

# Butterfly species vary in sex-specific sodium accumulation from larval diets

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## Abstract

1. Sodium is essential for animals, and its heterogeneous distribution can cause a range of phenomena, from sodium-seeking behaviours to impacting their performance. Although sodium content in soils and plants is relatively well documented, data for higher trophic levels are limited. Knowledge of the variation in sodium in lower trophic levels could have implications for understanding the behaviour and physiology of species at higher levels.
2. We investigated the variation in tissue sodium concentration between males and females of four butterfly species. Puddling behaviour of Lepidoptera suggests sodium needs of males are generally greater than females, thus, we predicted males would accumulate more sodium than females on a given diet.
3. Larvae were reared on plants (for *Battus philenor*, *Chlosyne lacinia* and *Danaus plexippus*) and an artificial diet (for *Pieris rapae*) under Low Na (no added sodium) and High Na (sodium added) conditions. Among species and sexes, we quantified and compared adult absolute tissue sodium concentrations and bioconcentration factors, which indicate net sodium accumulation or excretion relative to individuals' diets.
4. On average, individuals on low-sodium diets had higher bioconcentration values across all species. Male butterflies accumulated significantly higher sodium concentrations than females in two sodium treatments for *B. philenor*, and *P. rapae* and only in the High Na treatment for *C. lacinia*. However, in *D. plexippus*, individuals accumulate sodium in the High Na treatment, but males and females responded in the same way.
5. Our study revealed sex- and species-specific patterns of butterfly sodium accumulation, which could be linked to variations in behaviour and/or performance. Differences in sodium content across species have implications for variation in predation and trophic-level interactions, an interesting avenue for future ecological and evolutionary research.

## KEYWORDS

bioconcentration factor, chemical analysis, ICP-MS, larval diet, Lepidoptera

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## INTRODUCTION

Although sodium is toxic to animals at high levels, it is an essential micronutrient required for multiple physiological functions, including neural and muscular development and water homeostasis (Boné, 1947; National Research Council (U.S.), 2005; Soetan et al., 2010; Liebeskind et al., 2011; Snell-Rood et al., 2020). However, sodium concentrations are much lower in plants than in animal tissues, and herbivores often encounter substantial variation in the availability of dietary sodium across their geographic range (Borer et al., 2019; Bravo & Harms, 2017; Clay et al., 2022; Santiago-Rosario et al., 2021, 2022; Shephard et al., 2023). Thus, animals often exhibit focused sodium-seeking behaviours, such as puddling, salt lick visitation and sometimes even cannibalism (Burger & Gochfeld, 2003; Clay et al., 2014, 2017, 2023; Kaspari, 2020; Peterson et al., 2021; Santiago-Rosario et al., 2023; Simpson et al., 2006). In insect herbivores, sodium intake sometimes occurs in a sex-specific manner (Molleman et al., 2005; Watanabe & Kamikubo, 2005; Zarić et al., 2024). For example, in Lepidoptera, males of many species puddle to obtain sodium, which is a crucial component of the nuptial gift that males donate to females during copulation (Boggs & Dau, 2004; Mitra et al., 2016; Smedley & Eisner, 1996).

Sex differences in lepidopteran sodium foraging ecology raise the possibility that male and female butterflies might differ in their degree of accumulation of sodium in their diets (Molleman et al., 2005; Watanabe & Kamikubo, 2005). For example, in species in which male butterflies present sodium-rich nuptial gifts to mates, these males might employ additional sodium-accumulation tactics, such as concentrating dietary sodium in their own tissues. However, data on sex-specific sodium accumulation patterns across butterfly species are lacking, especially when animals are raised under controlled feeding conditions. Understanding whether dietary sodium accumulation in butterflies differs consistently between sexes would broaden our understanding of the ecology of sodium limitation in insect herbivores and the mechanisms by which sodium moves through trophic levels.

