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Rearing the Cabbage White Butterfly (*Pieris rapae*) in Controlled Conditions: A Case Study with Heavy Metal Tolerance

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Abstract

The cabbage white butterfly (*Pieris rapae*) is an important system for applied pest control research and basic research in behavioral and nutritional ecology. Cabbage whites can be easily reared in controlled conditions on an artificial diet, making them a model organism of the butterfly world. In this paper, a manipulation of heavy metal exposure is used to illustrate basic methods for rearing this species. The general protocol illustrates how butterflies can be caught in the field, induced to lay eggs in greenhouse cages, and transferred as larvae to artificial diets. The methods show how butterflies can be marked, measured, and studied for a variety of research questions. The representative results give an idea of how artificial diets that vary in components can be used to assess butterfly performance relative to a control diet. More specifically, butterflies were most tolerant to nickel and least tolerant to copper, with a tolerance of zinc somewhere in the middle. Possible explanations for these results are discussed, including nickel hyper-accumulation in some mustard host plants and recent evidence in insects that copper may be more toxic than previously appreciated. Finally, the discussion first reviews variations to the protocol and directions for troubleshooting these methods, before considering how future research might further optimize the artificial diet used in this study. Overall, by providing a detailed video overview of the rearing and measurement of cabbage whites on artificial diets, this protocol provides a resource for using this system across a wide range of studies.

Introduction

The small cabbage white butterfly (*Pieris rapae*, hereafter "cabbage white") is a cosmopolitan pest species of mustard crops, such as cabbage, broccoli, and canola[1,2,3]. At the same time, the cabbage white is a powerful system for research in biology and a commonly used butterfly model, as they can be easily reared and manipulated in controlled lab experiments[4,5]. Research on cabbage white butterflies has provided critical insights with respect to host

searching[6,7,8], nectar resource use[9,10,11], mate choice and sexual selection[12,13,14], wing pattern development and evolution[15,16,17], and responses to novel and changing environments[18,19]. Many of these insights rely on the fact that cabbage whites can be reared on artificial diets[4,20,21], which can be precisely manipulated to reflect poor nutritional conditions[22,23], ecologically relevant pollutant levels[24,25,26,27], or transitions to novel host plants[28,29]. The present study uses an experiment on exposure to heavy metals to illustrate basic methods for rearing cabbage white butterflies on an artificial diet in the laboratory and key performance measures of larvae and adults. Many aspects of these methods apply to other butterflies[30,31] and moths[32,33,34] that can be reared on an artificial diet.

In this paper, an experiment on metal tolerance is used to illustrate the general methods of rearing cabbage white butterflies. Heavy metals are a common anthropogenic pollutant stemming from the degradation of human products, industrial processes, and legacy contamination from historical use in pesticides, paints, and other products[35,36,37,38]. Many heavy metals, including lead, copper, zinc, and nickel, can move from soil and water into plant tissue[39,40,41,42], and metals in dust can be deposited on plant leaves[43,44,45], resulting in multiple routes of exposure to phytophagous insect larvae. Heavy metal exposure early in life can have negative effects on animal development, especially on neural tissue, and high levels can be lethal[35,36,46,47,48]. A number of studies have shown the negative effects of metal exposure on developing insects, including both pests and beneficial insects[49,50,51]. The large number of heavy metal pollutants, and the fact that they often co-occur in human environments[52], means that precise lab methods are needed in which researchers can expose developing insects to different levels and combinations of diverse metals to understand and mitigate their environmental effects.

The present work contrasts the impacts of common metals on cabbage white survival and development, focusing on copper (Cu), zinc (Zn), and nickel (Ni), three common pollutants in human environments. For instance, forbs from rural Minnesota roadsides contain up to 71 ppm Zn, 28 ppm Cu, and 5 ppm Ni[53]. This experiment manipulates the levels of these metals in artificial diets of cabbage white butterflies at levels corresponding to, and exceeding, the levels seen in the environment. An artificial diet is used to contrast the relative toxicity of these metals, predicting that cabbage whites would be more sensitive to metal pollutants that are not an integral part of their physiology (nickel) relative to those that occur, albeit at small levels, in enzymes and tissue (copper and zinc; **Figure 1**). Throughout, this text provides methodological details and accompanying video visualizations to illustrate the rearing and research methods of this important butterfly model system.

Protocol

This research was conducted under USDA APHIS permit P526P-13-02979.

1. Collection of experimental butterflies

1. Catch adult female butterflies with an aerial insect net. Cabbage whites are generally found in open, disturbed habitats with nectar plants and host plants (in the family Brassicaceae) present.
    1. Search when the sun is out and the temperature is warm. To target females, look for individuals fluttering slowly, close to the ground, and landing on plants to "drum" (taste) the leaves with their foretarsi.

2. Set up females in cages to harvest eggs.

   NOTE: Field-collected females, on average, have mated with one or two males[12], and should start laying fertile eggs shortly after capture. Wild-caught females need natural light to oviposit and mate, so place the cage in a greenhouse or windowsill.

