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IMPACT OF ARTIFICIAL DIET ON DEVELOPMENT AND COLORATION IN *ZERENE CESONIA*

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ABSTRACT. The development of artificial diets for Lepidopteran species is instrumental for the study of natural biological variation in controlled laboratory conditions. In this study, we test the ability to rear *Zerene cesonia*, a pierid species, on artificial diets containing varying amounts of two plant species: *Dalea purpurea* and *Trifolium pratense*. We evaluate the quality of each diet based on survivorship, developmental timing, and analyses of larval and adult coloration. This study concludes that diets that include the preferred host plant, *D. purpurea*, are best for rearing *Z. cesonia* based on survivorship and the ability to recapitulate the natural

color variation observed in the wild. **Additional key words:** Diet, Development, Ultraviolet

In the pursuit to understand and characterize the biological variation we see around us, it is imperative that organisms can be reared and sustained in a laboratory setting. Model organisms that are easily reared in the laboratory such as fruit flies and mice have facilitated groundbreaking advances in biology across both basic and applied science. However, these model organisms lack much of the biodiversity found in nature and offer limited insights into the biological processes we seek to understand. The ability to rear a diversity of organisms in controlled settings continues to be fundamentally important for many biological inquiries. Many successful rearing programs have been established for insects, as their short life cycles, small individual sizes, and high fecundity can be very amenable to controlled rearing. One of the key aspects in establishing controlled rearing conditions for insects is identifying a stable and costeffective food resource. Many insect herbivores consume large quantities of plant material that are unfeasible to maintain, which has driven the development of "artificial diets" that offer a costeffective means for rearing large, controlled populations.

The development and optimization of artificial diets for insects has been of interest to researchers since the early 1900s (Vanderzant 1974). In 1979, A.C. Morton published an extensive study on the use of artificial diets for butterfly rearing (1979). Morton demonstrated that over 50 species of butterflies were able to survive on a single, generic artificial diet recipe. Since then, there have been attempts to further develop artificial diets for a limited number of butterfly species (Taylor et al. 1981, Genc & Nation 2004). Many of the butterfly species most heavily studied in biological sciences, including *Bicylus anynana, Danaus plexippus, Junonia coenia, ~~and~~ Vanessa cardui*, have wellestablished artificial diets. However, these species all belong to the same family of Lepidoptera (Nymphalid), which gives a limited and phylogenetically biased perspective on butterfly diversity. In order to facilitate a broader, more comparative characterization of the biological variation in butterflies, it is important to develop resources such as artificial diets for additional butterfly species.

Here we test an artificial diet for controlled rearing of a Pierid butterfly, *Zerene cesonia*. The family, Pieridae, is a diverse clade of butterflies most commonly known for their white and yellow wing colorations. Several species are considered agricultural pests, as the larvae of species such as *Pieris rapae* and *Colias eurytheme* feed on cabbage and alfalfa, respectively. However, it is still not costeffective to farm large quantities of these foods needed for rearing populous laboratory colonies, although previous attempts have been made to develop artificial diet recipes (Taylor et al. 1981, Troetschler et al. 1985). We built on this previous work to develop an artificial diet for captive rearing of a nonpest Pierid species, *Zerene cesonia*, the Southern Dogface butterfly (Fig. 1). The genus *Zerene* consists of only two species and is the sister genus to *Colias*. *Zerene* and *Colias* are sulfur butterflies characterized by bright, yelloworange colored wings. Males of *Zerene* and several *Colias* species also have bright ultraviolet (UV) patterns on their wings due the development of highly ornate nanostructures on the wing scales. In *Colias*, the brightness of the UV reflectance can be influenced by larval conditions, with stressed larvae showing duller UV reflectance on the adult wings (Kemp et al. 2006). This wing color pattern variation and diversity in *Colias* and *Zerene* offers a comparative framework to study pattern development and evolution.

Building from a diet developed for *Colias* butterflies, we explored the use of alternative artificial diets for

FIG. 1. *Zerene cesonia*, male, collected at ~~Osborne~~ Prairie, Mississippi, vouchered at the Mississippi Entomological Museum. The common name, Southern Dogface, is based on the doglike head profile on the forewing.

rearing *Zerene cesonia*. In addition to the *Colias* diet, we tested diet recipes that included varying amounts of putative *Z. cesonia* host plants. Specifically, we tested the addition of *Dalea purpurea*, purple prairie clover, to the diet. Previously described as a host plant generalist, *Z. cesonia* was recently shown to have a strong oviposition preference for *D. purpurea* (Fenner et al. 2018). In addition, we tested a more economical host plant, *Trifolium pratense*, commonly known as red clover, on which *Z. cesonia* have been reported to feed (Brock & Kaufman 2003). To evaluate the efficacy of these diets towards the study of natural color pattern variation in *Z. cesonia*, we compared the coloration of larvae and adults from the wild population and reared individuals. We discuss our results in the context of *Colias* color patterning and offer a recommended diet for laboratory rearing of *Z. cesonia* butterflies.