We tested the hypothesis that males accumulate more sodium than females across four butterfly species reared on diets containing controlled sodium concentrations. We used species that differ in geographic ranges and natural history: the bordered patch, *Chlosyne lacinia* (Geyer, 1837: Nymphalidae), the cabbage white, *Pieris rapae* L., 1758 (Pieridae), the monarch, *Danaus plexippus* (L., 1758; Nymphalidae) and the pipevine swallowtail, *Battus philenor* (L., 1771; Papilionidae). We reared caterpillars on low and high sodium concentrations (hereafter regarded as Low Na and High Na, respectively), and the feeding conditions consisted of either live host plant material or an artificial diet. For each individual of each species and sex, we quantified the absolute tissue sodium concentration as well as the bioconcentration factor, a metric indicating whether the individual had net bioaccumulation or excretion of sodium relative to its diet (Hodson & Hilton, 1983). To our knowledge, this study represents one of the few investigations on sodium accumulation in caterpillars across multiple butterfly species, incorporating different larval diets. This research provides novel insights into sodium variation across species and

**TABLE 1** Mean and standard error of dietary sodium concentrations (ppm) across butterfly species considered in the study.

Butterfly	Diet	Low Na	High Na
<i>Battus philenor</i>	<i>Aristolochia fimbriata</i>	1632 ± 132.15	2115 ± 145
<i>Chlosyne lacinia</i>	<i>Helianthus annuus</i>	568 ± 215	2265 ± 1451
<i>Danaus plexippus</i>	<i>Asclepias syriaca</i>	77 ± 36.4	2087 ± 36.4
<i>Pieris rapae</i>	Artificial diet	520 ± 1.65	3666 ± 49.26

between sexes, thereby enhancing our understanding of sodium-related differences among species and sexes.

## METHODS

### Study species and provenance of individuals considered

Our study specimens originated from populations in Dilley, Sullivan City and Mission, TX (*C. lacinia*); Twin Cities, MN (*D. plexippus* and *P. rapae*); and San Marcos, TX (*B. philenor*). These species were chosen based on the ease of raising them and their host plants in captivity and availability from prior studies (Santiago-Rosario et al., 2023; Shephard et al., 2021, 2023; Snell-Rood et al., 2020).

### Dietary sodium manipulations

*Chlosyne lacinia* larvae were reared on hydroponically grown *Helianthus annuus* L. (Asteraceae), cultivar Sunspot, within controlled environmental conditions comprising a 16-h photoperiod, temperature set at 23°C ± 3°C and relative humidity maintained at 60% ± 10%. Sodium concentrations in the growth media were adjusted using a Hoagland solution. Two distinct treatments were employed: Low Na treatment, in which plants were grown only in Hoagland solution, and High Na treatment, which entailed the addition of 1 g NaCl per litre of Hoagland solution, equating to a final concentration of 1% NaCl (Santiago-Rosario et al., 2023).

*Danaus plexippus* individuals were raised on *Asclepias syriaca* L. (Apocynaceae) subjected to two sodium treatments: Low Na treatment, in which plants were sprayed with distilled water, and High Na treatment, in which plants were sprayed with 2 g NaCl per 946 mL distilled water (Shephard et al., 2021). *Battus philenor* larvae were reared on *Aristolochia fimbriata* Cham. (Aristolochiaceae) under Low Na treatment (plants sprayed with distilled water) and High Na treatment (plants sprayed with 2 g NaCl per 946 mL), as also detailed in Snell-Rood et al. (2020). *Pieris rapae* individuals were cultured on artificial diets manipulated to modulate sodium concentrations, for Low Na (Wesson salt mix without sodium addition) and High Na treatments (addition of 1 g of NaCl to the diet; refer to Shephard

**TABLE 2** Sample sizes by species and sexes across treatments (for sodium measurements).

	<i>Battus philenor</i>	<i>Chlosyne lacinia</i>	<i>Danaus plexippus</i>	<i>Pieris rapae</i>
Low Na treatment				
Females	3	4	5	6
Males	3	3	7	6
High Na treatment				
Females	4	3	5	7
Males	5	5	6	6

et al., 2023). The dietary sodium concentrations are summarized in Table 1.

