Diet-based Sodium Regulation in 6th Instar Grasshoppers, *Schistocerca americana* (Drury) (Orthoptera: Acrididae)

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Abstract: This study analyzed sodium intake by *Schistocerca americana* (Drury) (Orthoptera: Acrididae) grasshoppers using three different seedling wheatgrass based diet treatments to simulate a natural food source. Sodium is a key nutrient for grasshopper cells, nerves, and reproduction. Grasshoppers acquire sodium from plants that they consume. However, it is unclear if grasshoppers self-regulate their sodium intake. Additionally, if grasshoppers self-regulate their sodium intake, the extent to which they do is uncertain. Newly molted 6th instar grasshoppers were fed one of three diets in which the level of sodium that they had access to was varied. *Schistocerca americana* consumed significantly less of the 0.5 M added sodium only diet when presented with an option to choose between this diet and a no-sodium-added diet ($t = 9.6026$; df = 7; $P < 0.0001$). Grasshoppers in the 0.5 M added sodium only treatment consumed a significantly lower amount of food ($P < 0.0001$) as well as gained a significantly lower average mass ($P < 0.0001$) compared to the grasshoppers in the no-sodium-added only treatment. Our results generally correlated with previous studies on *Locusta migratoria* (L.) (Orthoptera: Acrididae) and information about the ecological tolerances and nutritional requirements of grasshoppers. Our data suggests that *S. americana* are capable of self-regulating their sodium intake. Additionally, we show that high concentrations of sodium in grasshopper diets have a negative effect on body mass. Our study illustrates that diet-based sodium regulation is a factor in the relationship between insect herbivores and their environments.

Keywords: physiology, nutrition, tolerance, salt, insect herbivory

For the majority of terrestrial plants, sodium (Na) is not required for growth, development, or reproduction (Maathuis 2014). Sodium can adversely affect plant communities by reducing the amount of water, other nutrients, and oxygen available to plants (Hasegawa 2013). Gradients of sodium concentrations occur on land as a result of magma flows, ocean water levels, and human activity such as fertilizing and watering crops or salting roadways (Kaspari et al. 2014). Sodium is an important nutrient for grasshoppers and other insects as well as plants (Boswell et al. 2008; Clarkson and Hanson 1980; Hasegawa 2013; Pedersen and Zachariassen 2002; Pontes et al. 2017). The sodium ion, Na^+, is essential for cell and nerve function in grasshoppers as well as egg production in females (Boswell et al. 2008).

Grasshoppers are primarily herbivorous insects and rely on plants for vital nutrients such as sodium. In one study, it was found that grasshoppers grazed in areas fertilized with nitrogen and phosphorus more than unfertilized areas and even identified the areas of higher fertilization and migrated to them within a single season (Sparks and Cebrian 2015). Insects also modify their behavior to regulate salt intake (Arms et al. 1974; Boggs and Jackson 1991; Chambers et al. 1997; Pontes et al. 2017; Trumper and Simpson 1993; Trumper and Simpson 1994). Depending on the chemical complexity of the food it is applied to and its concentration, sodium chloride can either stimulate or deter feeding in grasshoppers (Trumper and Simpson 1994). Locust nymphs, *Locusta migratoria* (L.) (Orthoptera: Acrididae), exhibit different feeding patterns within hours of being fed either a 2.48% Wesson's salts by dry weight diet or a no-added-sodium diet, including indications of increased foraging behavior in the locusts provided with the no-added-sodium diet (Trumper and Simpson 1994). In a more extreme case of behavioral regulation of sodium intake, Mormon crickets, *Anabrus simplex* Haldeman (Orthoptera: Tettigoniidae), forage for salt when marching and will even cannibalize each other to obtain this limiting nutrient (Simpson et al. 2006).

Animals, including insect herbivores, are known for selecting foods based on nutrient intake targets to support their biological processes (Behmer 2009). In one study, *A. simplex* preferred a 0.25 M solution of sodium chloride over other offered concentrations ranging from 0.0 M to 2.0 M (Simpson et al. 2006). Additionally, when *L. migratoria* nymphs are presented with two different food sources, a diet with no-added-sodium and a diet with added salt above their optimal level, they would be expected to eat amounts of both diets to reach a level of sodium intake close to or at their level of requirement (Simpson 1994). *Schistocerca americana* would be expected to behave similarly in this situation as they are also orthopterans and require sodium in their diet like most other insects.

