

Anthropogenic changes in sodium affect neural and muscle development in butterflies

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The development of organisms is changing drastically because of anthropogenic changes in once-limited nutrients. Although the importance of changing macronutrients, such as nitrogen and phosphorus, is well-established, it is less clear how anthropogenic changes in micronutrients will affect organismal development, potentially changing dynamics of selection. We use butterflies as a study system to test whether changes in sodium availability due to road salt runoff have significant effects on the development of sodium-limited traits, such as neural and muscle tissue. We first document how road salt runoff can elevate sodium concentrations in the tissue of some plant groups by 1.5–30 times. Using monarch butterflies reared on roadside- and prairie-collected milkweed, we then show that road salt runoff can result in increased muscle mass (in males) and neural investment (in females). Finally, we use an artificial diet manipulation in cabbage white butterflies to show that variation in sodium chloride per se positively affects male flight muscle and female brain size. Variation in sodium not only has different effects depending on sex, but also can have opposing effects on the same tissue: across both species, males increase investment in flight muscle with increasing sodium, whereas females show the opposite pattern. Taken together, our results show that anthropogenic changes in sodium availability can affect the development of traits in roadside-feeding herbivores. This research suggests that changing micronutrient availability could alter selection on foraging behavior for some roadside-developing invertebrates.

nutritional ecology | *Danaus plexippus* | *Pieris rapae* | ecological stoichiometry

The development of fitness-related traits is closely tied to nutrition—from fecundity being influenced by protein availability (1, 2) to ornament coloration being linked to carotenoid abundance (3, 4). However, humans are having a major impact on the availability of many nutrients important in the development of these traits. For instance, nitrogen and phosphorus availability has increased dramatically because of fertilizer application (5, 6), with drastic consequences for biomass and nutrient content of producers and consumers (7–9). Although the effects of changing macronutrients have been well-studied, the importance of human-induced changes in micronutrients is less established. Are anthropogenic changes in once-limited micronutrients enough to drive differences in trait development, potentially altering selection dynamics?

This research focuses on changing availability of an important micronutrient: sodium. Sodium is a key component of animal development, important for the function of neural and muscle tissue (10–12) and affecting the development of traits, such as brain size (13–16). However, sodium availability is limited in most ecosystems (17–19), which is thought to have led to the evolution of sodium cravings (20, 21) and specific foraging behavior to acquire sodium (22–25). Humans are increasing sodium availability, particularly through the application of road salt (26–29) but also, through agricultural activity (30). In the metropolitan area of Minneapolis and St. Paul, Minnesota, ~300,000 tons of sodium chloride are applied to roads each winter (31). Research on the ecological impact of road salt has mostly focused on the negative effects of chloride entering waterways (32–34).

However, road salt application can also increase the availability of dietary sodium for animals. A handful of studies suggest that road salt application may affect sodium foraging in animals from ants to moose (35, 36). We know little about whether local increases in sodium along roadsides have significant effects on development of fitness-related traits for species feeding along roadsides, thus altering evolutionary dynamics in the anthropocene.

Butterflies are an excellent study system to test the consequences of changing sodium availability. Sodium availability has been shown to affect the development and activity of flight muscle in male Lepidoptera (37–39). Many adult male Lepidoptera actively forage for sodium through puddling, transferring much of this sodium to females during mating (23, 40–43). In addition, host plants of many butterfly species commonly grow along roadsides and would be affected by roadside runoff. Butterfly larvae also have limited movement (44, 45), such that the spatially restricted effects of salt runoff are biologically relevant. This work starts by documenting the effects of roadside salt runoff on sodium availability in common butterfly host plants. Two rearing experiments—one using roadside-collected and control host plants and the other using a controlled artificial diet manipulation—show the importance of changing sodium availability on trait development. In particular, we focus on two fitness-related traits—muscle and neural tissue—where sodium availability has a shown importance in trait development and function.

