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RECEIVED 02 December 2022

ACCEPTED 03 April 2023

PUBLISHED 02 May 2023

CITATION

Whitaker MRL, Banack SA, Mescher MC, Cox PA
and De Moraes CM (2023) BMAA in
cycad-feeding Lepidoptera: defensive
sequestration or bioaccumulation?
Front. Ecol. Evol. 11:1114636.
doi: 10.3389/fevo.2023.1114636

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BMAA in cycad-feeding Lepidoptera: defensive sequestration or bioaccumulation?

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Many herbivorous Lepidoptera accumulate plant toxins within their own tissues as a defensive strategy. Pioneering research in this area was conducted by Miriam Rothschild and Deane Bowers, who showed that the cycad-feeding butterfly *Eumaeus atala* sequester the toxic plant compound cycasin and thereby deter vertebrate and invertebrate predators. The current study focuses on another cycad compound, β -methylamino-L-alanine (BMAA), that is known to accumulate in the tissues of insects and other herbivores, and which has been shown to have neurotoxic effects in humans. Chemical analyses revealed that BMAA accumulates in both immature and adult tissues of *E. atala*, as well as adult tissues of another cycad-feeding lepidopteran, *Seirarctia echo*. However, the distribution of BMAA across life stages and tissues did not conform to patterns predicted for defensive sequestration, and subsequent behavioral experiments with ants showed that these invertebrate predators were not deterred by BMAA. Our results suggest that high levels of BMAA in the tissues of cycad-feeding insects likely reflect passive bioaccumulation rather than defensive sequestration. Combined with the previous work by Rothschild and Bowers, these results provide an example in which two different plant toxins accumulate within the tissues of a single herbivore species via different mechanisms and with different implications for ecology and evolution. They thereby lay the groundwork for further investigation into the processes underlying active sequestration and non-adaptive bioaccumulation.

KEYWORDS

Miriam Rothschild, Deane Bowers, β -methylamino-L-alanine (BMAA), chemical defense, non-protein amino acid, *Eumaeus atala*, *Seirarctia echo*, aposemetism

1. Introduction

The study of sequestration of plant defensive compounds by herbivorous insects is a cornerstone of chemical ecology. Sequestration is the ability to selectively accumulate plant compounds, often into specialized tissues, to gain a fitness advantage (Opitz and Müller, 2009; Beran and Petschenka, 2022), and defensive sequestration is a special case in which the fitness advantages of accumulating compounds are realized through protection from natural enemies. Historically, defensive sequestration among moths and butterflies (Lepidoptera) has provided particularly useful study systems for elucidating such important concepts as co-evolution, tri-trophic interactions, mimicry, and natural selection (Nishida, 2002). Knowledge of defensive sequestration among Lepidoptera has grown considerably in the past decades, though the field still suffers from a few widespread limitations. Many putative

cases of defensive sequestration lack experimental evidence of deterrence among ecologically relevant predators, such that defensive sequestration often is not sufficiently distinguished from other adaptive functions or even non-adaptive bioaccumulation. Furthermore, there is significant taxonomic and chemical bias in defensive sequestration studies, with most studies focused on a handful of well-studied lepidopteran groups (e.g., danaiids) and plant compound classes (e.g., cardenolides), leaving a huge portion of lepidopteran diversity and plant chemistry unexplored. Finally, sequestration is typically studied in terms of single compounds or compound classes, despite the fact that most plants produce multiple defensive compounds that could be co-sequestered. The present study addresses these limitations by investigating the accumulation and defensive potential of an unusual plant compound by a butterfly from a large family that is underrepresented in defensive sequestration studies.

Our study was motivated by the research of two pioneering chemical ecologists, Miriam Rothschild and Deane Bowers. These influential scientists investigated the chemical ecology of diverse lepidopteran groups (as well as other organisms), but in the late 1980's their scientific interests converged on a somewhat unusual butterfly, *Eumaeus atala* Poey 1968 (Lycaenidae). *Eumaeus atala* is a Caribbean species whose larvae feed solely on cycads, a group of highly toxic gymnosperms. The larval, pupal, and adult stages of *E. atala* all have bright warning coloration (Koi and Daniels, 2015), and even the eggs are conspicuous due to the bright butterfly scales deposited on them during oviposition (Rothschild, 1992). Warning coloration, in addition to the gregarious larvae and slow-flying adults, indicate that *E. atala* is aposematic, a somewhat rare characteristic among lycaenid butterflies.

Through a series of experiments performed in the late 1980's, Rothschild, Bowers, and their colleagues were able to elucidate the chemical basis of aposematism in *E. atala*. First, Rothschild and colleagues showed that *E. atala* sequester cycasin, a glycosylated form of the cycad toxin, methylazoxymethanol (MAM), and that cycasin accumulates in the insect's tissues throughout its lifespan (Rothschild et al., 1986). Bowers and colleagues subsequently demonstrated that *E. atala* adults are distasteful to birds (Bowers and Farley, 1990) and that both the butterflies and pure cycasin are avoided by ants (Bowers and Larin, 1989), suggesting that cycasin sequestration confers protection against both vertebrate and invertebrate predators. Based on their combined work, *Eumaeus* butterflies are the only lycaenids that are included in Nishida's (2002) seminal review of sequestration by Lepidoptera, despite the Lycaenidae being one of the largest and most trophically diverse butterfly families.

Recent years have seen a renewed interest in the chemical ecology of cycad-insect interactions, including in *E. atala* and its congeners. All six *Eumaeus* species are obligate cycad specialists and genomic evidence suggests that toxin tolerance is likely a key innovation underlying the evolution of the genus (Robbins et al., 2021), possibly through an "escape and radiate" process (Ehrlich and Raven, 1964; Thompson, 1989) in which the insects diversified after evolving tolerance to cycad toxins facilitated their shift to these chemically distinctive host plants. However, the importance of individual cycad toxins in this process is unknown. In addition to cycasin and other MAM-glycosides, cycads produce

sterol glycosides, the non-protein amino acid, β -N-methylamino-L-alanine (BMAA), and likely other toxic compounds (Bell, 2009; Liu et al., 2022). BMAA is a particularly interesting compound due to its toxicity to diverse organisms and its role in human neurodegenerative disease (Cox et al., 2003). Recent work suggests that BMAA may not be an effective anti-herbivore compound for cycads (Whitaker et al., 2022), but its potential as a defensive compound for cycad-feeding insects has never been explored. *Eumaeus* butterflies and other cycad herbivores encounter BMAA in their larval host plants, which is produced by endophytic cyanobacteria in specialized roots (Banack and Cox, 2003; Cox et al., 2003), but aside from evidence that BMAA is present in the larvae and pupal casings of the cycad-pollinating weevil, *Rhopalotria mollis* (Vovides et al., 1993), nothing is known about BMAA sequestration by insects.