In all cases, the adult individuals were sacrificed immediately after eclosion for sodium concentration analysis. The sample sizes used in this study are summarized in Table 2.

### Inductively coupled plasma with mass spectrometry of sodium in butterfly tissues

Inductively coupled plasma with mass spectrometry (ICP-MS) was used to measure abdominal ionic sodium concentrations. Analyses were conducted using a Thermo iCap Qc quadrupole ICP-MS system in the Department of Geology and Geophysics at Louisiana State University, Baton Rouge, LA, for *C. lacinia*, and at the Quantitative Bioelement Imaging Center, Northwestern University, Evanston, IL, for *B. philenor*, *D. plexippus* and *P. rapae*. Before chemically extracting sodium, all the tissues were thoroughly dried at 60°C and weighed. Abdomens were digested in 1 mL trace metal-free HNO<sub>3</sub> acid (Fisher Scientific©) and 0.5 mL trace metal-free H<sub>2</sub>O<sub>2</sub> (VWR©) overnight in trace metal-cleaned Teflon vials. Completely digested tissues were prepared for ICP-MS analyses using trace metal clean 15 mL centrifuge tubes. Each of the two ICP-MS systems was coupled to its own ESI SC-DX2 autosampler, equipped with a fast valve with Sample-Sense. Sample uptake and introduction were optimized to yield optimal signal strength and stability for all the analytes. Rinse solutions were prepared using trace metal-free HNO<sub>3</sub> acid and Milli-Q water to prevent sample contamination.

For the sodium measurements, we used a dilution of 1/10 with trace metal-free water. This level of dilution was necessary given the high concentrations of sodium, which otherwise prevented an accurate analysis. The diluted samples were then processed. All tissues were compared to an elemental standard (IV-ICPMS-71A, Inorganic Ventures©) to generate a calibration curve. All measurements of sodium concentrations are reported in parts per million (ppm).

### Bioconcentration factor and statistical analysis

Sodium bioconcentration values allowed us to compare patterns of sodium accumulation in butterfly tissues relative to the sodium

concentrations of their diets (Fu et al., 2009; Hodson & Hilton, 1983). Values exceeding zero indicate net accumulation of sodium in consumer tissues, whereas values less than zero indicate net excretion. We calculated the sodium bioconcentration as follows:

$$\text{Bioconcentration factor} = \log \left( \frac{\text{Tissue sodium concentration (ppm)}}{\text{Diet sodium concentration (ppm)}} \right)$$

Statistical analyses were performed using R Studio Version 3.6.3 (R Core Team, 2023). Linear models were used to analyse the effects of larval dietary sodium treatment, sex, species, and all two-way interactions and the three-way interaction on butterfly sodium concentration and sodium bioconcentration factor. For each model, we tested for the significance of main and interactive effects using Type III sums of squares tests with the 'Anova' function in the *car* package (Fox & Weisberg, 2019). To explore the nature of the significant interactive effects, we performed planned contrasts using the *emmeans* package (Lenth, 2023) and adjusted p-values using the Bonferroni method Table 3.