3. House females with a host plant to harvest eggs.

   NOTE: Females will accept a variety of host plants, including green cabbage, radish, kale, collards, and *Arabidopsis*, but ensure that the plants have not been treated with any pesticides.

   1. Present the host plants either in pots or in containers with water to maintain the leaf pressure, such as stems of kale in floral water tubes.

   2. If the researcher wishes to collect eggs and transfer them directly to diet, first use a rubber band to attach a host plant leaf to the top of a plastic cup of water, and then stretch a piece of parafilm around the edge-females touching the leaf will oviposit onto the parafilm (see[5]).

4. Ensure that the cage also contains something to keep the relative humidity high, for instance, through daily watering of a potted plant or wetting of a towel, especially in dry conditions. If potted plants are watered in the cage, ensure a towel is under the pot, as butterflies can get stuck in pooling water.

5. Feed the butterflies with a 10% diluted honey water solution presented on a yellow sponge, which butterflies quickly learn to use, especially if housed with experienced individuals.

   1. To encourage butterflies to feed from sponges, place them directly on the feeder, especially after lightly spraying with a water bottle, which often causes them to stick out their proboscis.

   2. To set up the feeder, first thoroughly rinse the yellow or orange sponges, and then cut them into small squares that fit into 60 mm plastic Petri dishes. Change the feeders daily and clean the sponges in a mild bleach solution, followed by thorough rinsing to prevent mold growth.

2. Making artificial diets

1. First, use the recipe in **Table 1**, or other relevant sources, to determine the relevant recipe for an experiment. Make necessary modifications specific to the focal species or experiment. Print out a recipe to follow while weighing ingredients.

2. Weigh all the dry ingredients, except for the agar, into one container. Ensure the ingredients are put back into their respective storage location, noting that several ingredients are stored at 4° C. Place the pre-weighed, dry ingredient mixture into a blender with 5 mL of flaxseed oil.

3. For each diet batch, follow the steps detailed below.

   1. Mix 15 g of fine-mesh agar with 400 mL of distilled water in a beaker at least 1 L in size. Microwave until the agar is close to boiling, with fine bubbles throughout the mixture, stirring the mixture every 30-60 s to prevent boiling over.

   2. To this hot agar mixture, add 400 mL of faucet-temperature distilled water to bring it to an appropriate temperature to mix with the dry ingredients, as the vitamin mixture is heat-sensitive.

   3. Add the agar mixture to the blender and thoroughly mix, scraping the edges of the blender if necessary.

4. As the agar is heating, place at least seventy four-ounce diet cups on the counter with the edges touching. After thoroughly mixing the diet, pour the mixture from the blender into diet cups, ensuring the diet covers the bottom of each cup.
5. After the diet cools, place lids on the cups, label the diet cups with diet type, stack them on trays, and store for up to 1 month at 4 °C until use.

3. Transfer and rearing on artificial diets

1. House host plant leaves with butterfly eggs in 30 oz deli cups with a mesh cover in a 24 °C climate chamber. After 1 week, check the cups-ensuring that the larvae are hatched and in the late first or early second instar stage, a good time for transfer to the artificial diet.
2. Transfer the larvae to the artificial diet with a paintbrush, disinfecting it with bleach spray and a water rinse in between containers of larvae. Transfer three larvae into each 4 oz cup. While the artificial diet is energy dense and can support high densities of larvae, avoid packing larvae into cups, as diseases and mold can spread in cups with high larval density.
3. Place the cups into a plastic bin on their sides so that frass falls to the bottom of the cups and away from the diet, reducing mold and disease risk.
    1. House the diet cups in controlled temperature conditions with low to moderate light levels. Monitor the cups for mold and disease every 1-2 days by peeking through the clear cup lids.
    2. Cups with mold or disease can be quarantined or frozen to prevent spreading to other cups.

4. Adult emergence and handling

1. Allow the larvae to pupate and emerge in the diet cups. When adults emerge, give them a few hours for their wings to harden before removing them for marking. Remove adult butterflies from the cups with clean hands by gently grasping their wings, noting that grabbing all four wings closer to their body is a more stable hold.
2. To mark the butterflies, hold dry individuals by the head and thorax and use a fine-tipped sharpie to lightly mark a number on their hindwing.
    1. Sex individuals using a combination of wing markings and genitalia; females generally have two black spots on their dorsal forewing and darker, more yellowish hindwings, while males generally have one smaller black spot on the dorsal forewing on a brighter white background[54].
    2. Given that this coloration shows individual and seasonal variation, confirm the sex using abdominal traits-males have two claspers at the distal end of their abdomen and a narrower abdomen in general, while females have a single genital opening.
3. Transfer the adults to wax glassine envelopes by opening the envelope with one hand, holding the butterfly by the head and thorax, sliding it into the envelope, and grabbing the wings through the envelope with the other hand.
    1. Make sure all four wings are closed normally within the envelope.
    2. Maintain the butterflies in cold conditions (5-6 °C) for up to 1 week prior to experimentation, but allow at least 1 day to acclimate when taken out of the fridge.