MATERIALS AND METHODS

Butterfly Rearing. *Z. cesonia* adults were collected with hand nets from Osborn prairie MS (33°30'36.98"N, 88°44'14.57"W) from June through August 2017. Wildcaught adults were released into 12" mesh cubical cages that contained petri dishes of a 20% sugar solution. Each cage housed 7–10 females and 7–10 males per cage, not exceeding 20 individuals per cage. Cages of adults were kept in temperature, humidity, and light controlled chambers (26°C/50% humidity/16hour light cycle). *D. purpurea* plants for the experiment were purchased from Prairie Nursery Inc in 2016 and reared in temperaturecontrolled greenhouses. To initiate oviposition, individual *D. purpurea* plants were placed in cages for 24 hours. Eggs were collected from the *D. purpurea* host plants within 24 hours of oviposition. Upon hatching, larvae were equally distributed among the six experimental diets. Larvae were kept in clear plastic cups, with only one individual per cup, due to a high frequency of cannibalism during the first larval instar. Cups and food were changed daily. Early experiments revealed that larvae need the diet to be scored after solidifying in order to initiate feeding. We used spatulas to place small scoops of artificial diet into every larval cup. To prevent resource limitation, individuals were given an amount of food that was greater than their current body size. Toothpicks were placed in cups with fifth instar larvae, where they will frequently pupate. After the hardening of the pupal casing, pupae were moved and hung using the toothpicks in screened cages. After eclosion and the adult wings had dried, individuals were moved to flight cages.

Artificial Diet Recipes and Preparation. Our diet was largely adapted from a diet developed and tested in *Colias* butterflies by Taylor et al. (1981). All ingredients, excluding any dried plant materials, remained the same for all diets. The recipe in Table 1 makes 200 ml of diet. Six experimental diets were tested that varied in the amount and source of host plant material. The first diet is based on the *Colias* diet and does not include any host plant material for *Z. cesonia* ('no plant'). We then tested the inclusion of *D. purpurea* using 'low' (0.5 g) and 'high' (3 g) amounts of dried leaf tissue. In addition, we tested the inclusion of *T. pratense*, a more economical and readily available host plant source, using 'low' (0.5 g) and 'high' (3 g) amounts of dried leaf tissue. Lastly,

TABLE 1. Artificial Diet Base Recipe

Ingredient	Amount
Sorbic acid	0.2 g
Lepidoptera vitamin mix (BioServ #722)	0.5 g
Asorbic acid	0.7 g
Methyl phydroxybenzoate powder	0.8 g
Agar	3.5 g
Brewers yeast	6.4 g
Wheat germ	10 g
Dried lima bean powder	17.5 g
10% formaldehyde	3.2 ml
Deionized water	100 ml (fill to 200 ml)

Treatment	Survivorship t
No Plant (n=105)	6.7%
D. purpurea low (n=109)	45.0%
D. purpurea high (n=138)	58.7%
T. pratense low (n=116)	31.9%
T. pratense high (n=45)	0.0%
50:50 mix (n= 40)	27.5 %

we tested a '50:50 Mix' diet of *D. purpurea* and *T. pratense*, using intermediate amounts (1.5 g) of both dried plant tissues. *D. purpurea* tissue was collected from the greenhouse plants typically used for oviposition. *T. pratense* plants were grown from seed for the experiment and leaves were harvested from sprouts 3–4 inches tall. Both types of plant leaves were dried in an oven at 115°C for 24 hours, blended at highspeed, ground into a fine powder using a mortar and pestle, and stored in amber-colored bottles at room temperature.

The preparation of the diets was in the following order: water, agar, ascorbic acid, wheat germ, lepidoptera vitamin mix (purchased from BioServ ©), dried lima bean powder, and plant material (if used for the specific diet). Next, methyl phydroxybenzoate powder, 10% formaldehyde, and sorbic acid were added to the mixture for anti-microbial purposes. All ingredients were heated on a hot plate and continually stirred until the mixture was thick, continual stirring was necessary to prevent burning of the food. Once thickened, the mixture was removed from the heat source and allowed to cool to 64°C. Lastly, brewer's yeast was stirred evenly into the mixture. The food was cooled to room temperature and stored at 4°C.