Results

Sodium Concentration in Plants and Control Butterfly Tissue. *Sodium of roadside plants.* For two of four plant species collected, specimens collected adjacent to roadways tended to have higher

Significance

Sodium is an essential micronutrient that is often limited in animal diets. It is important in the development of neural tissue and thought to have driven the evolution of specific foraging behavior. Human activity is drastically altering patterns of sodium availability, particularly through road salt application, but we know little about the consequences of such anthropogenic change on the development and evolution of wild animals. Here, we show that road salt runoff affects sodium concentrations of roadside plants, which in turn, have significant, (sometimes positive) effects on neural and muscular development of herbivores. These results suggest that anthropogenic changes in sodium can have major consequences for both behavioral development and selection on foraging behavior.

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sodium concentrations in leaves relative to specimens collected at control sites 100 m away. There were significant differences between sites for the milkweed and the oak species but not the grass or the mustard (Table 1). There were no significant differences in leaf nitrogen concentration between the sites (Table 1). **Sodium in control butterfly tissue.** Sodium was the most concentrated micronutrient measured in butterfly tissue relative to plant tissue, being more limited in availability than potassium, calcium, and phosphorus (Table 2). *Pieris rapae* reared on bok choy had, on average, 5,096 ppm sodium in their tissue relative to 112 ppm in wild-collected mustards (Table 2). Similarly, sodium bioaccumulated in monarch tissue was, on average, six times more concentrated than in control host plant tissue (Table 2). Element concentration varied somewhat with tissue type (e.g., head or abdomen) and sex (Tables S1–S3).

Experiment 1: Host Plant Diet Manipulation with Monarchs. Survival. Survival rates of monarch caterpillars were significantly lower on the roadside-collected leaves (40.5%) than the prairie-collected leaves (58.2%; $\chi^2 = 5.2$, $P = 0.02$); sex ratio did not differ on the two diet types ($\chi^2 = 0.03$, $P = 0.85$).

Eye and muscle investment. Because there were significant interactions between sex and diet, we analyzed male and female monarchs separately. There was a significant effect of diet on thoracic muscle investment in males but not females (Fig. 1A and Table 3). Males reared on roadside-collected milkweed had greater thoracic protein investment than those reared on prairie-collected milkweed; this pattern was reversed in females but was not significant. There was a significant effect of diet on relative eye size in females but not males (Fig. 1B and Table 3): females reared on roadside milkweed had significantly larger eyes (controlling for body size) than those reared on prairie-collected milkweed.

Tissue sodium. Monarchs reared on roadside-collected milkweed contained significantly more sodium in their abdomens than prairie-reared monarchs, regardless of sex [mean (SE): prairie-reared = 129.7 ppm (89.7); roadside-reared = 636.6 ppm (82.5); diet: $F_{1,22} = 18.3$, $P = 0.0003$; sex: $F_{1,22} = 0.44$, $P = 0.5$]. Concentrations of other elements are reported in Table S3.

Experiment 2: Artificial Diet Manipulation with Cabbage Whites.

Survival. Cabbage white butterfly survival was significantly lower on the high-sodium artificial diet than the medium- or low-sodium diet, although there was no survival difference between the latter two diet types (high: 10.9%; medium: 34.3%; low: 41.7%; $\chi^2_{2,525} = 48.8$, $P < 0.0001$; difference between medium and low: $\chi^2_{1,351} = 2.05$, $P = 0.15$). Because of the low survival on the high-sodium diet, all subsequent analyses contrast the low and medium diet types only. Development time was longer by 1 d on the medium-sodium diet relative to the low-sodium diet, but this difference was only marginally significant (low: 36 d; medium: 37.1 d; $F_{1,131} = 3.23$, $P = 0.07$ in a model controlling for sex).