It is therefore unclear whether cycad-feeding insects sequester BMAA, in addition to cycasin, for defense against natural enemies. Although many insects sequester multiple defensive compounds from their host plants, these compounds tend to be structurally similar and belong to the same broad compound classes (e.g., cardenolides, pyrolyzidine alkaloids). In contrast, BMAA and cycasin are chemically dissimilar and likely require distinct adaptations for sequestration. Although mixed or dual-sequestration defense strategies appear to be relatively uncommon, they have been reported among insects—for example, *Platyphora* leaf beetles that sequester both amyryns and pyrolyzidine alkaloids from their hosts (Termonia et al., 2002). The circumstances under which such dual defense strategies are adaptive are unclear, though Termonia et al. (2002) argue that dual chemical defense increases the chances of host transitions among chemically specialized insects. If *E. atala* and other cycad herbivores sequester multiple cycad compounds, the selective pressures giving rise to sequestration among cycad-feeding Lepidoptera may be more complex than previous research suggests.

Our study aimed to investigate the role of BMAA in interactions between cycads and their specialized lepidopteran herbivores by addressing the following questions: (1) Do cycad-feeding Lepidoptera sequester BMAA from cycads, and if so, in which life stages and body parts? (2) Is sequestered BMAA likely to confer protection from predators? and (3) How does BMAA sequestration compare to cycasin sequestration? If BMAA is sequestered as a defensive compound, we predict that (1) it will be distributed across the insects' body parts in a way that would maximize a predator's exposure to BMAA while minimizing fatal attacks and (2) both pure BMAA and butterfly extracts containing BMAA will be deterrent to ants, a common predator of lepidopterans. By testing these predictions, we build on Miriam Rothschild's and Deane Bowers' prior research on cycasin in *Eumaeus atala* to better understand the chemical ecology of cycad-herbivore interactions.

2. Methods

2.1. Study organisms

Eumaeus atala specimens were collected from the Montgomery Botanical Center in Coral Gables, Florida, a research and

conservation garden cultivating more than 200 species of native and exotic cycads that support a robust *E. atala* population (Whitaker et al., 2021). Third-5th instar larvae were collected from accessioned *Zamia integrifolia* (Zamiaceae) and *Z. aff. lucayana* plants and starved for 24 h before storage at -80°C . Pupae were collected from accessioned *Z. sp.* Andros and *Z. aff. lucayana* plants; a subset of pupae were immediately frozen, and the remaining pupae were kept under controlled conditions (27°C , 60% RH, 16:8 L:D cycle) until eclosion, after which the freshly emerged adults were sexed and immediately frozen. Cycad leaflets from which larvae and pupae were collected were also frozen.

For comparison to *E. atala*, which is an obligate cycad specialist, we also analyzed specimens of *Seirarctia echo*, the only known lepidopteran herbivore that feeds facultatively on cycad leaves (Whitaker and Salzman, 2020). Final instar *S. echo* larvae were collected from *Z. integrifolia* cycads in Naples, Florida, and reared in captivity under controlled conditions (27°C , 60% RH, 16:8 L:D cycle) through pupation and eclosion. Adults were housed in mesh cages and allowed to mate and oviposit, and adults found dead in the cages were frozen. Eggs were collected daily and F1 larvae were reared on a combination of artificial diet (Frontier Insect Diets, product F9772) and store-bought lettuce (following Packard, 1890). F1 adults were sexed and preserved immediately after eclosion. All cycad and insect samples were stored at -80°C and lyophilized for 24 h prior to blinded analysis.

2.2. BMAA quantification in plant and insect tissues

2.2.1. Total BMAA

Six *E. atala* adult males and 6 adult females were dissected into wings, heads (including antennae), thoraxes (including legs), and abdomens. Due to the butterflies' small size, the dissected tissues did not meet minimum starting weights (10 mg) for the analysis and were therefore pooled by sex and tissue. Twelve *S. echo* adult males and 12 females (half of which were reared on cycads and half on lettuce/artificial diet) were also dissected into separate tissues as above, each of which were massive enough to be analyzed separately with the exception of wings, which were pooled by sex and larval diet. Fifteen *E. atala* larvae and 5 pupae were left whole and were large enough to be processed individually. For both species, pooled wing samples were not massive enough to be weighed using the analytical balance available to us, so wing BMAA was quantified per individual (2 wings) rather than per gram.

Whole pupae, larvae, and adult abdomens were homogenized in 600 μL cold 20% w/v TCA in Eppendorf tubes with a sonicator probe on ice. Samples were then transferred to glass hydrolysis vials by washing each Eppendorf tube with an equal volume of 12M HCl. Leaf, head, thorax and wing samples did not require sonification; instead, heads and thoraxes were manually crushed with forceps, wings were left whole, and cycad leaves were homogenized in 50 mL Falcon tubes containing stainless steel beads in a Genogrinder. These pre-weighed tissues were then transferred directly to individual glass hydrolysis vials, to which 600-3000 μL (depending on sample volume, see Supplementary Table S1) of 6M HCl was added. All samples were heated to 110°C for 16 h,

after which time 100 μL of the hydrolysate was transferred to a 0.22 μm centrifugal filter (Ultrafree-MC GV PVDF, Sigma-Aldrich #UFC30GV) and centrifuged at 14,000 x g for 3 min.