5. Performance measures

1. To measure wing traits on dead individuals, remove the wings of the butterfly by holding the thorax in one hand and using forceps to remove each wing at its base. Place the wings flat in a lightbox and take photographs for later measurements.
2. To get estimates of fecundity, house the adults in mating cages, allowing at least 1 day for reproductive maturation of males and 1 day for mating. Sacrifice females at set time points for egg counts through dissection, or collect eggs each day on host plants.
3. To estimate egg loads, remove the abdomen of the female, place it in 1x PBS buffer, and cut a slit along the ventral side.
    1. Use forceps to separate the innards from the cuticle, then pull the ovaries away from the gut, trachae, and other contents of the abdomen.
    2. Uncurl the four curled ovarioles within each of the two ovaries, noting where mature, yolked, and shelled eggs transition to immature follicles. Use a counter to help tally the total mature eggs, generally ranging from 0-200 eggs.
4. To determine the mating status of a dissected female, open the bursa copulatrix and separate the spermatophores within. As spermatophores are digested, they generally develop a "tail" and are nested within each other.

6. Case study

NOTE: Adult female cabbage white butterflies were collected from the wild in 2014 to found the experimental populations. Adult females originated from near Davis, California (N = 8 founding females).

1. Housing the butterflies
    1. House the females in "BugDorm" mesh cages (61 cm x 61 cm x 61 cm) under natural light in a greenhouse. Provide an organic leaf of the host plant cabbage (*Brassica oleracea*) for ovipositioning.
    2. To maintain humidity in the cages, include a small potted plant (*Cosmos*), watered daily, placed on top of a towel within each cage.
    3. Collect eggs daily by transferring leaves with new eggs to 473 mL plastic cups with holes in the lid and place in a climate chamber.
    4. Provide butterflies with *ad libitum* access to a 10% honey water solution (made by diluting organic honey with distilled water), accessible through a yellow sponge in a small Petri dish that is changed daily.
2. Preparing artificial diets
    1. Prepare artificial diets for cabbage white larvae using modifications of previously developed Lepidoptera diets[4]. One batch of diet contained 50 g of wheat germ, 27 g of casein, 10 g of cellulose, 24 g of sucrose, 15 g of cabbage flour, 9 g of Wesson salt mix, 12 g of Torula yeast, 3.6 g of cholesterol, 10.5 g of Vanderzant vitamin mix, 1.1 g of methyl paraben, 1.5 g of sorbic acid, 3 g of ascorbic acid, and 0.175 g of streptomycin (see **Table of Materials**).
    2. Pre-weigh the dry ingredients for multiple diet batches (**Table 1**) and mix thoroughly to increase the homogeneity across diet types before being

subdivided into separate batches for mixing with metal solutions.

3. Place the dry ingredients in a blender with linseed oil and the corresponding metal mixture.
   NOTE: Linseed oil was used in the present experiment as it was sold by a past provider of insect diets. Now, organic flaxseed oil is used exclusively, which is made from the same plant, but is less likely to contain any additives as commercial providers of linseed oil.
4. Pour the prepared diet into 118 mL (4 oz) plastic deli cups. Use soluble metal salts to add focal metals to artificial diets. Aim for metal concentrations based on prior observations of the metal content of plants (e.g., nickel accumulation[55,56,57] or roadside contamination of plants[58,59,60]) and tolerance of metals in other Lepidoptera[49,50,51].
5. Dissolve metal salts in 500-1,000 mL of distilled water prior to taking the corresponding amounts to add to artificial diets. For example, to make the 100 ppm nickel diet, add 317.6 mL of 1 M $NiCl_2$ solution to the artificial diet before blending to give a final diet concentration of 100 mg/g Ni dry weight (approximately 53 mg/g wet weight). This amount translates into an average measured concentration of 109.6 ppm (**Table 2**) based on inductively-coupled plasma atomic emission spectroscopy.
   NOTE: The levels of metals were estimated by the University of Minnesota's Research Analytical Labs with six samples.

3. Maintenance

1. Maintain the eggs harvested on host plants in climate chambers at 23 °C on 14:10 photoperiods for 7 days. After this, transfer the early second instar larvae to the artificial diet.
2. At transfer, evenly divide the larvae from a given plant across the four diet types to avoid confounding batches of larvae with diet type. Transfer the larvae (N = 346 total) as two individuals per 118 mL diet cup to reduce disease incidence from overcrowding and allow for ample space for adults to eventually eclose.
3. Punch holes (three per lid) in the lids of the rearing cups. Place the cups in shoebox-sized plastic bins for rearing, with the different diets interspersed to avoid any systematic effects of location in the rearing chamber.
4. House the cups of larvae in climate chambers at 23 °C on 14:10 photoperiods (with bins of water at the bottom of the chamber to keep the humidity around 50%-60%, monitored with a home humidity sensor). In the event that the cups became moldy (about eight total cups in this case study), remove the cups from the chamber and remove those individuals from the experiment.
5. Allow larvae to pupate and emerge in the rearing cups (N = 162 total).
   NOTE: For the rearing conditions in this study, the development time from egg collection to adult emergence averaged around 25-30 days (ranging from 20-40 days, e.g.,[25,28]).
6. As the pupae approach adult emergence, check the cups daily for newly-eclosed individuals and remove adults with dried wings. Label the adults on their hindwings with their corresponding individual number (assigned at larval transfer) using a fine-tipped black sharpie. Determine the sex of each

individual and mark on a glassine envelope along with their number and emergence date. Place the adult butterflies in glassine envelopes and store at -20° C until further processing.