Survivorship and Developmental Timing. The number of eggs laid each day was recorded. Upon hatching, each larval individual was given an identification number for tracking. The number of hatchlings produced from each clutch of eggs was recorded. From hatching to pupation, observations on growth and development were recorded daily, as were the specific individual deaths and any obvious causes of death (failure to thrive or disease). Sex determination was done for individuals that survived to fifth instar by using a method described by Underwood (1994), which identifies sexspecific pores located on the 8th and 9th abdominal segments on the ventral side of the body. Date of pupation was also recorded for individuals, as well as the date of eclosion. Individuals from each treatment were dissected and preserved for future studies.

Statistical Analyse**s** fo**r** Survivorship**.** Pairwise tests were done at a significance level of $\alpha = 0.05$ to evaluate the differences in the frequency of successful individuals for three comparisons: 'low' D*.* purpure*a* vs. 'high' D*.* purpurea, 'low' D*.* purpure*a* vs. 'low' T*.* pratense, and 'high' D*.* purpure*a* vs. '50:50 Mix'. Pairwise Chisquare tests were done to evaluate differences in the average lengths of larval and pupation periods between treatments. Also, differences in survival based on sex was evaluated between the abovementioned treatments using a pairwise Chisquare test at a significance level of $\alpha = 0.05$. For the survival analyses between males and females, only the individuals whose sex was confirmed were used. Because individuals were sexed during the fifth instar, all individuals who died before being sexed, as well as fifth instar individuals who could not be sexed due to ambiguouslooking genital pores, were excluded from the analysis (n= 53, n= 65).

males females

Larval Color Pattern Analysis. Photos were taken from 5th instar larval individuals from the wild and diet treatments with a Nikon D7000 camera and AFS Micro Nikkor 105 mm lens (wild caught n=23; 'low' *T. pratense* n=15 'low' *D. purpurea* n= 10; 'high' *D. purpurea* n=16). *Z. cesonia* larvae vary in the presence of segmental black and yellow lines, which typically emerge during the third instar. Individuals were placed on their side with a ruler and photographed from directly above to capture the black striping. To assay the impact of the experimental diets on the presence of the stripe patterns, we compared the number of striped individuals among wild caught and experimental individuals. Wild caught larvae were collected from four locations in northeast Mississippi (33°55'52.28"N, 88°51'27.18"W; 33°30'36.98"N, 88°44'14.57"W; 33°18'2.94"N, 88°36'35.94"W; 33°27'47.23"N, 88°45'36.24"W) and photographed. Wild caught larvae were fed fresh, locally collected *D. purpurea*. At fifth instar, photos of wildcaught larvae were taken. Although these individuals were fed on wild *D. purpurea*, they were collected at varying instars. This resulted in wildcaught individuals developing for different lengths of times in the controlled laboratory conditions, which could impact later adult wing coloration. Therefore, no wildcaught larvae were included in any analyses on survivorship or adult wing coloration. The ggplot package in R studio was used to construct stacked bar charts of the data from the experimental treatments and from the data gathered from wild caught individuals (Wickham 2016).

Wing UV Reflectance Measurements. To assay the impact of diet on UV reflectance in adult wings, an HR 2000+ ES Ocean Optics spectrometer with a Halogen Deuterium light source (DH2000) was used to measure reflectance spectra from male forewings. Reflectance measurements were standardized with a magnesium oxide standard (Ocean Optics). Reflectance spectra were measured from a 0.5 cm region near the melanic eyespot on the dorsal forewing, which is highly UV reflective in male *Z. cesonia*. Spectroscopy measurements were taken in triplicate for the surviving males reared on the *D. purpurea* ('low', n=10; 'high', n=9) and *T. pratense* ('low', n=10) diets. The other three diet treatments were not included due to the lack of

TABLE 2. Survivorship from hatchling to eclosion across diets

Ingredient	Amount
Sorbic acid	0.2 g
Lepidoptera vitamin mix (BioServ #722)	0.5 g
Asorbic acid	0.7 g
Methyl phydroxybenzoate powder	0.8 g
Agar	3.5 g
Brewers yeast	6.4 g
Wheat germ	10 g
Dried lima bean powder	17.5 g
10% formaldehyde	3.2 ml
Deionized water	100 ml (fill to 200 ml)

Treatment	Survivorship to pupation	Survivorship to eclosion
No Plant (n=105)	6.7%	3.8%
D. purpurea low (n=109)	45.0%	42.2%
D. purpurea high (n=138)	58.7%	55.1%

male survivorship. Raw spectra files were trimmed to only focus on the UV wavelength (300–450 nm). UV brightness, Rmax, was defined as the point of maximum reflectance along the UV wavelength. The reflectance measurements were averaged for each treatment and plotted with 95% confidence intervals in R studio.

