. Experiment 3

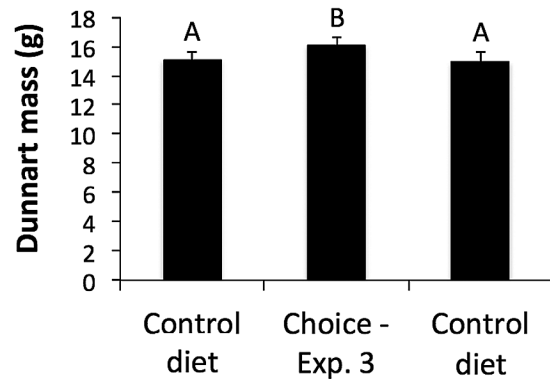
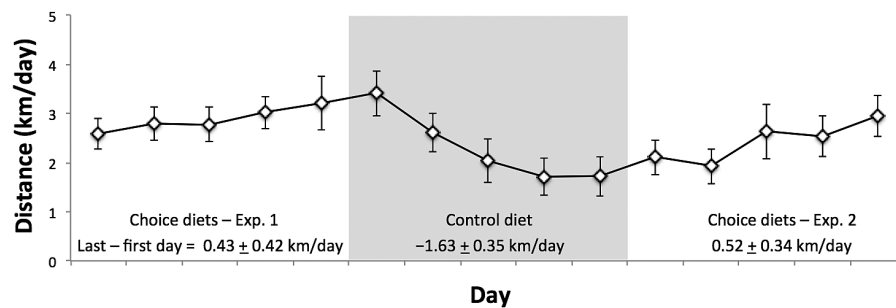


Fig. 2.—Body mass of fat-tailed dunnarts at the end of choice and no-choice periods for A) experiments 1 and 2 and B) experiment 3. Letters above bars identify treatments that were significantly different.

wisdom that carnivores prefer or are highly adapted to high-protein diets but supports similar studies investigating dietary preferences in placental carnivores, where relatively low-protein and high-lipid diets also were selected (Erlenbach et al. 2014). For example, mink, *Neovison vison*, self-selected a diet with 35% protein and 50% lipid by metabolizable energy (Mayntz et al. 2009). Cats self-selected a diet with relatively higher protein content but protein still was only around one-half of their total metabolizable energy intake (40–50% protein, 40% lipid, 10–20% carbohydrate—Hewson-Hughes et al. 2011, 2013). While the protein intake target of carnivores is relatively low compared to available foods in the experiments (Fig. 1), this protein intake is also relatively high compared to herbivores and some omnivores. Furthermore, low protein and high lipid intake may be preferred because high protein consumption can result in shorter lifespan and because protein metabolism yields far less energy and more potentially damaging metabolic byproducts than does lipid metabolism (Simpson and Raubenheimer 2009; Solon-Biet et al. 2014, 2015). Presumably, the higher mass-specific metabolic rates of these small mammals (i.e., dunnarts and mink) relative to cats may help explain why they selected diets with higher concentrations of energy-dense lipids (Nagy et al. 1988; Gillooly et al. 2001).

Studies of animal intake targets often include multiple diet combinations, as we did in this study, to test 1) if animals consistently feed nonrandomly and 2) if they defend a particular mixture of nutrients (Simpson and Raubenheimer 2012). In our experiments, dunnarts fed nonrandomly and consistently preferred low protein, low carbohydrate, and high lipid intakes. However, the target intake that they selected differed among diet experiments, which is likely because the available nutrient spaces differed among experiments and were not completely overlapping. In other words, experiment 1 had the broadest