NOTE: A small fraction of emerging adults show wing deformities that would interfere with flight and adult survival (5%-8%); these individuals are excluded from survival analyses for these experiments.

4. Measurement and data analysis

1. Measure the survival as survival from second instar (when the caterpillars were placed on diet) to adult emergence.

NOTE: The present study focused on survival and development time as measures of performance on the different diets.

2. Measure the development time as the number of days between transfer to diets and adult emergence in the climate chamber.

3. For data analysis, run two sets of models that included interactions between the metal and concentration.

NOTE: As both interactions were significant ($F_{2,194}$ = 4.56, p = 0.01 for development time and X^2 = 12.1, p = 0.002 for survival), the study proceeded with separate analysis of each metal.

4. To analyze survival, run chi-square tests for each metal to test the effects of metal dose (treated as four categories) on survival to adulthood with wings fully intact.

5. When a significant effect of dose is detected, perform a follow-up chi-square to compare each level to the control diet. To analyze the development time (from time of transfer to emergence as an adult), test for effects of sex on development time.

NOTE: As there was no effect of sex on development time, (p > 0.10) for any metal in this experiment, we dropped it from consideration in the model.

6. Run a separate ANOVA for each metal to test for the effect of the four concentrations on development time. Additionally, run t-tests for each concentration relative to the control to determine the minimum concentration where a performance effect is seen.

NOTE: In this study, JMP v16 was used for all analyses. All raw data are available on Mendeley[61].

Representative Results

Overview

Artificial diet can be used to raise cabbage white butterflies in standard conditions to test the effects of certain diet ingredients on butterfly performance. In the present work, artificial diets were used to study the toxicity of different metals found in host plants growing in polluted areas (**Figure 1**). Larvae were raised on diets containing increasing concentrations of three different metals (**Figure 2**; specific methodological details presented in section 6 of the protocol). Butterfly survival and development were more impacted by copper and zinc and least impacted by nickel (**Figure 3** and **Figure 4**), with a sensitivity comparable to other studies with butterflies and moths raised on artificial diets (**Figure 5**).

Survival

Butterfly larvae were transferred to artificial diets containing copper, nickel, zinc, or control, where each metal type varied in concentration at three levels (**Table 3**). A representative image of larvae at an increasing dosage of toxin is shown in **Figure 2**. There was no effect of metal concentration on survival for nickel, but there was a significant effect for both

copper and zinc (**Table 3** and **Figure 3**). Post-hoc chi-square comparisons demonstrated that zinc showed a decline in survival relative to the control diet at only the highest level of zinc (1,000 ppm, post-hoc comparison X_1^2 = 8.41, p = 0.004; **Figure 1**). Copper also showed a significant decline in survival only at the highest levels used (500 ppm, X_1^2 = 7.00, p = 0.008), although there was a non-significant beneficial increase in survival at the two lowest levels (50 ppm and 100 ppm; **Figure 3**).

Development time

There was a significant effect of copper and zinc concentration on development time (**Table 4** and **Figure 4**). As copper concentration increased, there was an increase in development time, with a significant deviation from the control starting at 50 ppm (p = 0.027; **Figure 3**). As zinc concentration increased, there was an increase in development time, with a significant deviation from the control starting at 100 ppm (p = 0.03; **Figure 4**). There was a trend for increasing nickel to result in longer developmental times (p = 0.08; **Table 4**), and comparisons of each diet with the control showed significant effects starting at 100 ppm (p = 0.022; **Figure 4**).