RESULTS **Survivorship across larval diets.** Survivorship varied greatly among the diet treatments. The *D. purpurea* diets had the highest survivorship rates, with 46 out of 109 individuals surviving from the 'low' diet and 76 out of 138 individuals surviving from the 'high' diet (Table 2). The '50:50 Mix' diet and 'low' *T. pratense* diets had much lower survivorship with only 8 out of 40, and 32 out of 116 surviving, respectively. The 'high' *T. pratense* diet produced no adult survivors (0 out of 45). The 'No Plant' diet had the second lowest survivorship (4 out of 105). Tests for the impact of diet on survivorship showed that diets with relatively more *D. purpurea* always showed greater survivorship. Pairwise tests to evaluate survivorship based on diet for three comparisons, 'low' vs. 'high' *D. purpurea*, 'low' *D. purpurea* vs. 'low' *T. pratense*, and '50:50 Mix' vs. 'high' *D. purpurea*, showed significant associations between diet and survivorship (p = 0.0445, 0.0213, and <0.0001, respectively). Pairwise Chisquare tests showed no significant difference in survivorship between sexes for the *D. purpurea* diets or *T. pratense* diet ('low' versus 'high' *D. purpurea* p= 0.1249 and 'low' *T. pratense* and 'low' *D. purpurea* P=0.1949) .

Developmental Time Observations. Developmental time from 1st instar larvae to eclosion varied among the diet treatments from 22.5–25.75 days. The D. purpur*ea* diet individuals had significantly longer larval development relative to the T. pratens*e* diet individuals (pairwise chisquare test, p < 0.0001). Among D. purpur*ea* diets, individuals reared on the 'high' D. purpur*ea* diet had significantly longer larval periods than individuals reared on the 'low' diet (pairwise chisquare test, p = 0.0003). The 'No Plant' diet individuals had the longest total development time, as well as the longest larval period (Fig. 2).

Frequency of Larval color patterns. Among the wild caught larvae (n=23), 35% had a striped pattern and 65% were solid green. Among the laboratory reared experimental treatments, both larval color patterns were observed at varying frequencies ('low' *T. pratense* 80% striped/20% solid; 'low' *D. purpurea* 50% striped/50% solid; 'high' *D. purpurea* 75% striped/ 25% solid) (Fig. 3).

UV Reflectance across diets. Among the experimental diets, the males reared on the 'high' *D. purpurea* diet were the brightest with the highest Rmax occurring at 373 nm. Males reared on the 'low' *T. pratense* diet and the 'low' *D. purpurea* diets were similar in their brightness. The 'low' *T. pratense* diet males had an Rmax located, on average, at 362 nm similar to plant fed individuals. However, the Rmax of 'high' and 'low' *D. purpurea* diet males were shifted towards 370 nm (Fig. 4).

There was a significant difference between the Rmax values of males reared on the 'low' *D. purpurea* diet and the 'high' *D. purpurea* diet (p = 0.040, one tailed ttest). There was no significant difference between the Rmax values of males reared on the 'high' *D. purpurea* diet and those reared on *D. purpurea* plant leaves only (p = 0.975, one tailed ttest). There was not a significant difference between the Rmax values of males reared on the 'low' *T. pratense* diet and the 'low' *D. purpurea* diet (p = 0.091, one tailed ttest).