Table 1. Sodium concentration in roadside plants

Species	Sodium (ppm)		Nitrogen (%)	
	Roadside (control)	$F_{1,4}$	Roadside (control)	$F_{1,4}$
Milkweed	2,065 (62)	11.1*	4.87 (4.75)	0.03
Mustard	115 (112)	0.07	3.73 (3.35)	1.65
Oak	50.9 (35.8)	33.9†	2.60 (2.37)	1.06
Grass	71.6 (54.7)	0.65	3.21 (2.97)	0.20

Shown are nutrient concentrations in plants collected along roadsides or from control sites over 100 m away. For sodium values, statistics were performed on log-transformed data (raw values are shown as means).

* $P < 0.05$.

† $P < 0.01$.

Table 2. Element concentration in butterfly tissue and their respective host plants

Tissue	Calcium	Potassium	Sodium	Phosphorus
<i>Pieris</i> tissue	755	12,024	5,097	11,579
Mustard leaves*	14,208	28,870	112	9,657
Monarch tissue	2,055	18,084	383	8,852
Milkweed leaves	14,208	33,955	62	9,020

Shown are mean concentrations (in milligrams per kilogram or parts per million) of different elements in cabbage white (*Pieris*; $n = 38$) and monarch tissue (mean concentration across head, thorax, and abdomen tissue; $n = 27$). Leaf samples ($n = 3$) came from control sites at Cedar Creek Ecosystem Science Reserve. Full analysis (by sex and tissue) is in Tables S1–S3.

*For further measures of sodium leaf concentration of mustards, see Table S4.

Neural and muscle investment. Because there were significant sex by diet interactions, males and females were analyzed separately. There was a significant effect of diet on thoracic protein for both males and females, but the direction of that effect differed: males on the medium-sodium diet had greater thoracic protein than males on the low-sodium diet, whereas females on the low-sodium diet had greater thoracic protein than females on the medium-sodium diet (Fig. 2A and Table 4).

Females raised on the low-sodium diet had significantly smaller total brain volume than those raised on the medium-sodium diet in a model that included body size (Fig. 2B and Table 4). There was no difference in brain size of males reared on the two diets (Fig. 2B and Table 4).

Discussion

Road Salt Runoff Affects Sodium Nutrition in Diverse Ways. We found that some species of plants—but not all—had higher leaf sodium levels along roadsides than in nearby control sites. In two of four species assayed (milkweed and oak), sodium concentration of roadside plants was significantly higher relative to plants at least 100 m from the road (Table 1) (1.5–30 times greater concentration). This effect on sodium concentration of roadside leaves is likely conservative given that we were focused on an area with sandy, rapid-leaching soil and a county road with only moderate levels of traffic and salt application. We might see more pronounced differences along interstates or in areas with clay-rich soil. Sodium from road salt runoff did not seem to stress the plants in this study, because nitrogen content in leaves did not differ between sites.

There were no significant effects of road salt runoff on sodium levels in the mustard and grass species that we measured. The lack of a response in the mustard was particularly surprising, because several existing laboratory studies have shown that salt treatments significantly elevate sodium concentration in *Brassica* leaf tissue (from 1.3 to 20 times) (Table S4). It is possible that the lack of an effect is specific to the mustard species that we measured, which tends to have lower sodium levels than other mustards (Table S4) and is not a preferred host of *Pieris* butterflies (46, 47). The variation that we observed between plant species in sodium bioaccumulation could stem from phylogenetic variation in sodium physiology (48, 49). Alternatively, because we did not wash collected leaves (as we were interested in what a caterpillar would consume), it is also possible that differences stem from variation in leaf stickiness. For instance, common milkweed has pubescent leaves covered with many soft short hairs (50), which could potentially trap salt precipitate. Regardless, these results suggest that salt application will affect the nutrition of at least some roadside herbivores, but more research is needed to determine exactly how changing sodium translates into nutritional differences for specific herbivores.