BMAA was quantified following validated methods (Banack, 2021) that allow for discrimination between BMAA and its isomers, *N*-(2 aminoethyl)glycine (AEG), 2,4-diaminobutyric acid (DAB), and β -amino-L-methylalanine. Briefly, filtered sample extracts were diluted 1:50 with ultrapure water and derivitized using the AccQ•Tag Ultra Derivatization Kit (Waters WAT052880) for amino acids according to the manufacturer's instructions, with the addition of an internal standard (D_3 $^{15}\text{N}_2$ -BMAA) to the kit's buffer solution. Derivatized samples were injected in triplicate on a Thermo TSQ Quantiva triple quadrupole mass spectrometer run in positive mode (settings available in Banack, 2021) with water blanks between samples, and the three measurements per sample were averaged for final values. BMAA concentrations ($\mu\text{g/g}$) were calculated based on tissue starting weights and the reagent volumes used in each step of the preparation workflow (Supplementary Table S1).

2.2.2. BMAA fractions

BMAA can occur in biological samples in different forms: freely circulating BMAA, BMAA that is associated with small peptides, and protein-bound BMAA. Free BMAA is immediately toxic to cells, while protein-bound BMAA can be detrimental to the organism by interfering with protein folding and function (Dunlop et al., 2013). To quantify these BMAA fractions separately, we processed 8 *E. atala* specimens (6 larvae and 2 pupae) using a modified version of the above protocol. Samples were sonicated in 600 μL cold 20% w/v TCA and allowed to sit for 2 h at room temperature. Extracts were then centrifuged at 14,000 x g for 3 min (0.22 μm) to pellet out the proteins. The supernatants were decanted into fresh Eppendorf tubes, and an additional 600 μL cold 20% w/v TCA was added to each protein pellet. The pellets were re-sonicated and allowed to sit for 1 h at room temperature, then re-centrifuged. The supernatants were again removed and pooled, for a total of 1200 μL per sample. 600 μL of each pooled supernatant was added to 600 μL chloroform in new Eppendorf tubes, then vortexed and allowed to separate. 100 μL of the TCA layer was centrifuge-filtered at 14,000 x g for 3 min, diluted 1:100 in ultrapure water, then derivitized and analyzed as above. 200 μL of the remaining supernatant was transferred to glass hydrolysis vials, to which 200 μL 12M HCl was added. Protein pellets were transferred to separate glass hydrolysis vials by rinsing the sample tubes twice with 200 μL 6M HCl, for a total volume of 400 μL . Samples were hydrolyzed at 110°C for 16 h, and 200 μL of the hydrolysate was filtered, diluted, derivitized, and analyzed as above. Based on initial derivitization efficiency, some samples needed to be diluted with ultrapure water then re-derivitized and re-analyzed to optimize quantification efficiency. Because the hydrolyzed supernatant contained both free and small peptide-associated BMAA, free BMAA values were subtracted from hydrolyzed supernatant values to avoid double counting free BMAA. Samples subjected to fraction-specific analysis are not included in measurements of "Total BMAA."

2.3. Ant behavioral experiments

Behavioral experiments followed the methods of Bowers and Larin (1989) with some modifications. We used 4 captive colonies of *Lasius niger*, which, like the *Camponotus abdominalis* used by Bowers & Larin, are generalist foragers that feed on scavenged insects, plant nectar, and insect honeydew. Colonies were individually housed in 32.5 x 17.6 x 15 cm plastic containers with plaster floors and Fluon[®]-coated walls, such that ants could forage freely without escaping. Glass test tubes wrapped in aluminum foil were provided as nesting spaces, and colonies were maintained on a diet of dried mealworms and 10% honey water. Colonies were starved for 48 h prior to testing.

Three solutions were tested simultaneously: a control solution of 25% sucrose, an experimental solution of 150 µg L-BMAA hydrochloride (Sigma-Aldrich) dissolved in 1 mL 25% sucrose solution, and an experimental solution containing a single dried, ground female *E. atala* adult (8.5 mg) suspended in 1 mL 25% sucrose solution, such that the BMAA- and butterfly-spiked solutions contained roughly equal concentrations of BMAA. A single droplet of each solution was placed on a glass microscope slide inside each colony box, equidistant to the nesting chambers. Ants were allowed to forage freely on the droplets for 1 h, over the course of which the number of ants feeding on each droplet was counted at 1-min intervals.

To account for correlations among time points, raw data were de-correlated by partitioning them into four 15-min time chunks (partitions), and averaging each partition into a single observation. We fit a linear mixed-effects model with the log-transformed means as the response variable, experimental solution as a fixed effect, and partition and colony as random effects. We confirmed that the model residuals were normally distributed, though partitioning the raw data in this way likely renders our model somewhat underpowered due to the reduction in observations. After finding that overall preference for the butterfly-spiked solution differed significantly from control and BMAA-spiked solutions, we performed *post-hoc* tests for pairwise differences between solutions by comparing estimated marginal means (using the *emmeans* package in R). We used a multivariate time series regression on the raw (un-partitioned) data to test whether ant preferences changed over the course of the experiment. R code for the statistical analyses is provided in the [Supplementary material](#).

3. Results

BMAA was detected in all plant and insect samples, with the exception of F1 *S. echo* adults that had been reared on lettuce and artificial diet, and the head of one *S. echo* adult that has been reared on cycads. BMAA concentrations in the leaves of three species of *Zamia* cycads ranged from 8.8–77 µg/g, within the range of previously published values from other cycad species (Figure 1). Total BMAA concentrations differed across *E. atala* life stages: larvae contained the highest concentration of BMAA (comparable to concentrations found in larval foodplants), followed by adults then pupae. This pattern is quite different from what Rothschild et al. (1986) reported for cycasin in *E. atala* (Figure 2). The majority of BMAA in *E. atala* larvae and pupae was present in its free form,