In addition to behavioral aspects of sodium regulation, the physiological effects of sodium on grasshoppers include body mass, development time, the concentration of sodium in tissue, and the concentration of sodium in feces. However there has been little research on these specific effects related to sodium on grasshopper physiology (Boswell et al. 2008; Simpson et al. 2006; Trumper and Simpson 1994). It has been reported that when reared on a standardized diet, the concentration of sodium related to body dry mass increases linearly as grasshoppers age (Boswell et al. 2008).

The objectives of our study were to determine if *S. americana* self-regulate sodium intake as well as analyze how different concentrations of sodium in the diet affect *S. americana* body mass. We hypothesized that the grasshoppers would display evidence of sodium regulation based on the previous studies described. We also expected that body mass in *S. americana* would be reduced when fed a diet with a high concentration of sodium.

Materials and Methods

Sodium intake was measured between three groups of eight 6th instar *S. americana* grasshoppers by providing each of them with a different variation of a seedling wheatgrass based treatment to mimic what a grasshopper might eat in a natural environment. The amounts of food consumed and mass gained were analyzed. Three different treatments were used: no-added-sodium only (control treatment), 0.5 M added sodium only, or a block of each of two prepared diets.

Study Insects. The *S. americana* grasshoppers used in this study were reared in a colony setting at the Behmer Lab of Insect Physiology and Behavior at Texas A&M University, College Station, Texas, USA. Individual 6th instar nymphs were chosen from groups that were newly molted within 24 h. The grasshoppers were released back into the colony at the conclusion of the experiment.

Diets. Two different diets were prepared, a diet without added sodium to represent a standard wheatgrass based diet and a diet with 0.5 M added sodium. The main component of each diet was freeze dried seedling wheatgrass which contains 0.5% sodium by dry weight (S.T.B., unpublished data).

Freeze-dried seedling wheatgrass preparation. Wheatgrass seeds were planted in an equal mixture of potting soil and vermiculite in a germination tray and covered until they sprouted. Once sprouted, the wheat grass was transferred to the Norman Borlaug Institute for International Agriculture, Texas A&M University, College Station, Texas, USA, greenhouse and grown until they were between 15-20 cm tall. During this time, the wheatgrass was watered as needed to prevent desiccation and promote growth. The wheat grass was harvested by cutting the grass blades at their bases. The grass was wrapped in paper towels and placed in a FreeZone Plus 6 Liter Cascade Console Freeze Dry System (Labconco, Kansas City, MO) freeze dryer until the wheatgrass was completely dry or up to about a week. The freeze-dried seedling wheatgrass was ground into a fine powder with a fine sieve fitted in an MF 10 basic Microfine grinder drive (IKA Werke, Wilmington, NC) and stored in a -40°C freezer until it was needed.

Diet preparation. The no-added-sodium diet was prepared by combining 1 g of freeze-dried seedling wheatgrass powder (Wilmington, NC) with 10 mL of distilled water (H_2O) and 100 mg of melted agar (Sigma-Aldrich, Inc., Saint Louis, MO). The solution was poured into a 100 mm by 15 mm plastic petri dish (VWR, Radnor, Pennsylvania) for solidification. This resulted in a final diet that was 8.79% sodium by dry weight. Blocks of diet ranging from about 400 mg and 1300 mg in weight were cut out of the petri dishes and weighed to the nearest 0.10 mg using an electronic bench scale immediately before being presented to grasshoppers (Fig. 1).