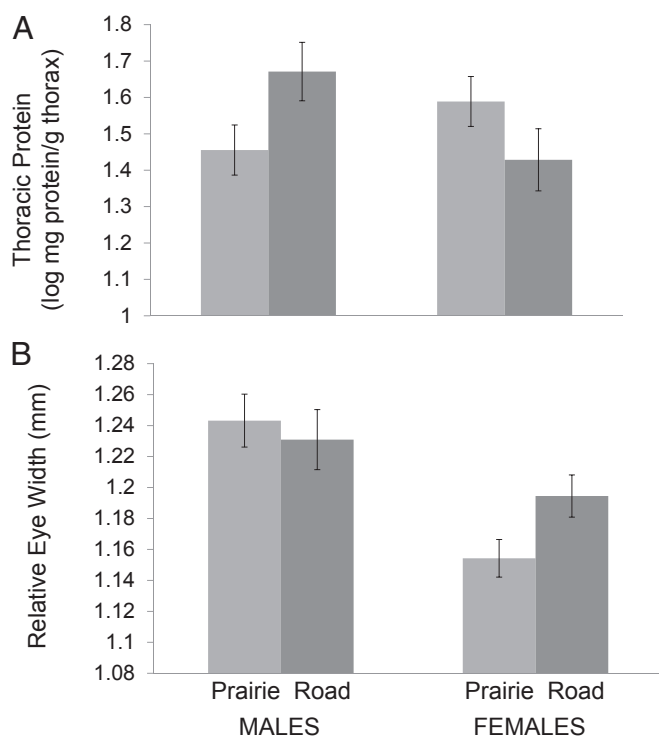


Fig. 1. Effects of sodium manipulation on eye size and thoracic protein in monarchs. Monarchs reared on roadside-collected milkweed had 16 times the amount of sodium in their larval diet than those reared on milkweed collected from prairies 500 m away. Shown are differences in (A) thoracic protein and (B) relative eye width for adult males and females. For eye width, least square means (and SEs) are plotted from a model that also included body size (wing length) and head alignment accuracy (statistics in Table 3).

Changes in Sodium Affect Butterfly Muscle and Neural Development.

Sodium was the most limited micronutrient in the diet of butterflies relative to its concentration in butterfly tissue (Table 2 and Tables S1–S3). Increases in sodium availability from road salt runoff affected the development of neural and muscle tissue in butterflies. Monarchs reared on roadside-collected milkweed, which had 16 times more sodium than control milkweed, developed greater flight muscle in males and greater relative eye size in females (Fig. 1 and Table 3). We were interested in whether trait differences were being driven specifically by variation in sodium. We used an artificial diet manipulation with cabbage white butterflies to specifically measure the effects of sodium, contrasting two diets where sodium was limited (with respect to butterfly tissue) but fell within the range of variation in host plant sodium values (Tables 1 and 2 and Table S4) (51). Results of this artificial diet experiment paralleled the results from the plant-rearing experiment: males in the enriched sodium treatment invested significantly more in thoracic protein, and females invested significantly more in neural tissue (Fig. 2 and Table 4). These data are consistent with observations from both vertebrates and invertebrates that sodium is a key micronutrient for brain (14) and muscular development (38). Although our manipulations were unable to eliminate the importance of chloride, other diet manipulations have determined the importance of sodium over chloride in driving insect foraging behavior (18, 52). In addition, as a cation, sodium in road salt runoff is more likely than chloride to be retained in negatively charged soils (33).

Somewhat unexpectedly, female butterflies reared on relatively higher-sodium diets invested less in thoracic protein than those reared on lower-sodium diets. Although this difference was

significant only for cabbage whites reared on artificial diet, the same pattern was seen in monarchs reared on field-collected plants (Figs. 1 and 2 and Tables 3 and 4). It is possible that this sex-specific effect reflects differences between males and females in what levels of sodium are stressful (see below). Alternatively, it is possible that females are plastically altering dispersal strategies depending on the quality of their larval diet, which has been seen in other species of butterflies (53, 54). If females reared on higher-sodium diets perceive these resources as higher quality, it may pay to invest less in flight and dispersal and instead, invest more in searching locally (55, 56). However, experiments are needed to determine whether the pattern in females is adaptive or simply a stress response.