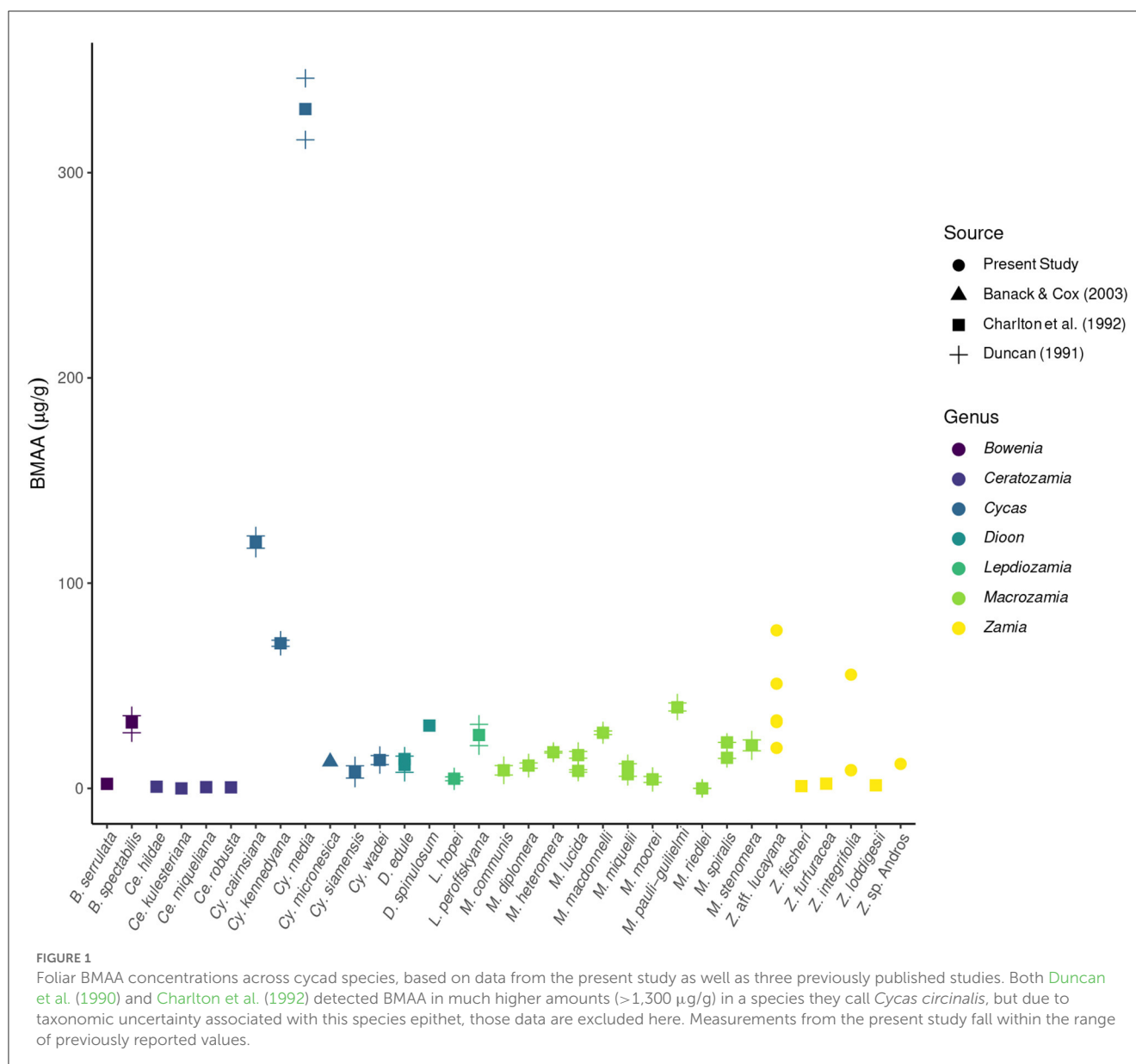
with protein-bound BMAA making up the smallest fraction of total BMAA in these samples (Figure 3). In both *E. atala* and cycad-reared *S. echo* adults, BMAA was least concentrated in the insects' wings relative to their heads, thoraxes, and abdomens (Figure 4), and although BMAA was detected in the wings of both male and female *E. atala* butterflies, it was present in amounts below the lower limit of quantification [LOQ = 0.037 ng/mL; limit of detection (LOD) = 0.01 ng/mL].

Ants fed on all three experimental solutions (Figure 5) and exhibited no obvious aversive behavior toward droplets containing BMAA or butterfly extracts. However, the overall preference for the butterfly-spiked solution differed significantly from control and BMAA-spiked solutions ($t = -2.321$, $p = 0.025$), and *post-hoc* tests showed an overall aversion to the sucrose solution containing whole *E. atala* extracts: ant feeding was significantly greater on BMAA-spiked solution than butterfly-spiked solution ($t = 3.71$, $p = 0.0018$), slightly greater on control solution over butterfly-spiked solution ($t = 2.32$, $p = 0.0645$), and no different between control and BMAA-spiked solutions ($t = -1.393$, $p = 0.3544$). Over the course of the 60-min experiment, the number of ants feeding on the butterfly-spiked solution declined significantly (slope = -0.1041, $t = -7.15$, $p < 0.001$) whereas the number of ants feeding on control and BMAA-spiked diets did not significantly change (slope = -0.0159, $t = -1.09$, $p = 0.279$; and slope = 0.0282, $t = 1.40$, $p = 0.166$, respectively), indicating that ants' attraction to the butterfly-spiked solution declined as they gained experience feeding on the three solutions.

4. Discussion

Our results show that BMAA accumulates in cycad-feeding insects' tissues, but we did not find evidence of dual defensive sequestration. We detected BMAA in all insects that were fed on cycads, though quantities varied across life stages and tissues. We did not detect BMAA in *S. echo* adults that had been reared on a larval diet without cycads, indicating that BMAA is sequestered from cycads and is not synthesized *de novo* by the insect, at least in this species.