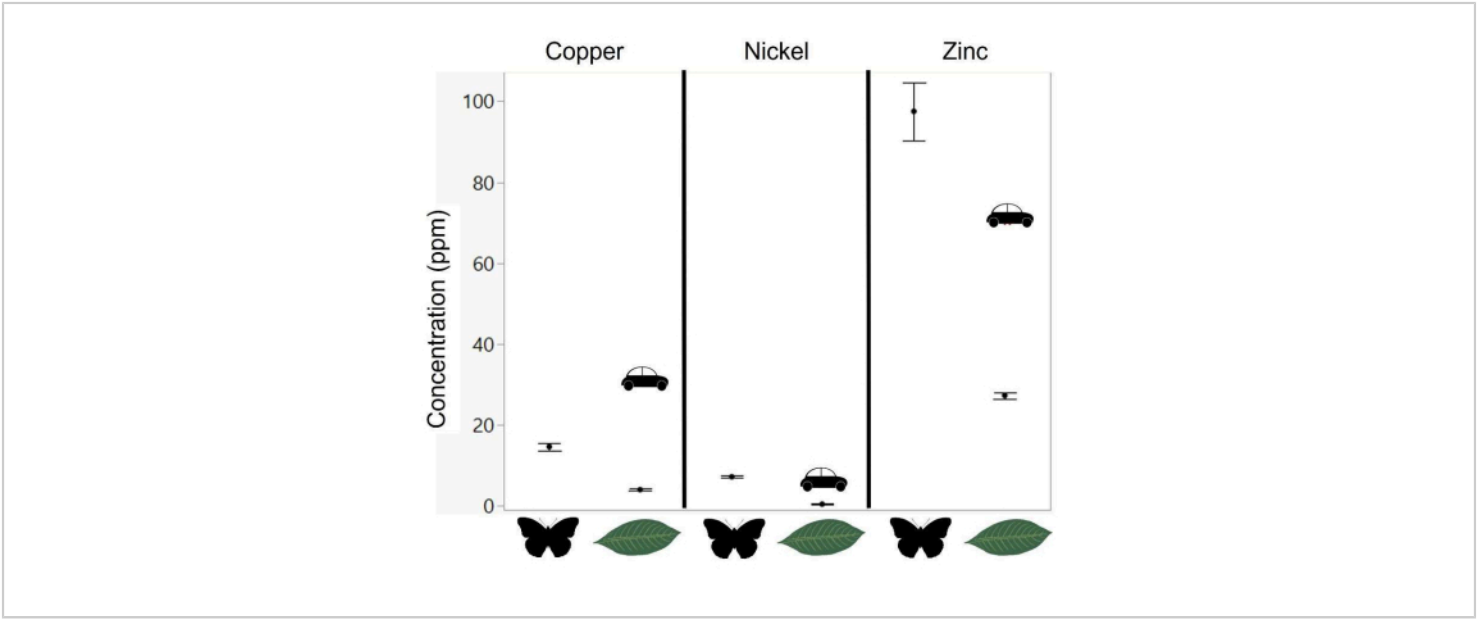

Figure 1: Observed levels of focal metals in butterfly tissue and host plants. (Data from[62].) Levels of copper, nickel, and zinc are shown for Pieris butterfly tissue (reared on bok choy in the lab) and wild-collected mustards (*Bertorea* sp.). Cars indicate the levels seen in plant leaves along high-traffic roads[53]. The levels of metals in artificial diets used in this study are reported in **Table 1**; points represent means, and error bars represent standard error. Please click here to view a larger version of this figure.

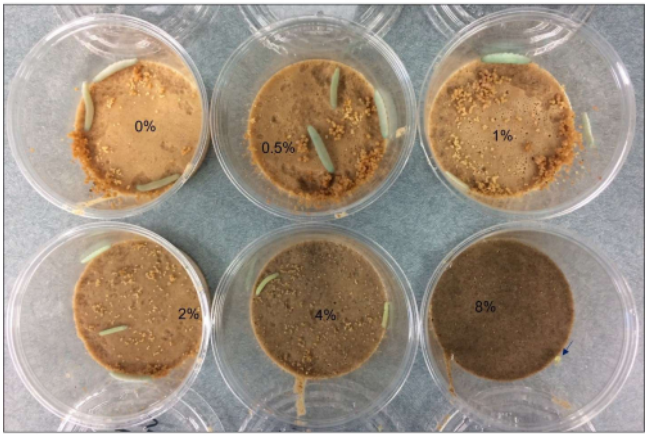

Figure 2: Image of cabbage white larvae transferred on the same day to artificial diets of increasing concentration of a toxin. This image shows larvae from a dose-response study (presented in [28] using dried plant material for the toxic plant Aristolochia). Photo by ESR. Please click here to view a larger version of this figure.

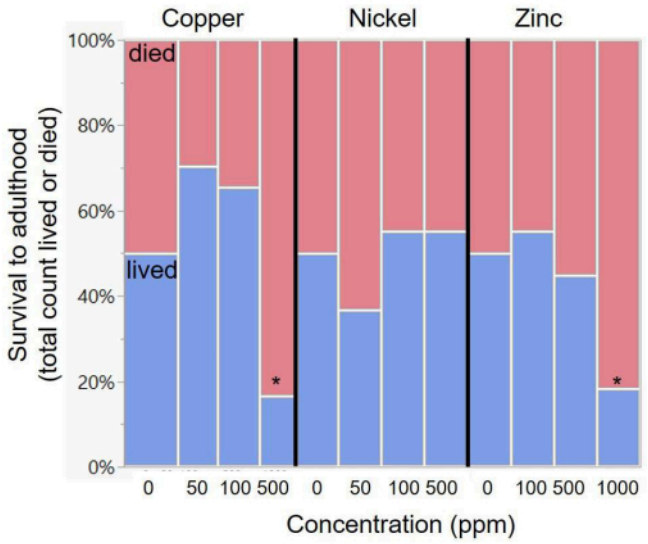

Figure 3: Variation in survival across metal diets of increasing concentrations. Asterisks indicate significant deviation in survival relative to the control diet. The exact metal concentrations in the diets are listed in **Table 2**. Please click here to view a larger version of this figure.