Linear Regression Equation. A linear regression model was created to convert the wet mass of each diet to its final dry mass using Microsoft® Excel for Mac, version 2013 (Microsoft, Redmond, WA). The data for the linear regression model was obtained by completely drying 12 blocks of each diet ranging between 400 mg and 1300 mg in weight in a Model 20 Gravity Convection

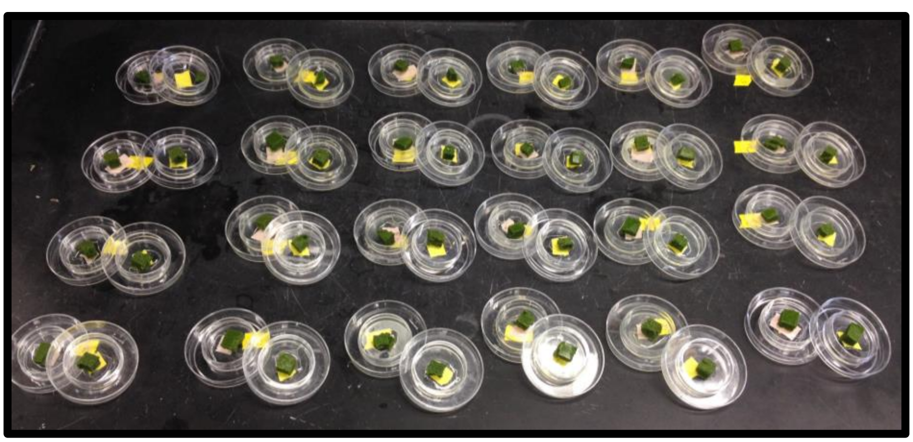

Fig. 1. Food dishes prepared prior to being presented to the grasshoppers during the experiment.

(GC) Lab Oven

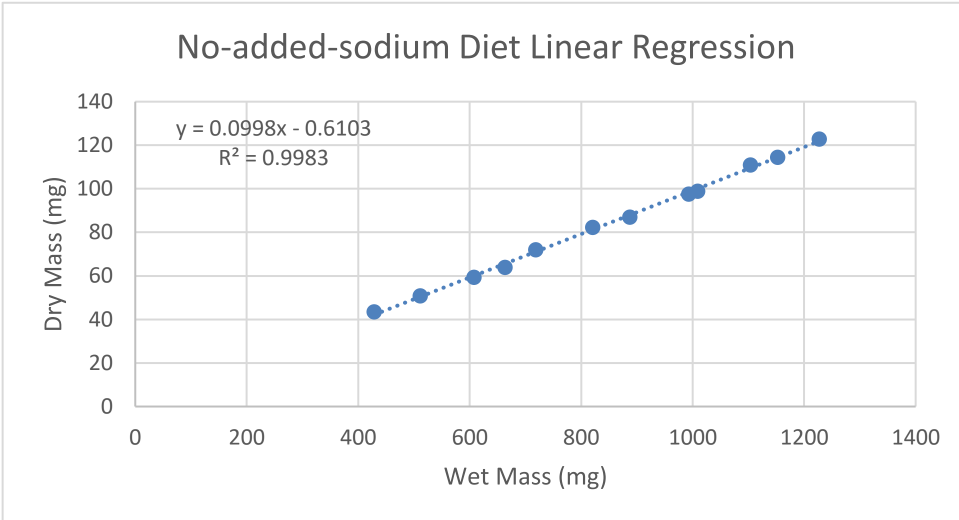

Fig. 2. The linear regression data for the no-added-sodium diet showing dry mass (mg) by wet mass (mg).