Specific patterns of toxin localization—i.e., the life stages, tissues, and body parts in which insects store defensive compounds—influence the costs and benefits of sequestration, yet surprisingly few studies have compared the concentrations of sequestered plant toxins across different insect tissues. In one study, Mason and Deane Bowers (2017) investigated the distribution of sequestered iridoid glycosides in two *Euphydryas* butterfly species and framed their results in the context of three hypotheses regarding localization of sequestered compounds. According to their “differential risk hypothesis,” insects' defensive compounds should be most concentrated in tissues that can be easily and non-lethally sampled by birds and other pursuing predators, such as wings. In a classic example, Brower and Glazier (1975) found that monarch butterflies (*Danaus plexippus*) sequester very high concentrations of cardiac glycosides in their wings relative to other adult body parts, which the authors interpreted as evidence of an adaptive anti-predatory strategy. The results of the present study, however, do not match the predictions of this hypothesis: although we detected BMAA in all adult body parts of cycad-fed *S. echo* and

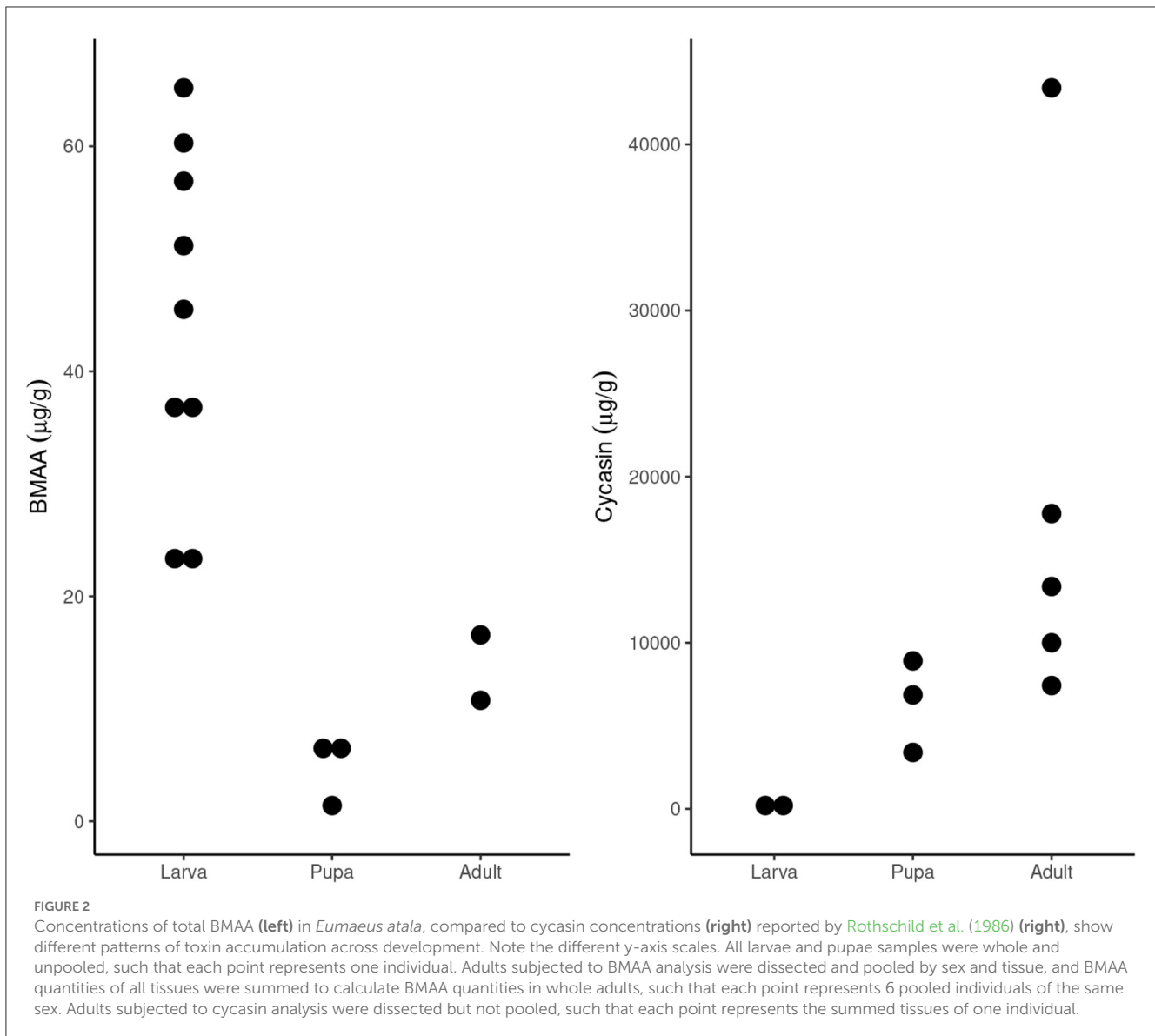


E. atala, quantities were very low in the wings of both species—too low, even, to be quantified in the wings of *E. atala* (Figure 4). This may be due to the low starting mass of wings, but even so, birds or other predators that ingest the wings of these insects will therefore not immediately encounter appreciable quantities of BMAA and are unlikely to be deterred.

Alternatively, rather than storing compounds in the body parts that are most vulnerable to predator attack, Mason & Bowers' "differential costs hypothesis" posits that insects should concentrate toxins away from tissues and organ systems that could be damaged by exposure to sequestered compounds. Under this hypothesis, the costs of sequestration, rather than its benefits, drive the adaptive localization of toxins. Because many important organ systems are located in the insect abdomen, [Mason and Deane Bowers \(2017\)](#) predicted that iridoid glycosides should be least concentrated in the abdomens of *Euphydryas* butterflies relative to other body parts, for which the authors found mixed evidence. Of course,

one might expect different localization patterns for compounds with differing modes of toxicity, and in the case of a neurotoxin like BMAA, nervous tissues might warrant the most protection. However, BMAA concentrations were moderate to high in the heads and thoraxes of *S. echo* and *E. atala* adults (though with considerable individual variation), suggesting that these insects do not sequester BMAA in a way that would prevent exposure to their cerebral or thoracic ganglia.