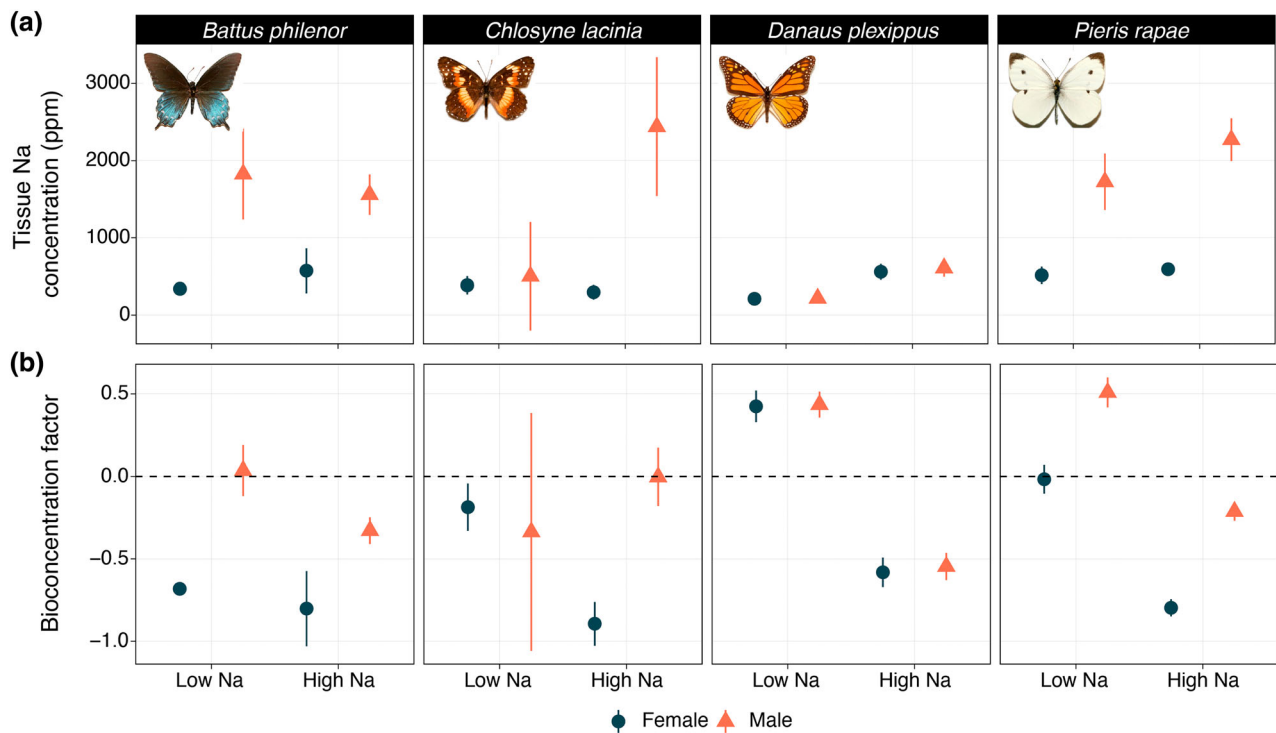
## RESULTS

Regardless of the larval dietary sodium treatment, we found that males accumulated significantly more sodium than females in *B. philenor* ( $t = 6.83$ ,  $df = 1$ ,  $p < 0.001$ ) and *P. rapae* ( $t = 8.40$ ,  $df = 1$ ,  $p < 0.001$ ; Figure 1a). In *B. philenor* and *P. rapae* reared on the Low Na diet, males accumulated approximately 5.37 and 3.36 times more sodium than females, respectively. On the High Na diet, males accumulated 2.72 and 3.84 times more sodium than females, respectively. In *C. lacinia* that developed on the High Na diet, males accumulated 8.22 times more sodium than females ( $t = 7.40$ ,  $df = 1$ ,  $p < 0.001$ ); however, on the Low Na diet, males and females did not differ in sodium concentration ( $t = 1.19$ ,  $df = 1$ ,  $p = 0.23$ ; Figure 1a). Sodium concentration did not differ significantly between the sexes in *D. plexippus* ( $t = 0.33$ ,  $df = 1$ ,  $p = 0.74$ ), yet, for this species, sodium concentration was higher on the High Na diet relative to the Low Na diet for both males ( $t = 4.88$ ,  $df = 1$ ,  $p < 0.001$ ) and females ( $t = 4.05$ ,  $df = 1$ ,  $p < 0.001$ ; Figure 1a).

Sodium bioconcentration factors were significantly higher in males than in females for both *B. philenor* ( $t = 6.83$ ,  $df = 1$ ,

**TABLE 3** Summary of the linear model results for the effects of sodium diet, sex, species, table and their interactions on tissue sodium concentration (log-transformed) and sodium bioconcentration factor in four butterfly species.