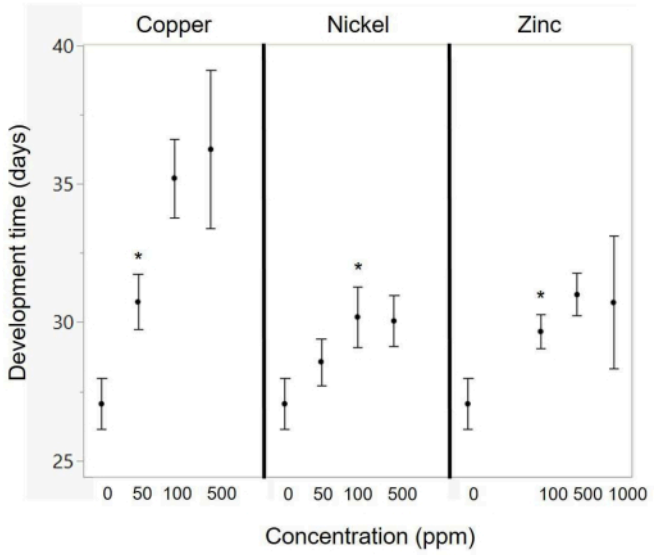

Figure 4: Effects of metal concentration on development time. The asterisks indicate the lowest metal concentration for which there is a significant difference relative to the control (using a t-test). The exact metal concentrations in the diets are listed in **Table 2**. Points represent means, and error bars represent standard error. Please click here to view a larger version of this figure.

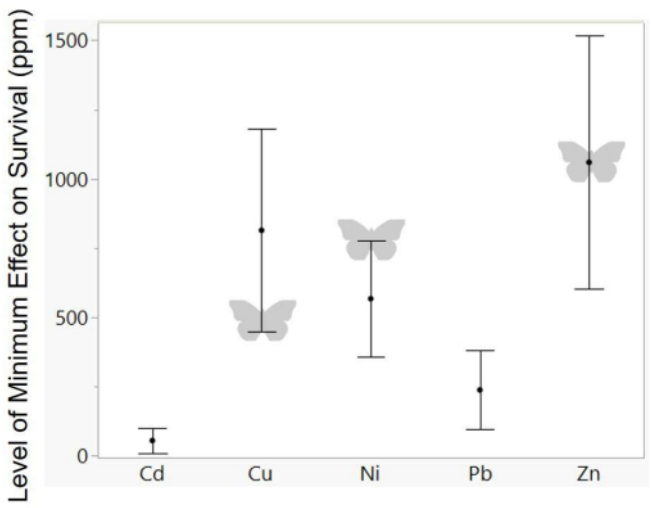

Figure 5: Summary of metal tolerance in other Lepidoptera. Shown are composite survival data plotted from 11 existing studies[49,50,51,56,63,64,65,66,67,68]. The response variable is the level (in ppm) of metal concentration where negative effects on survival are first seen. Butterflies indicate results from this study, noting that the tolerance values for nickel were higher than those measured in this study. Points represent means, and error bars represent standard error. Please click here to view a larger version of this figure.

Ingredient	Weigh as	g	mL
Wheat Germ	dry ingredients	50	
Cellulose	dry ingredients	10	
Cabbage flour	dry ingredients	15	
Casein	dry ingredients	27	
Sucrose	dry ingredients	24	
Wesson Salt Mix	dry ingredients	9	
Torula Yeast	dry ingredients	12	
Cholesterol	dry ingredients	3.6	
Vitamin Mix	dry ingredients	10.5	
Methyl Paraben	dry ingredients	0.75	
Sorbic Acid	dry ingredients	1.5	
Ascorbic Acid	dry ingredients	3	
Streptomycin	dry ingredients	0.175	
Flaxseed oil	wet ingredients		5
Agar	agar	15	

Table 1: Recipe for artificial diet. Shown are the weights (and volumes) of ingredients in one batch of cabbage white butterfly diet. The dry ingredients (and flaxseed oil) are prepared separately from the agar mixture (dissolved in 400 mL of boiling water, then brought to a cooler temperature with 400 mL of room temperature water).

Diet type	Copper (ppm)	Nickel (ppm)	Zinc (ppm)
Copper-“100 ppm”	**96.1**	1.75	69.9
Nickel-“100 ppm”	7.29	**109.6**	68.9
Zinc-“100 ppm”	7.96	1.06	**186.2**
Zinc-“500 ppm”	6.51	1.16	**708**
Control	5.89	0.59	59.3

Table 2: Measures of metals in diets. Shown are the mean levels of copper, nickel, and zinc in a subset of the artificial diets used in the study. The diet name ("type" in the analysis) is shown on the left, with values in quotes being the calculated level. The target concentration is shown in quotation marks. A subset of diets used in the study was analyzed to ensure the calculated values were on target with realized values; it should be noted that there is often some small degree of variation in the composition of diet components, and each line reported represents only one replicate.