Finally, in contrast to the differential risk and differential costs hypotheses, both of which treat toxin localization as an adaptive process, the "tissue bias hypothesis" predicts that localization is driven by a sequestered compound's physiochemical properties that influence its affinity for different insect tissues. While some degree of tissue bias is likely always present, it will be most evident in the absence of significant adaptive drivers of toxin localization. [Hay-Roe and Nation \(2007\)](#) measured concentrations of cyanide released from adult tissues of *Heliconius* butterflies fed on various

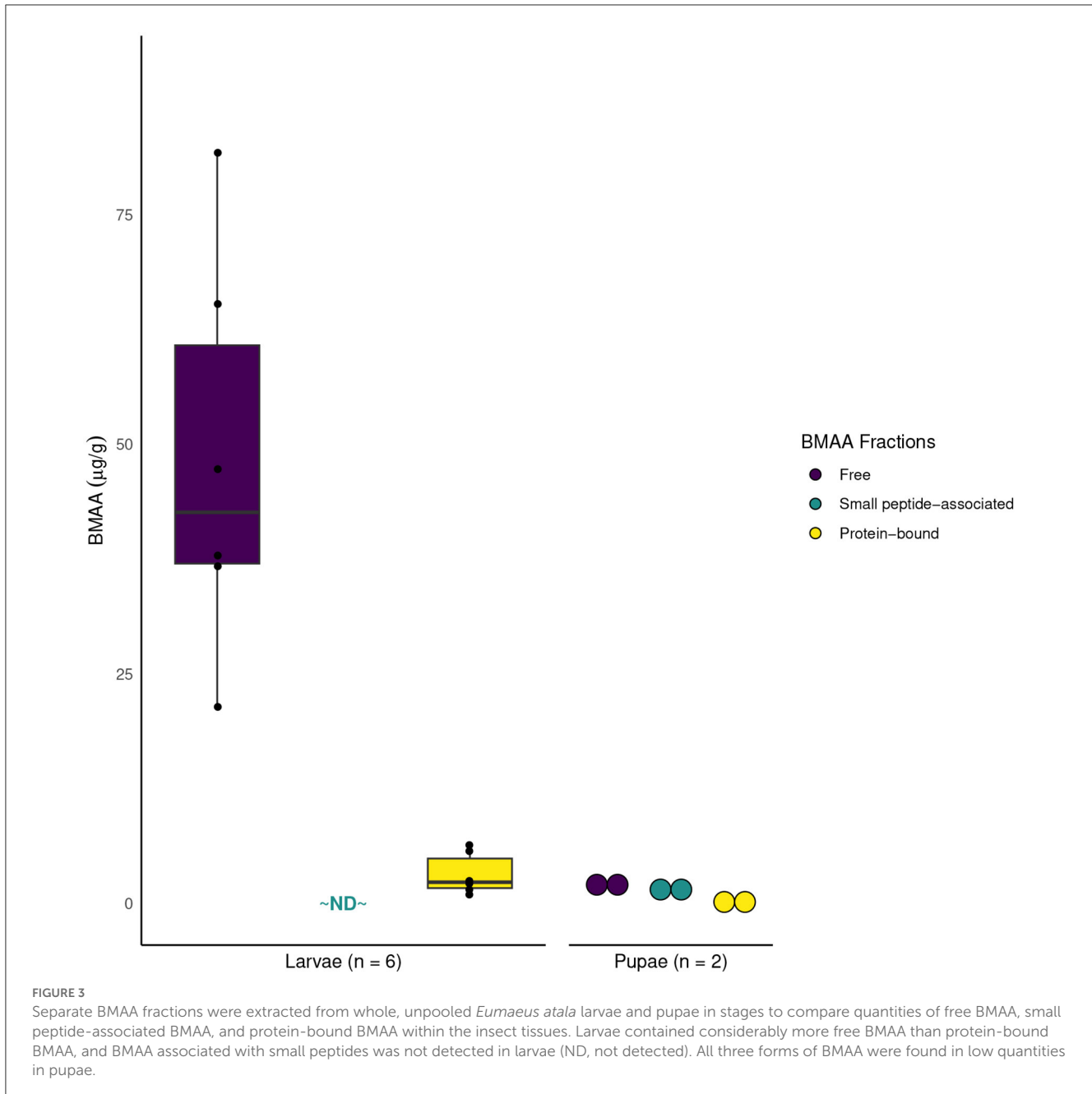


Passiflora hosts and found evidence that cyanogenic compounds were least concentrated in the butterflies' wings relative to other tissues, which they suggest is a result of low levels of β -glucosidase in the wings. In our case, the physiochemical properties of BMAA or of butterfly wings (or both) may similarly preclude storage of BMAA in these tissues.

In addition to evaluating BMAA localization in adult tissues, we also investigated BMAA sequestration in juvenile stages of *E. atala* and compared our findings to patterns of cycasin sequestration in different life stages. Cycasin has been found in all lifestages of *E. atala* (Rothschild et al., 1986), as well as *S. echo* larvae (Teas et al., 1966; Teas, 1967) and the adults of several other cycad-feeding Lepidoptera (Nash et al., 1992). In *E. atala*, cycasin accumulates over the course of development, with the lowest concentrations in larvae and the highest concentrations in adults (Rothschild et al., 1986). We observed a very different pattern for BMAA, which was most concentrated in larvae and least concentrated in pupae, with low to intermediate concentrations in adults. A large portion

of sequestered BMAA appears to be lost, excreted, or chemically altered between the larval and pupal stages. Adult butterflies do not feed on cycad tissues, so it is somewhat peculiar that concentrations of both BMAA and cycasin increase from the pupal to adult stage.