Although increasing sodium availability can assuage the need for a limited micronutrient, it is also clear that increasing nutrients beyond some level can be stressful. Rearing cabbage whites on a high-sodium diet (6,000 ppm) resulted in significantly lower survival than the medium- and low-sodium levels. Monarch survival was also lower on roadside-collected milkweed, but it is unclear whether that was because of differences in sodium or some other factor (e.g., car exhaust contamination). Thus, it is possible that more extreme road salt runoff (e.g., along interstates) could result in more negative impacts on herbivores feeding alongside the road. Although caterpillars may sometimes be able to move away from host plants with toxic sodium levels and choose more nutritious host plants (57), their ability to disperse and make adaptive diet choices is likely quite limited (44, 45, 58, 59). Studies on the movement and food preferences of caterpillars will be necessary to determine the extent to which corridors of roadside plants with very high sodium levels may negatively affect caterpillar survival.

Implications: Changes in Limited Nutrients and Selection on Foraging Behavior.

Taken together, these data suggest that anthropogenic changes in nutrient availability may affect trait development in some species. Our data suggest that increases in sodium from road salt runoff have the potential to affect diverse species (e.g., both monarchs and cabbage whites), but the effects will depend on whether salt runoff affects the diet of a given species. Even within plant families, we see variation in the effects of salt on plant sodium levels (Table 1 vs. Table S4).

Our results leave open the extent to which road salt runoff affects the net fitness of roadside-feeding herbivores. Monarchs reared on roadside milkweed had significantly more sodium in their abdomen than those reared on control milkweed. Given the importance of sodium in Lepidopteran mating (41–43) and egg production (60), it is possible that the increased abdominal sodium may directly translate into differences in fitness, such as spermatophore size or egg number (61). Although the levels of sodium that we measured in butterfly tissue were similar to those

Table 3. Effects of roadside vs. prairie plants on monarch development

	Males	Females
Thoracic protein		
Diet	$t_{29} = 2.06^*$	$t_{12} = -1.32$
Eye width		
Diet	$F_{1,16} = 0.18$	$F_{1,14} = 4.75^*$
Wing length	$F_{1,16} = 0.23$	$F_{1,14} = 10.1^\dagger$
Alignment	$F_{1,16} = 0.01$	$F_{1,14} = 2.61$

Shown are results from *t* tests and ANOVAs testing for effects of diet on thoracic protein (mass protein per mass dry thorax) and eye width. The model for eye width also included a measure of body size (wing length) and head alignment error.

* $P < 0.05$.

† $P < 0.01$.

reported in other studies (60), the results of these studies emphasize that sodium concentrations vary significantly in butterfly tissue, and we do not fully understand the fitness consequences of such variation (if any). Future experiments will have to determine the net effects of sodium on fitness given that we also observed a decline in survival with high dietary sodium.

If increased sodium does, indeed, impact fitness, it is possible that anthropogenic changes in nutrient availability will change selection on foraging behavior in animals. We already see evidence of altered foraging behavior in some species—ants that live closer to roads forage less actively for sodium than those farther from the road (36), and moose also show some preference for roadside ponds because of salt runoff (35). Given that butterflies and moths can detect sodium concentration through contact chemoreceptors (62, 63), it is possible that they are also showing changes in foraging behavior. This observation raises the question of whether a thirst for a micronutrient, such as sodium, could lead to increased preference for roadside habitats. This preference might come with other changes, such as increased mortality because of car collisions [as seen in moose (64)] and subsequent selection on flight patterns to avoid cars [as seen in birds (65)]. Animals, including insects and humans, can choose to feed on sodium to the point of toxicity (66, 67), suggesting that roadside sodium runoff may function as an evolutionary trap (68) or select for resistance to high-sodium levels (69, 70). However, consistent preferences for roadside plants with higher sodium levels could potentially lead to decreases in adult puddling behavior or even evolutionary changes in brain size (71).