A. Experiments 1 and 2



B. Experiment 3

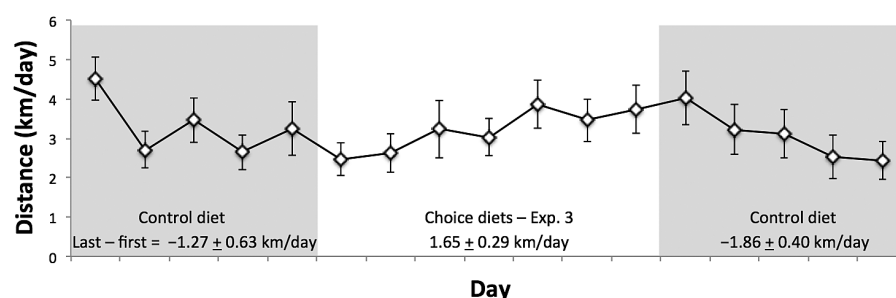


Fig. 3.—Mean ± 1 SE distance travelled by individual dunnarts over the course of the choice and no-choice periods of the experiment. For each choice or no-choice period, we also calculated the change in activity level over the period (daily distance travelled on the last day – daily distance travelled on the first day).

nutrient space triangle and experiments 2 and 3 had triangles with progressively higher minimum protein concentrations. Hence, animals in experiments 2 and 3 were unable to regulate a nutrient target with as low a protein content as could the animals in experiment 1. Another explanation for the differences in nutrient intake targets is that dunnarts may have flexibility in their nutrient intake. Flexibility in nutrient intake would be advantageous for small animals with higher relative metabolic rates, like dunnarts, that eat almost their body weight in food each day and may experience spatial and seasonal variation in food supply (Nagy et al. 1988). Fat-tailed dunnarts also are flexible in their use of torpor to reduce their metabolic rate, which may be further evidence that spatial or temporal variation in food availability is a strong selective factor for flexibility in feeding and metabolism in this species (Munn et al. 2010).

Feeding during free-choice periods resulted in higher total energy consumption relative to periods of no choice. However, this higher energy intake did not result in a consistent increase in body mass. In the 1st and 2nd experiments, there was an increase in dunnart weight with time, which may be related to seasonal patterns of weight gain. In the 3rd experiment, the mass of dunnarts was 7% higher during the choice period relative to either no-choice period. The small differences in body mass between choice and no-choice despite greater energy intake during choice periods may be related to the consistent increase in activity of dunnarts during choice periods. Dunnarts increased their daily distance traveled by 24–39% over the course of the 5- to 7-day choice trials. The change in distance traveled (0.4–1.7 km/day) was likely a large change in energy use for such a small (~12 g) animal. These results have important implications for maintaining animals in captivity. A challenge in captive maintenance and breeding programs is maintaining optimum animal weight and encouraging exploration and activity, which can provide enrichment and increase metabolic health (Wilson 1982; Dishman et al. 2009). These goals often are pursued separately by limiting the quantity and lipid content of diets and also providing activities to encourage exploration. Our data suggest that diet intake and activity level in dunnarts are linked and that allowing increased intake or a different balance of nutrients could result in increased activity of animals. Further work is needed to explore the link between diet and activity level and if changing opportunities for activity (e.g., removing a running wheel) affects the ability of animals to balance energy intake and expenditure.

Torpor is another response that is commonly used by dunnarts and many other small mammals to balance their energy intake and expenditure (Munn et al. 2010; McAllan et al. 2012). Dunnarts are very flexible in the onset and duration of bouts of torpor and will use it when food is in low abundance and sometimes when food is present but of a nonpreferred type (Geiser et al. 2005; Munn et al. 2010; McAllan et al. 2012). We rarely observed animals engaging in torpor during the course of this study, which is likely because animals were always provided with sufficient food to maintain baseline activity. Yet, on more restricted diets both use of torpor and decreases in activity could be used to conserve energy.

Many studies of diet regulation focus primarily or exclusively on the ratio and total intake of nutrients. Yet, diet regulation is a process that integrates among many different components of physiology and behavior. Our result suggests that dunnarts balance their intake and activity level to maintain stable body weight. Future work on diet balancing should focus more on multiple aspects of nutrient intake and expenditure simultaneously to provide a more comprehensive and integrative understanding of diet balancing. In addition, further work is needed to understand how animals respond to diets with fixed compositions. There is a range of behavioral decisions that animals may make when feeding on imbalanced diets (e.g., rules of compromise, lipid ceiling, etc.) that can influence their total energy intake or intake of particular key nutrients and have consequences for life history traits (e.g., growth and reproduction) or activity level (Simpson et al. 2004; Hewson-Hughes et al. 2011, 2013).

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