Metal	**Pearson X_3^2**	***P***
Copper (N = 118)	17.82	0.0005
Nickel (N = 152)	3.45	0.33
Zinc (N = 152)	12.52	0.006

Table 3: Effects of metal concentration on survival. Shown are the results of a chi-square test for each metal, contrasting three concentrations of metal relative to a control diet.

Metal	**F**	***P***
Copper (N = 61)	$F_{3,57} = 9.84$	<0.0001
Nickel (N = 75)	$F_{3,71} = 2.35$	0.079
Zinc (N = 64)	$F_{3,60} = 3.79$	0.015

Table 4: Effects of metal concentration on development time. Shown are the results of individual ANOVAs for each metal.

Data Availability: All data are available on Mendeley[61].

Discussion

In this research, cabbage white butterflies (*Pieris rapae*) were raised on an artificial diet to examine differences in heavy metal toxicity. In doing so, this study provides general methods for rearing and laboratory studies of this easy-to-manipulate butterfly system. This discussion first considers more general questions about the methods reviewed here, then reviews our scientific findings before concluding with reflections on the components of the artificial diet.

The protocol reviewed here gives steps of a general rearing method for cabbage white butterflies, but there are many points within this protocol that can be tweaked. For instance, while the case study presented here uses sponges for feeding, other researchers have had luck with dental wicks and silk flowers filled with honey water[5]. While the present study uses honey water as food, other researchers have used sugar solutions and even Gatorade. If pupae need to be weighed, or moved to other conditions for emergence (e.g., inducing diapause and needing to cold store for 1 month), the researcher can easily remove them from the cups by spritzing them with water to moisten their silk attachments and grab them with feather forceps, then re-hanging them using double-sided tape. If researchers need more flexibility in terms of when adult butterflies are moved into cages for adult behavior, they can be held in the refrigerator for several weeks, but they need to be fed. Every several days, the butterflies should be taken out to be fed a dilute honey water solution. Under indoor lighting, this can be done by using a pin to unroll their proboscis into the food. On the adult performance end, a wide range of fitness measures can be taken on cabbage white butterflies. Body size can be measured as the wet or dry mass of larvae at certain stages, pupae, or adults (sacrificed, or held in glassine envelopes), or through the measurement of wing length in the program ImageJ (see[12,24,25,28]). The lifetime fecundity of females can be measured through daily egg collections on host plants[25,69,70], and the size of specific traits can be measured as a metric of performance; for instance, the mass or volume of the brain or individual brain regions[62,71,72], or the mass or protein content of the thorax or flight muscle[62,70]. Finally, adults can be used in behavioral studies to test any number of questions examining the effect of diet manipulation on foraging or oviposition choice[27,73].

If the rearing protocol is not working as expected, there are a few aspects to troubleshoot. First, one can ask whether the light levels are high enough to elicit normal adult behavior. While lab-adapted lines of *Pieris* will lay eggs under fluorescent light, the only artificial light that works for wild-type lines are powerful broad-spectrum greenhouse lights. Natural light in greenhouses, windowsills, or outdoors works best to elicit mating and egg-laying behavior. Second, if eggs are not hatching or if larvae are dying early in development, there are a few things to consider. The host plant material must be organic, noting that "organic" plants from stores are sometimes treated with chemicals that can kill larvae, so raising one's own host plants is often best. If the host acceptance rate is lower, younger leaves with higher nitrogen content can be attempted, presenting potted plants instead of individual leaves and ensuring females are mated. Females will accept seeding *Brassica*, even small sprouts that are 2 weeks of age. The paraffin method works well to transfer eggs to different conditions, but it should be noted that the acceptance rate tends to be lower than whole plants. Third, all the components of the diet must be of high quality and not expired. Flaxseed oil should be replaced annually and stored in the fridge[24,25]. Wheat germ, the vitamin mix, and antibiotics should also be kept cool. Fourth, one can consider tweaking the diet cup setup. Any number of disposable plastic

cup types can be used for rearing, from 1 oz to 15 oz. We have found that 4 oz is a good size to allow for adult emergence and packs nicely into our climate chambers. Holes poked in the lids allow for airflow, but too many holes can dry the diet in low humidity conditions, so this number may need to be adjusted. Fifth, the conditions in the climate chamber may need to be adjusted in combination with the cup conditions. If the conditions are too dry, host plants with eggs may dry out before larvae can be transferred, and cups with diet may dry out before butterflies emerge. On the other hand, if the conditions are too wet, the cups can harbor mold and disease. Researchers may need to adjust the airflow in cups through the use of mesh lids, or more or less holes in the lids. Another common issue is chamber lights that are bright enough to cause temperature swings in the cups and a build-up of condensation; using dimmer lights is an easy option for larval rearing.