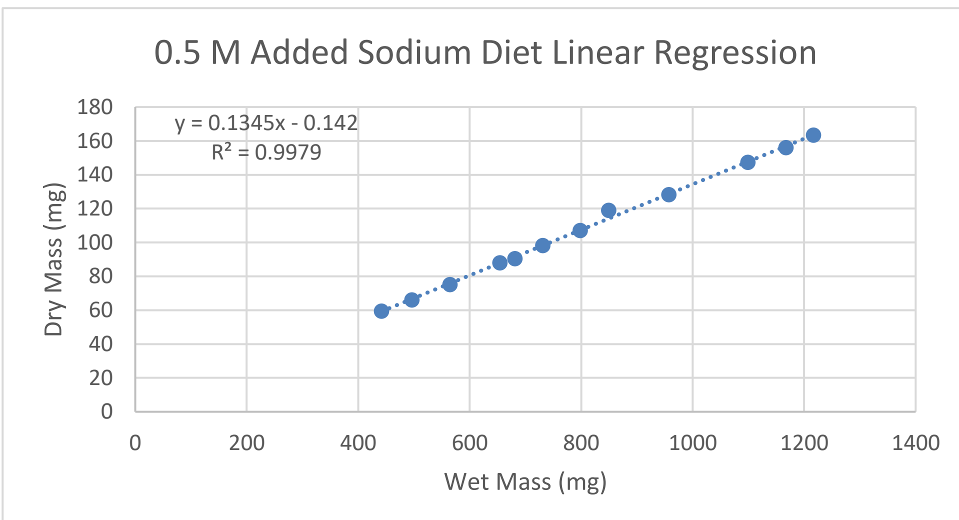

Fig. 3. The linear regression data for the 0.5 M added sodium diet showing dry mass (mg) by wet mass (mg).

(Quincy Lab Inc., Chicago, IL) set at 40°C. The no-added-sodium diet linear regression equation was $y = 0.0998x - 0.6103$ ($R^2 = 0.99828$) (Fig. 2). The 0.5 M added sodium diet linear regression equation was $y = 0.1345x - 0.1420$ ($R^2 = 0.99794$) (Fig. 3). These equations were applied to the wet masses of each block of the respective diet presented to the grasshoppers throughout the study so that the mass of food consumed by the grasshoppers could be calculated.

Study Design. Arenas were created with clear plastic chambers measuring 19 cm long by 14 cm wide by 9 cm high with six, 5 mm air holes evenly spaced in the lid (Fig. 4). A wire mesh roost measuring 17 cm long (bent inwards at 4.5 cm from each side) and 4.5 cm high was placed at one end of the box while two clear plastic food dishes, each composed of an outer dish with a diameter of 5.7 cm and an inner dish with a diameter of 3.5 cm glued inside of it, were placed in each of the opposite corners of the box (Fig. 1; Fig. 4). The food dishes were replaced each time new food was presented to a grasshopper (Fig. 1). To minimize the effects of external influences such as the behavior of other grasshoppers, the bottom and sides of the boxes were wrapped with white printer paper which was secured with strips of clear tape. Additionally, the boxes were arbitrarily placed on shelves by treatment type in the Behmer Lab colony room (Fig. 5). They were maintained under standard laboratory conditions with a photoperiod of 14:10 (L:D) and under radiant heat at a temperature of 32-35°C during the light phase (supplied by 60W full-spectrum incandescent bulbs), and at 24-26°C during the dark phase.

Experimental Procedures. The three different treatments were randomly assigned to a total of 24 grasshoppers, with eight grasshoppers per treatment. The sex of each grasshopper as well as its initial weight was recorded to the nearest 0.10 mg using an electronic bench scale prior to treatment. The experiment was conducted for three days with the weighed food blocks being replaced every eight hours, at approximately 7:30 A.M., 3:30 P.M., and 11:30 P.M. each day. Upon the replacement of the food, any remaining food in each dish was placed in a small plastic cup in a Model 20 Gravity Convection (GC) Lab Oven (Quincy Lab Inc., Chicago, IL) set at 40°C and totaled at the conclusion of the experiment. Each grasshopper's final weight was also recorded to the nearest 0.10 mg at the conclusion of the experiment. The total dry mass of food that remained was compared to the mass of food expected based on the amount of wet food presented to the grasshopper over the course of the study and the linear regression equation described previously so that the actual mass of food consumed could be calculated and analyzed. Each grasshopper's final weight was also recorded to the nearest 0.10 mg at the conclusion of the experiment. The total dry mass of food that remained was compared to the mass of food expected based on the amount of wet food presented to the grasshopper over the course of the study and the linear regression equation described previously so that the actual mass of food consumed could be calculated and analyzed.