Overall, this work adds to a growing body of research suggesting that humans are changing the nutritional ecology of

Table 4. Effects of low- and medium-sodium artificial diets on *Pieris* development

	Males	Females
Thoracic protein		
Diet	$t_{19} = 2.45^*$	$t_{23} = 2.24^*$
Brain size		
Diet	$F_{1,4} = 0.41$	$F_{1,5} = 6.73^*$
Wing length	$F_{1,4} = 0.57$	$F_{1,5} = 1.16$

Shown are results from *t* tests and ANOVAs testing for effects of diet (low vs. medium sodium in an artificial diet) on thoracic protein (mass protein per mass dry thorax) and brain size. The model for brain size also included a measure of body size (wing length).

* $P < 0.05$.

species, which results in changes in competition and community ecology, life history traits and population cycles, and selection on foraging behavior. This work highlights that changes in micronutrients may be just as important as changes in macronutrients, such as carbon, nitrogen, and phosphorus, where the majority of the research has been focused. Humans are changing the availability of other micronutrients other than sodium, and it is likely that similar effects hold true for those micronutrients. For instance, given that calcium affects the abundance and development of birds, snails, and worms (72–74), anthropogenic changes in calcium (75, 76) may be particularly relevant for these taxa. Our results highlight the need for more comprehensive descriptions of how nutrition is changing in the face of humans and the diverse responses across traits and species to such changes.

Materials and Methods

Sodium in Roadside Plants. To determine how road salt runoff affects sodium availability in plant tissue, we collected plant samples from Cedar Creek Ecosystem Science Reserve, a 2,200-ha field site just north of Minneapolis and St. Paul, Minnesota. We focused on four common roadside species that represent four common host plant families of butterflies: a perennial grass (Poaceae: *Panicum oligosanthos*), a mustard (Brassicaceae: *Berteroa incana*), an oak (Fagaceae: *Quercus ellipsoidalis*), and a milkweed (Apocynaceae: *Asclepias syriaca*). At least three samples of each species were taken from roadside ditches (within 5 m of a county road), and at least three samples were taken from open field sites (at least 100 m from the paved road). Plant material was harvested on the same day about 6 wk into the growing season of these plants (June 14, 2013). Leaf material was dried in a drying oven (at 60 °C), and at least 1 g was submitted for analysis to the University of Minnesota Research Analytical Laboratory. Inductively coupled plasma atomic emission spectrometry (ICP-AES), dry ash method, was used to determine sodium concentration in leaves (77). Leaves were not washed before drying, because we were interested in the nutrients that a caterpillar would consume. Given that sodium can result in plant stress and lower nitrogen concentrations (78), a key macronutrient for herbivores (79, 80), we also assayed total nitrogen in these samples using the Dumas method (81).

Butterfly Rearing. Experiment 1: Rearing on roadside-collected plants. We compared monarchs (Nymphalidae: *Danaus plexippus*) reared on roadside- and prairie-collected host plants [in this case, common milkweed (Apocynaceae: *A. syriaca*)]. Monarchs were chosen, because milkweed is a common roadside plant, and investment in sodium-rich muscle should be important for a migratory species like monarchs. In 2011, milkweed was collected from the same locations as the plant measurements (see above). ICP-AES analysis of these leaves found that the roadside-collected milkweed was 16 times richer in sodium than prairie-collected milkweed (764 relative to 47.5 ppm) but that nitrogen levels were comparable (3.24% relative to 3.13%). Monarch eggs were obtained from mating cages of wild-derived monarchs that had been reared in the laboratory for one generation. Monarchs were reared in the laboratory (one or two per 15-oz cup) on a 14-h photoperiod at 24 °C. Field-collected milkweed was refreshed every 1–2 d. Adults were frozen at emergence and stored in sealed containers at –20 °C for additional analysis.