	Na tissue concentration			Na bioconcentration factor		
	F stat	df	p	F stat	df	p
Diet	2.08	1	0.15	0.9	1	0.35
Sex	28.4	1	<0.0001	28.4	1	<0.0001
Species	5.01	3	0.0036	29.4	3	<0.0001
Diet × Sex	1.97	1	0.16	1.96	1	0.17
Sex × Species	12.2	3	<0.0001	12.2	3	<0.0001
Diet × Species	3.96	3	0.012	10.2	3	<0.0001
Diet × Sex × Species	10.8	3	<0.0001	10.8	3	<0.0001

**FIGURE 1** (a) Mean tissue sodium concentrations, with 95% confidence intervals (ppm), in abdomens of males (dark blue) and females (orange) in Low Na and High Na sodium treatments across the four lepidopteran species. (b) Mean sodium bioconcentration factors, with 95% confidence intervals, for all the lepidopteran species considered in this study. The black dashed lines indicate the levels at which the diet and the lepidopteran abdomen had the same sodium concentration.

$p < 0.001$ ) and *P. rapae* ( $t = 8.40$ ,  $df = 1$ ,  $p < 0.001$ ), regardless of diet treatment (Figure 1b). In the High Na diet, males of *C. lacinia* had a significantly higher sodium bioconcentration factor than females ( $t = 7.40$ ,  $df = 1$ ,  $p < 0.001$ ); however, sodium bioconcentration factors did not significantly differ between males and females on the Low Na diet ( $t = 1.93$ ,  $df = 1$ ,  $p = 0.23$ ; Figure 1b). In *D. plexippus*, there was no significant effect of sex on the sodium bioconcentration factor ( $t = 0.33$ ,  $df = 1$ ,  $p = 0.74$ ; Figure 1b). *D. plexippus* sodium bioconcentration factor was lower on the Low Na diet than on the High Na diet in both males ( $t = 10.69$ ,  $df = 1$ ,  $p < 0.001$ ) and females ( $t = 9.64$ ,  $df = 1$ ,  $p < 0.001$ ; Figure 1b).

## DISCUSSION

We tested whether males accumulated more sodium than females across four butterfly species raised in low and high dietary sodium concentrations. We found that regardless of dietary sodium concentration, males accumulated more sodium than females in two species (*P. rapae* and *B. philenor*). In *C. lacinia*, males accumulated more sodium than females only on a High Na diet. However, in monarch butterflies (*D. plexippus*), we found no evidence that males accumulated more sodium than females (Figure 1a, Table 3).

Net bioaccumulation and excretion rates of dietary sodium among individuals help interpret the patterns of incorporation of sodium from

larval diets into adult tissues among the sexes and species of butterflies included. Bioaccumulation was observed exclusively in the Low Na treatments. Conversely, the most extreme cases of bioexcretion were predominantly found in females under High Na treatments, except for *B. philenor* females in the Low Na treatment, which still exhibited high sodium levels compared with other treatments (Figure 1b, Table 3).

Here, we suggest possible hypotheses that could explain these patterns and suggest directions for future work.

### In some butterfly species, males accumulate more sodium than females

Males of many lepidopteran species puddle as adults, and evidence suggests that sodium in nuptial gift spermatophores can increase their mating success (Mitra et al., 2016; Smedley & Eisner, 1996). Males of all three species in which sodium concentration was higher in males than females, at least when they were fed on High Na larval diets, are known to puddle in the wild (Boggs & Dau, 2004; Otis et al., 2006; Wahlberg et al., 2004). We hypothesize that in species in which males provide sodium to females during courtship or mating, males that have access to sodium in their larval diets can avoid or partially avoid costs associated with puddling (e.g., exposure to predators, energy expenditure) by accumulating sodium as larvae, in some cases possibly by consuming more food (Xiao et al., 2010). Furthermore, we hypothesize that females in these species avoid potential physiological costs (e.g., physiological stress) associated with high concentrations of sodium in their tissues by excreting or tightly regulating sodium from their larval diets. Finally, whenever sexes (or species) differ in sodium content, it is entirely possible that some butterfly predators would selectively choose sexes (or species) to obtain otherwise limiting sodium or to avoid or accumulating too much.