Statistical Analyses. T-tests were performed to determine statistical significance of the average mass of food consumed per dish per treatment. Analysis of variance (ANOVA) tests were performed to determine statistical significance on the average total mass of food consumed per treatment as well as the average mass gained by the grasshoppers per treatment. Dunnett's method was applied for *post hoc* analyses of the average total mass of food consumed per treatment as well as the average mass gained by the grasshoppers per treatment. Dunnett's method compared the control treatment (no-added-sodium only) to each of the other two treatments individually to determine the level of statistical difference between the treatments. Significant differences were measured at the $P < 0.05$ level. All statistical analyses were run using JMP®, version 7.0.2 (SAS Institute Inc., Cary, NC).

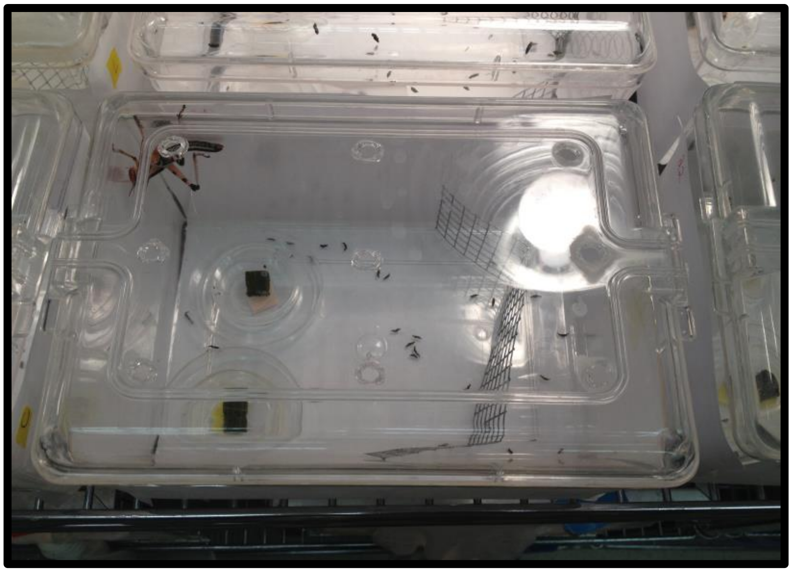

Fig. 4. Arena with a 6th instar *S. americana* grasshopper showing the relative positions of the wire mesh roost and food dishes.

Fig. 5. Randomized placement of the chambers under standard laboratory conditions during the experiment.

Results

Average Mass of Food Consumed per Dish per Treatment. The t-test showed that there was not a significant difference in the average amount of food consumed between the two food dishes in the no-added-sodium only treatment (t = 0.9317; df = 7; P = 0.3825) (Fig. 6). There was a significant difference in the average amount of food consumed between the two food dishes in the choice treatment (t = 9.6026; df = 7; $P < 0.0001$) (Fig. 6). Additionally, there was not a significant difference in the average amount of food consumed between the two food dishes in the 0.5 M added sodium only treatment (t = -1.0254; df = 7; P = 0.3393) (Fig. 6).