Experiment 2: Rearing on artificial diets. We used an artificial diet to vary the concentration of dietary sodium while holding other nutrients constant. We focused on cabbage white butterflies (Pieridae: *Pieris rapae*), because this species is easy to rear in large numbers on an artificial diet (82, 83). We used

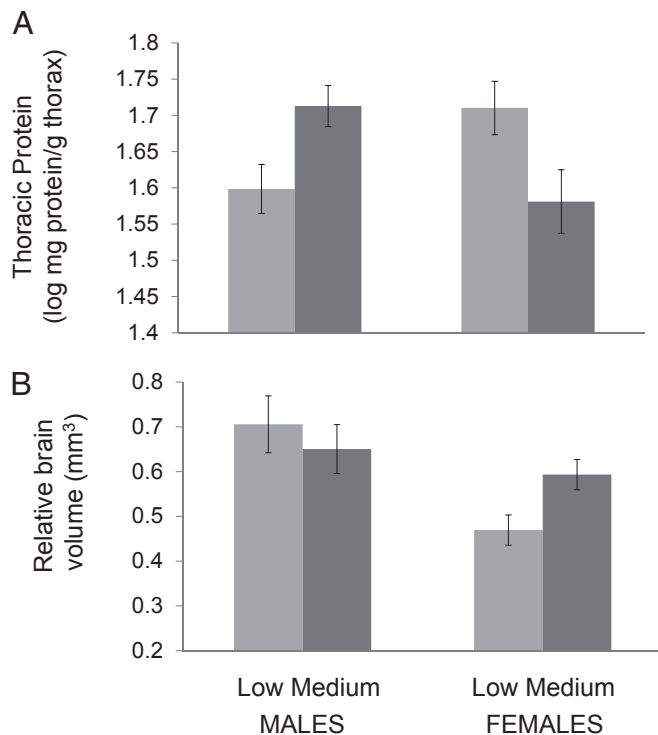


Fig. 2. Effects of sodium manipulation on brain size and thoracic protein in cabbage whites. Cabbage white butterflies were reared on low- or medium-sodium artificial diets (high-sodium diets resulted in high mortality and were not analyzed further). Shown are differences in (A) thoracic protein and (B) relative brain volume for adult males and females. For brain volume, least square means (and SEs) are plotted from a model that also included body size (wing length) (statistics in Table 4).

an existing diet recipe but varied the concentration of sodium chloride. We used ICP-AES to test the concentration of sodium in a set of pilot diet manipulations. Based on these values, we constructed NaCl diets that were ~400, 3,000, and 6,000 ppm sodium. These levels were chosen based on values from field and laboratory studies of sodium in Brassicaceae leaves, which show concentrations ranging from 69 to 14,900 ppm (median = 2,800 ppm) (Table S4). To make the artificial diet, we first constructed a base Wesson salt mix without NaCl. We based our salt mix on established mixes but used less calcium, because this mix has more calcium than is needed by insects (84, 85). The base salt mix (wt/wt) included 43% potassium phosphate monobasic, 17% potassium chloride, 14% calcium carbonate, 11% tricalcium phosphate, 13% magnesium sulfate, 1.5% ferric phosphate, and trace amounts (<0.05%) of copper sulfate, manganese sulfate, potassium aluminum sulfate, potassium iodide, and sodium fluoride. Per 800 mL water, the artificial diet was made with 60 g wheat germ, 15 g cabbage flour, 27 g casein, 24 g sucrose, 6.44 g base salt mix, 12 g torula yeast, 3.6 g cholesterol, 10.5 g vitamin mix, 0.75 g methyl paraben, 1.5 g sorbic acid, 3 g ascorbic acid, and 0.2 g streptomycin mixed with 15 g agar and 6 mL linseed oil. The low-, medium-, and high-sodium diets were constructed by adding 0.42, 6.1, and 12.65 g NaCl, respectively, to the base diet ingredients.