### Net bioaccumulation versus excretion

In *B. philenor*, *C. lacinia* and *P. rapae*, males apparently have a different tolerance for sodium accumulation than females. This higher tolerance in males may be a sexually selected trait. Males appear to maintain higher sodium concentrations in their tissues, but this does not necessarily imply that they are more or less limited in sodium use than females. Instead, males may face a higher risk of sodium-related stress. Conversely, females may utilize sodium in nuptial gifts during copulatory or postcopulatory mate choice. Maintaining low sodium levels in their tissues could help them better assess the ability of males to provide sodium and prevent the physiological stress associated with maintaining high levels of sodium in their own tissues.

### Why are monarch butterflies different from the others?

Monarch butterflies stand out from the other species studied in terms of the relative uniformity of sodium concentrations observed between

males and females, at levels similar to the lowest levels observed in the other taxa. Several hypotheses could explain this pattern. First, the literature lacks documentation of puddling behaviour in the very well-studied monarch butterflies, suggesting a potentially diminished use of sodium as a nuptial gift in this species, in contrast to other species (Mitra et al., 2016; Smedley & Eisner, 1996; Watanabe & Kamikubo, 2005), but is consistent with higher remating rates and more intense sexual selection in monarchs (Gage, 1997).

Second, monarch butterflies have a mechanism to tolerate cardiac glycosides from their main larval host plants, which otherwise have toxic effects on sodium-potassium pumps. Monarch-specific mutations in the Na/K-ATPase enzyme cause it to function despite the presence of cardiac glycosides in milkweed (*Asclepias* L.: Apocynaceae: Holzinger & Wink, 1996). It may be that pleiotropic effects of these mutations cause males and females to process sodium similarly in the presence of cardiac glycosides. Further comparative investigation is recommended into sodium processing of insects that differ in their tolerance to cardiac glycosides, or any toxin that influences the sodium-potassium pump, and to assess potential connections to host utilization, movement and other behaviours.

Finally, it is possible that the migratory behaviour of monarchs affects the patterns of sodium accumulation between the sexes. As both males and females must migrate to their overwintering grounds (Reppert & de Roode, 2018), there may be a strong selective advantage associated with sodium accumulation and its physiological influence on flight muscle development, movement and neural function (Kammer & Rheuben, 1981). This influence may apply to both sexes and could act in concert with the other explanations.

## CONCLUSION

Variation in sodium accumulation across sexes and species of butterflies revealed a tendency for males to accumulate more sodium than females. However, the implications of this sodium disparity and the mechanisms governing female homeostasis in maintaining stable sodium concentrations remain inadequately understood. Variation across males in their ability to accumulate sodium could influence fitness, for instance, the quality of their nuptial gift or the number of spermatophores they could produce (Lederhouse et al., 1990; Mitra et al., 2016; Molleman et al., 2004). It is possible that males that are more efficient at extracting sodium from their larval diet may be able to avoid puddling behaviour, but this idea remains to be tested. Beyond the biology of the present species, this study also has implications for predators, as the sodium content of their prey is not uniform, which could select for consumptive preferences for higher sodium species, sexes or individuals.

## AUTHOR CONTRIBUTIONS

**Luis Y. Santiago-Rosario:** Conceptualization; investigation; methodology; validation; formal analysis; data curation; project administration; visualization; funding acquisition; writing – original draft; writing – review and editing; resources. **Alexander M. Shephard:**



Conceptualization; methodology; formal analysis; resources; visualization; validation; investigation; writing – review and editing. **Emilie Snell-Rood**: Conceptualization; investigation; validation; resources; funding acquisition; writing – review and editing. **Achim D. Herrmann**: Methodology; validation; investigation; formal analysis; resources; writing – review and editing. **Kyle E. Harms**: Conceptualization; methodology; investigation; validation; supervision; resources; project administration; funding acquisition; writing – review and editing.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in Dryad at <https://doi.org/10.5061/dryad.c866t1ggb>.

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