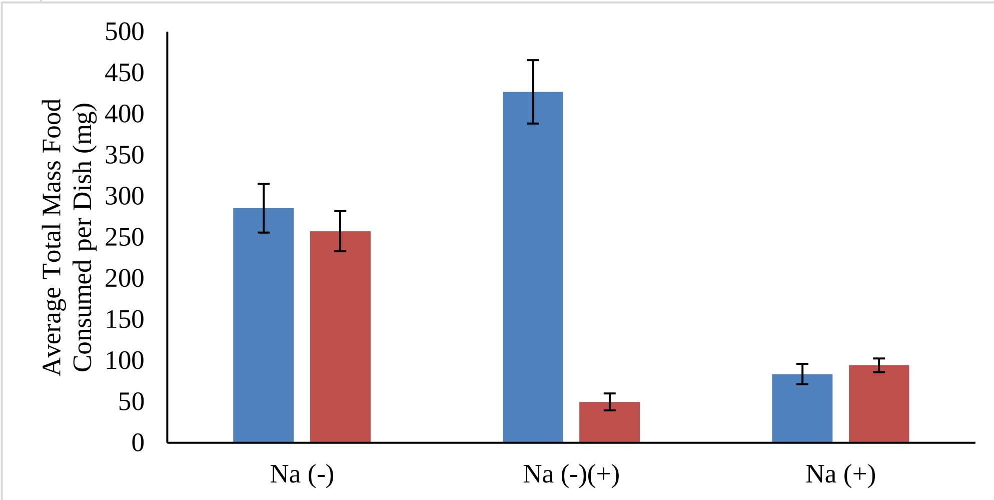

Fig. 6. The average total mass of food (mg) consumed per food dish per treatment type is presented. The no-added-sodium only treatment (Na (-)) dishes, the combination of diets (Na (-)(+)) with the no-added-sodium dish in blue and 0.5 M added sodium dish in red, and the 0.5 M added sodium only treatment (Na (+)) dishes are shown.

Fig. 7. The average total mass of food (mg) consumed per treatment type is presented. The no-added-sodium only treatment (Na (-)), the combination of diets (Na (-)(+)) treatment, and the 0.5 M added sodium only treatment (Na (+)) are shown.

Average Total Mass of Food Consumed per Treatments. The ANOVA test and the Dunnett's method *post hoc* analysis showed that the average total amount of food consumed in the choice treatment was not statistically different than the no-added-sodium only treatment ($P = 0.3544$) (Fig. 7). However, the average total amount of food consumed in the 0.5 M added sodium only treatment was statistically different than the no-added-sodium only treatment ($P < 0.0001$) (Fig. 7).

Average Grasshopper Mass Gained per Treatment. The ANOVA test and the Dunnett's method *post hoc* analysis showed that the average mass gained by the grasshoppers in the choice treatment was not statistically different than the no-added-sodium only treatment ($P = 0.9565$) (Fig. 8). However, the average mass gained by the grasshoppers in the 0.5 M added sodium only treatment was statistically different than the no-added-sodium only treatment (*P* < 0.0001) (Fig. 8).

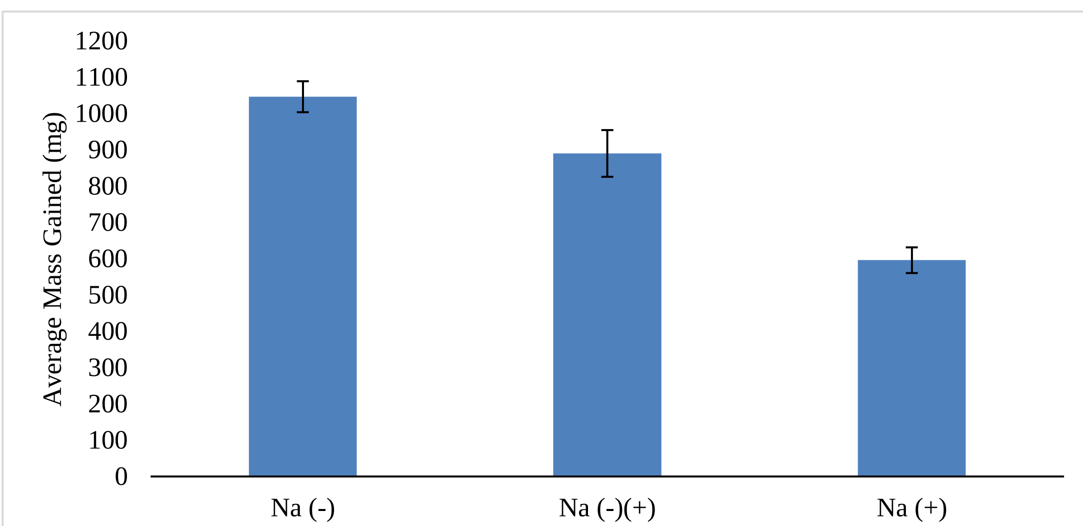

Fig. 8. The average mass (mg) gained by the grasshoppers per treatment type is presented. The no-added-sodium only treatment (Na (-)), the combination of diets (Na (-)(+)) treatment, and the 0.5 M added sodium only treatment (Na (+)) are shown.