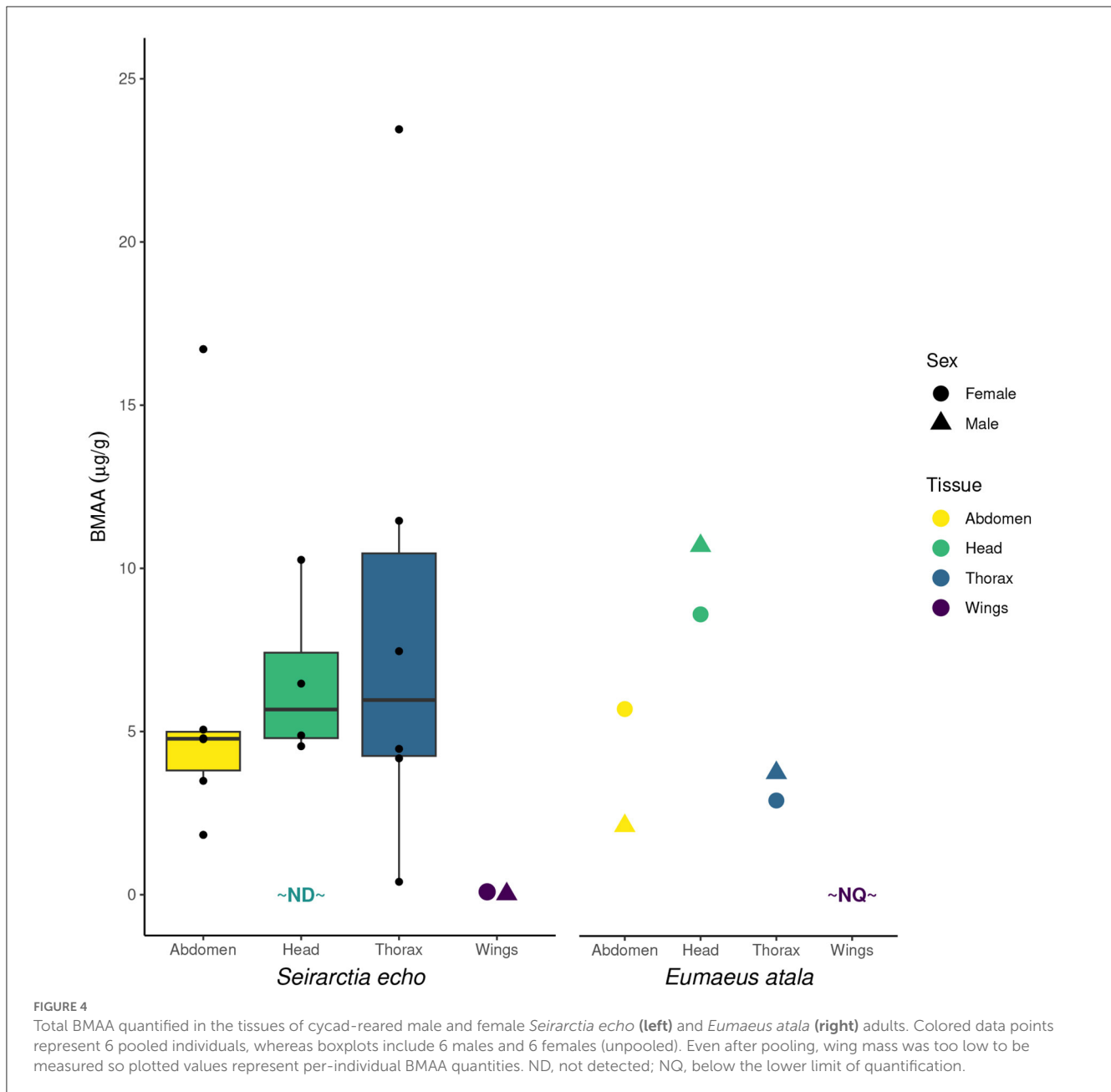
The biochemical fates of sequestered toxins during insect metamorphosis are often unknown, and because BMAA can incorporate into proteins and interfere with protein folding and function (Dunlop et al., 2013), we were interested in the different fractions of BMAA present in larval and pupal stages. We quantified each fraction of BMAA—protein-bound, small peptide-associated, and freely circulating—in *E. atala* larvae and pupae, and found that the majority of BMAA in larvae occurs in its free form, with comparatively little BMAA incorporated into proteins and no detectable BMAA interacting with small peptides. Pupae sample sizes were too small to draw general conclusions, especially since quantities of all BMAA fractions in pupae were low, but relative differences between BMAA fractions were smaller in these samples. While we did not measure individual fractions in adult samples,



these results suggest that *E. atala* may avoid some degree of toxicity by preventing the misincorporation of BMAA into proteins.

After establishing that two cycad-feeding insects accumulate BMAA in their tissues, we tested whether BMAA might confer a protective advantage for these insects. Lepidoptera have many natural enemies, including vertebrate and invertebrate predators, microbial pathogens, and parasitoids, and it is often not feasible to test the effects of a given compound on the preferences and performance of all potential enemies. Ants have been successfully used as a representative higher trophic level in butterfly palatability experiments, as the foraging preferences of ants are sensitive to prey palatability and nutritional value (Molleman et al., 2010). Bowers and Larin (1989) demonstrated that both cycasin and the extracts of whole *E. atala* adults were deterrent to ants, and concluded that

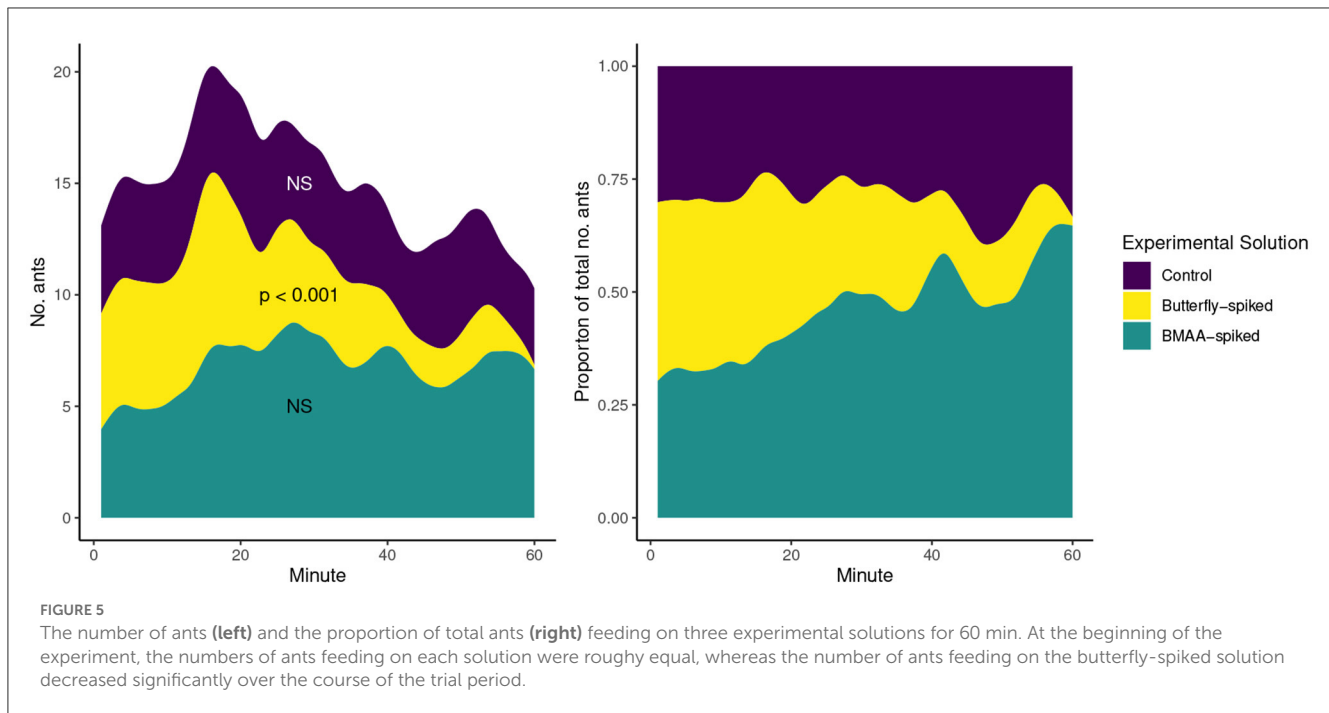
the accumulation of cycasin by *E. atala* butterflies is therefore a case of defensive sequestration. We also found that ants visited butterfly-spiked food the least out of the 3 experimental solutions, and that their attraction to butterfly-spiked food declined over the course of the experiment (Figure 5). We suspect that this aversion is driven by the presence of cycasin (and potentially other defensive compounds) within the adult tissues. However, we observed no significant differences in preference for control vs. BMAA-spiked solutions, nor did ants' preferences for either solution change significantly over the trial period, suggesting that BMAA was not deterrent to ants under these conditions. Though BMAA is commonly considered a defensive compound for cycads, the only experimental test of this hypothesis found that concentrations of BMAA comparable to amounts naturally occurring in cycad