Gravid female *P. rapae* were obtained from wild populations in Minnesota and Virginia. Eggs were collected from females on cabbage plants in greenhouse cages. Within 7–10 d of eggs being laid, larvae were transferred from cabbage to one of three artificial diets (randomly assigned). Larvae were reared in groups of three per 4-oz cup in climate chambers at 23 °C with a 14-h photoperiod. On emergence, the head of each individual was fixed for brain histology (see below). The rest of the butterfly was stored in sealed containers at –20 °C until additional analysis. No significant differences were seen between populations, and therefore, they were pooled for analyses. An additional set of *P. rapae* from Minnesota populations was reared (three to six per rearing cup) in the laboratory on a host plant (bok choy; changed daily) to test levels of sodium in butterfly tissue reared on plants. Adult butterfly tissue was analyzed using ICP-AES (three to four individuals per sample to increase measurement accuracy).

Trait Measurements. Development time, survival, and body size. Development time was measured as the number of days between the date that an egg was laid and the date that the adult butterfly emerged. Survival was measured across all individuals transferred from the original egg-laying substrate to their respective diet manipulation. Body size was measured as forewing length (from removed wings) from the articulation of the wing with the thorax to the wing apex.

Muscle tissue. A Bradford assay was used to determine the amount of protein in the thorax, which is primarily composed of flight muscle. Thoraxes of individual butterflies were separated and dried in a drying oven (at 60 °C) for at least 24 h. Individual thoraxes were weighed to the nearest 0.1 mg and then processed using established methods (86). Briefly, 2% sodium sulfate was added to the sample (800 μ L for monarchs and 400 μ L for *Pieris*), which was then finely ground before a 2- or 1- μ L aliquot was taken from each *Pieris*

and monarch sample, respectively. Samples (two technical replicates each) were compared against eight BSA standards (average R^2 of standard curve = 0.974). We controlled for variation across batches and measurement error by taking the least square mean for each individual in a model across all replicate measurements that included batch as a fixed effect. An individual's thoracic protein was quantified as the total protein from the Bradford assay divided by the dry mass of their thorax.

Neural investment—eye size and brain size. We used two methods to measure neural investment. First, we used standard histological techniques to measure the brain size of *P. rapae* reared on artificial diets (87) ($n = 8$ females and $n = 7$ males). Briefly, heads were fixed in formalin, stained with 1% osmium tetroxide, embedded in plastic, and sectioned using a carbide tungsten knife at 15- μ m thickness. Brains were imaged on a Leica DM 2500 microscope using differential interference contrast and measured in ImageJ (National Institutes of Health). Total brain volume was measured as neuropil from the appearance of the antennal lobes (anterior) to the end of the medulla (posterior). Second, given that brain processing is labor-intensive (5 h/individual) with high probability of sample loss because of damage, we used eye size as a measure of neural investment for monarchs. About 75% of the butterfly brain is dedicated to visual processing (88, 89), and relative eye size is correlated with visual processing areas and brain size in vertebrates and invertebrates (90–92). To measure eye size, we took frontal images of each individual's head (93) after orienting heads (with labial palps removed) in a Styrofoam holder. Any individuals with eye damage were eliminated. Eye width was measured for each eye. Measurement error (the absolute value of the difference in width between each eye) was included in each model to correct for alignment errors.

Sodium in butterfly tissue. We used ICP-AES to measure sodium in monarch abdomens. We focused on monarch abdomens, because they were large enough to accurately measure sodium in individual samples and given that they contain reproductive tissues, are presumably relevant for fitness.

Analyses. We used JMP 9.0 (SAS Institute, Cary, NC) for all statistical analyses. ANOVA and *t* tests were used to test for effects of diet on focal traits. For traits that were correlated with body size (e.g., eye size), we included body size (wing length) in the model. Thoracic protein, leaf sodium, and eye alignment measures were log-transformed for normality.

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