Discussion

Insect herbivores such as grasshoppers require sodium in their diets as well as several other nutrients in specific amounts (Behmer 2009; Boswell et al. 2008; Joern et al. 2012). Considering that the amounts of any particular nutrient may be found in low or high concentrations in a food source, insects must choose what and how much to eat in order to gain the greatest nutritional benefit without consuming too little or too much of what their bodies require. Due to the difference in amounts of food consumed between the three treatments, it is likely that the grasshoppers in our study were self-regulating their sodium intake (Fig. 7). This is further supported by the information provided by the comparison of the amount of food consumed between the two dishes in the other two treatments where there was no option between the diets (Fig. 6).

One reason why the grasshoppers did not eat as much food in the 0.5 M sodium added treatment compared to the other treatments is that consuming high levels of sodium can be toxic to organisms (Fig. 7; Trumper and Simpson 1994). Food with too much sodium can also deter grasshoppers from consuming prepared diets (Trumper and Simpson 1994). Sodium may act as a deterrent to grasshoppers because, when consuming food with high concentrations of sodium, they would likely get the amount of sodium that they require, but not enough carbohydrates, proteins, or other nutrients. The concentration of sodium in the 0.5 M added sodium food (8.79% sodium by dry weight) was high, nearly 20 times the amount of sodium found in seedling wheatgrass (0.5% sodium by dry weight).

There is also a trade-off in fitness when an insect is not able to meet each of its nutrient requirements in their preferred concentrations (Behmer 2009). Locusts show reduced growth within 24 h of salt deprivation (Trumper and Simpson 1994). Therefore, overall development time may increase when grasshoppers do not have adequate access to sodium. This could also explain why the grasshoppers eating the 0.5 M added sodium only diet gained less mass on average then the grasshoppers in either of the other treatments we tested in our study. It can be inferred that the grasshoppers fed only the no-added-sodium as well as the grasshoppers provided with a choice in diets were consuming sufficient amounts of the nutrients they needed to grow as they gained mass over the course of the study (Fig. 8). In contrast, the 0.5 M added sodium only treatment grasshoppers may have been deterred from consuming more nutrients due to either their satisfied intake of sodium or because the concentration of sodium in the food was slightly higher than their natural preference based on phagostimulation which would be congruent with Trumper and Simpson's (1994) findings with locusts. Our results also correlate with Trumper and Simpson's (1994) report in that their salt-deprived locusts ate significantly more than the non-deprived insects similar to our grasshoppers in our control (the no-added-sodium diet only) and choice treatments which ate more than the 0.5 M added sodium treatment group and gained greater average mass as a result (Fig. 7; Fig. 8).

Insect herbivores have been found to be associated with complex plant communities and nutrients found in plant foliage. These concentrations vary per local environmental condition (Joern et al. 2012). The diet of a generalist grasshopper species, *Melanoplus bivittatus* (Say) (Orthoptera: Acrididae), has been associated with intake selection of grasses and forbs based on carbon, nitrogen, and phosphorus levels **(**Jonas and Joern 2008). One of the limitations of this study was the minimal variety of diets and treatments tested. It would be expected that grasshoppers with more choices in their food options would have higher chances of meeting their nutrient requirements within the range of their food tolerance levels. Additionally, the relatively low number of test subjects for each treatment and study design made it impossible to quantify the specific level of sodium regulation in *S. americana*. Thus, additional research is required to better understand how the concentration of sodium in food effects consumption and overall grasshopper development. A possible study would consist of a wider range of diet options with varying levels of sodium concentration to see which one(s) the grasshoppers had a preference for. After this is determined, additional tests on the self-regulation of sodium and sodium's overall effects on development time, body composition, and sodium concentration in feces between instars and into adulthood can be conducted to potentially identify other methods of sodium regulation in *S. americana*. Data on sodium content in grasshopper feces has not been found but could be used to quantify sodium intake by comparing differences in the amount of sodium ingested versus the amount of sodium excreted from the body (Adler 1982; Raubenheimer and Simpson 1997).

In conclusion, *S. americana* grasshoppers show evidence of self-regulating their sodium intake and high concentrations of sodium in their diet negatively affect body mass. These findings have implications on the study of insect herbivores and how they interact with their natural environments.

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