With respect to the research questions in this paper, this study found that cabbage whites were relatively more sensitive to copper than to nickel or zinc. Copper had significant negative impacts on development time at concentrations as low as 50 ppm (**Figure 3** and **Table 3**) and on survival at 500 ppm (**Figure 4**, **Table 4**). In contrast, there were no negative effects of nickel on survival (up to 500 ppm; **Figure 3**) or negative effects on development time at 100 ppm (**Figure 4**). Cabbage whites were fairly tolerant of zinc, with survival effects seen only at 1,000 ppm (**Figure 3**) and negative effects on development time starting at 100 ppm (**Figure 4**). Based on the relatively greater concentrations of zinc in butterfly tissue and mustards (their host plant; **Figure 1**), it was expected that a relatively greater tolerance to zinc would be seen. However, the sensitivity to copper and the tolerance of nickel were somewhat unexpected given the very low levels of nickel in butterfly tissue (**Figure 1**) and the necessity of copper as a micronutrient. These unexpected findings are discussed below after considering the tolerance of these metals in other butterflies and moths.

To compare the present data with metal sensitivity measured in other Lepidoptera, data from existing studies were compiled on the minimum concentration, where heavy metals negatively impacted survival[49,50,51,56,63,64,65,66,67,68]; these studies focused on moths, especially pest species (*Galleria mellonella, Lymantria dispar, Plutella xylostella, Spodoptera sp.*). All of the measured sensitivity values in this study fall close to the range measured for these other species (**Figure 5**). However, the measure of nickel tolerance in this study does seem to be higher than expected-while there was not a significant effect of survival at 500 ppm, the previous study on *Pieris rapae* also found a very high tolerance for nickel (significant effects starting at 1,000 ppm[56]), despite low levels in their tissue naturally (**Figure 1**). The measure of copper sensitivity in this study also seems to be at the low end for studies of Lepidoptera. While the use of an artificial diet allows a convenient and controlled comparison of relative metal sensitivity, it is important to note that components of the diet could alter the measurement of absolute metal sensitivity. For instance, vitamin C in the diet could offset metal-induced oxidative stress[74], or antibiotics in the diet could alter any effects of microbes on the processing of metals[75]. An interesting line of future research would be to systematically manipulate such diet components to test effects on metal toxicity, especially given questions about the functional role of lepidopteran gut microbes[76,77] and nectar components that may have antioxidant properties[78]. In addition, variation in dietary requirements across species can make interspecific comparisons challenging, and artificial diet-based methods should be complemented with manipulations of host plants.

These butterflies are particularly tolerant of nickel and sensitive to copper. Previous research has noted that many plants in the mustard family, which includes plants favored by Pieridae, hyper-accumulate nickel as a defensive mechanism against herbivores[55,56,63,79,80,81]. This hyper-accumulation is over 1,000 ppm in plant tissue, which is orders of magnitude greater than what is seen in most plants (**Figure 1**). It is possible that *Pieris* have a particularly high tolerance for nickel due to past selection by such nickel accumulators, as previously speculated[26]. While copper has been less frequently studied as a micronutrient in insect diets, there is some evidence that it plays a small role in reproduction and immunity, although primarily in blood-feeding insects (e.g.,[82,83]). It is possible that copper plays a less important physiological role in butterflies than in other animals[84,85,86], consistent with recent work highlighting how copper may be as concerning of a pollutant for insects as lead, cadmium, and mercury (e.g.,[87,88,89]). While *Pieris* have been shown to avoid copper contamination at low levels[90], the mobility of copper in plants (e.g., moving into leaves and flowers) has also flagged it as a metal contaminant of concern[91].

While these results provide interesting data on the relative toxicity of these metals to cabbage white butterflies, this paper also aims to be of general use as a detailed visual illustration of methods for rearing this powerful system. Cabbage whites are easy to rear and manipulate in controlled lab experiments[4,5] facilitating studies of host searching[6,7,8], foraging[9,10,11], and sexual selection[12,13,14]. The ability to rear these butterflies on an artificial diet is key in creating common garden conditions for comparisons and to manipulate nutrients, toxins, and even novel host plants. However, it is important to note that this artificial diet is not necessarily the optimal artificial diet for this species, and could likely be improved with future manipulations. For instance, the salt mix in this diet (and other lepidopteran diets) was originally developed for vertebrates and has higher calcium levels than what most insects need[92,93]. Thus, some of our rearing efforts have made custom salt mixes with lower calcium levels (e.g.,[62]), and others make use of "Beck's salt mix", which may be more appropriate for many insect species[94]. In our own manipulations, we also found that butterflies performed better with relatively less wheat germ and relatively more cellulose compared to original concentrations[4]. One area in need of further attention is the lipid source and concentration in the diet. For instance, past work has shown that shifting from linseed oil (used in this study) to phospholipids increased the mating rates and growth rates of *Pieris* on artificial diets[95]. Supplementation of specific fatty acids in artificial diets may have additional positive effects[96,97]. Optimizing the artificial diet of *Pieris*[98,99] creates opportunities for addressing interesting questions about nutritional ecology[100,101,102], evolutionary ecology, and ecotoxicology. These artificial diet approaches allow researchers to address questions about the role of specific lipids in cognitive evolution[103], pre-adaptation to toxins[28], dietary components that reduce the toxicity of pollutants[104], or stoichiometric interactions between nutrients[105].

Disclosures

The authors have no conflicts of interest to declare.

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