leaves are insufficient to deter the generalist herbivore, *Spodoptera littoralis* (Whitaker et al., 2022). That BMAA is more concentrated in *E. atala* larvae than adults may indicate that the compound deters predators that specifically target the larval stages (e.g., parasitoids), though our data provide further evidence that BMAA may not be a feeding deterrent to invertebrates, in this case to a different trophic level.

Based on this combined evidence, the presence of BMAA in the tissues of cycad-feeding insects may be more accurately considered a case of bioaccumulation than defensive sequestration. While it remains possible that BMAA may protect insects from other natural enemies such as parasitoids or microbial pathogens, or that it confers some non-defensive (e.g. nutritional or reproductive) advantage, our results support a scenario of non-adaptive BMAA

accumulation in cycadivorous insects, as has been observed in diverse animals from both terrestrial and marine environments. For example, there is evidence that flying foxes (*Pteropus mariannus*) biomagnify BMAA from the cycad seeds on which they feed (Banack and Cox, 2003). BMAA has been found in the brains of experimental primates whose diets contained BMAA (Cox et al., 2016a), as well as humans with certain neurodegenerative diseases (Murch et al., 2004; Pablo et al., 2009). Marine BMAA—likely of microbial origin—bioaccumulates in the muscles and fins of sharks (Hammerschlag et al., 2016), the brains of dolphins (Davis et al., 2019), and the brains and other tissues of fish and shellfish (Jonasson et al., 2010). Similarly, BMAA has been detected in the tissues of *Drosophila melanogaster* adults fed on BMAA-spiked diet (Zhou et al., 2009). In none of these cases is BMAA



thought to confer a fitness advantage, and for many animals BMAA accumulation is associated with neurodegeneration and cognitive impairment.

Chronic dietary exposure to BMAA has been found to reduce lifespan, fertility, and motor function in *Drosophila* in a dose-dependent manner (Zhou et al., 2009, 2010; Nguyen et al., 2020; Cabrita et al., 2022). Chronic dietary exposure to BMAA has also been found to trigger neuropathology in non-human primates, including neurofibrillary tangles and sparse amyloid plaques in the brain, as well as TDP-43 +, FUS, and Ubiquitin proteinopathies in the spine (Cox et al., 2016a; Davis et al., 2020), whereas acute exposures do not produce such neuropathologies (Cox et al., 2016b). Since chronic exposure to BMAA is deleterious to vertebrates, further research is needed to determine if foraging on cycad-feeding insects reduces the fitness of birds and other insectivorous vertebrates.

It is possible that BMAA accumulation comes at a similar cost to cycad-feeding insects: even in established cases of adaptive sequestration, accumulating and storing defensive compounds is costly to specialized insects. But perhaps more interesting than the costs or benefits of BMAA accumulation is how cycad-feeding insects such as *E. atala* and *S. echo* tolerate their BMAA-rich diets. Our results show that these insects do not merely excrete BMAA, isolate it in non-vulnerable tissues, or detoxify it in the gut. Insects' ability to accumulate intact BMAA in their tissues while avoiding BMAA toxicity may therefore shed light on mechanisms of BMAA toxicity and resistance in other organisms. As we did not find evidence of a dual sequestration strategy among cycad-feeding Lepidoptera, we instead present a case in which defensive sequestration of one compound (cycasin) and likely bioaccumulation of another (BMAA) occur simultaneously within an individual. Given that most plants make multiple

defensive compounds, there are likely many similar cases in which herbivorous insects accumulate multiple plant toxins, each with different adaptive (or maladaptive) consequences. Understanding the selective significance of accumulating multiple compounds should therefore be a focus of future studies of defensive sequestration among Lepidoptera and other insects.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

MW and SB collected and analyzed the data. MW performed the statistical analyses and wrote the first draft of the manuscript with substantive input from all co-authors. All authors contributed to conception and design of the study and approved the final version of the manuscript.

Funding

The William Stamps Farish Fund provided funding to support this work. Open access funding was provided by ETH Zürich.

Acknowledgments

We thank William Tang for collecting the *Seirarctia echo* specimens used in this analysis; Diego Salazar for providing access

to a lyophilizer; the ETH Seminar for Statistics for assistance with analyzing ant preference data; the Montgomery Botanical Center for their continued support; and the entomologists, chemists, evolutionary biologists, and ecologists of all genders whose work advances gender equality in the field of chemical ecology. This manuscript was much improved by the thoughtful suggestions of two reviewers.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2023.1114636/full#supplementary-material>

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