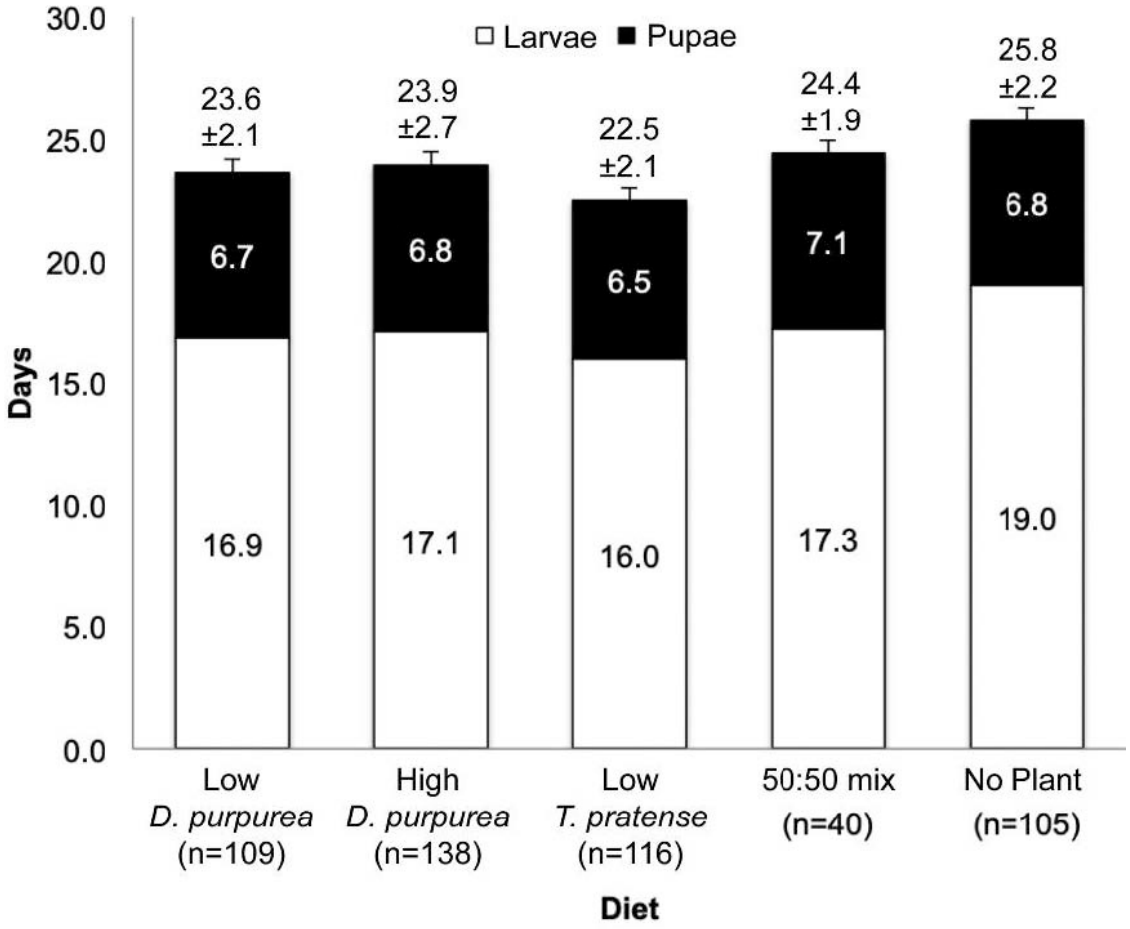

FIG. 2. Developmental timing across diets. The average total time and standard deviation in days for developmental timing until eclosion is noted at top columns for each treatment. The average number of days for larval and pupal duration are given in white and black shaded areas, respectively.

DISCUSSION This study provides a viable means to rear *Z. cesonia* in the laboratory for experimentation. Survivorship was positively related to the amount of *D. purpurea*, the preferred larval host plant of Mississippi *Z. cesonia* populations (Fenner et al. 2018). The highest survivorship achieved on the artificial diets was only 55%, with most loss occurring before pupation. Due to the large number of plants needed to determine survivorship when feeding solely on fresh *D. purpurea* tissue, we are unable to determine if 55% survivorship is atypical. However, based on our personal observations, there may be a higher incidence of contracting disease and larval death when feeding on *D. purpurea* grown in research greenhouses. Despite the rate of loss during larval development, individuals reared on artificial diet with *D. purpurea* have a very high probability of surviving to eclosion. We can also confirm that *T. pratense* does not appear to be a favorable alternative for *D. purpurea*, as survivorship was reduced to ~25%. Lastly, we can confirm that artificial diets that lack host plant supplement are not suitable as larval resources for *Z. cesonia*.

The development of artificial diets is a crucial step to establishing a laboratory model system. Here we present a tested and optimized artificial diet for *Z. cesonia*. The 'high' *D. purpurea* diet provides both ample survivorship and maintains the natural color variation observed in the wild. Butterflies can serve as a laboratory model system for many fundamental biological questions (Watt & Boggs 2003). In order to make the rearing process standardized and affordable for these model systems, the development of an artificial diet is a vital step. Although many artificial diet recipes have been published for Lepidoptera, no diet can be universally applied to moths and butterflies. In

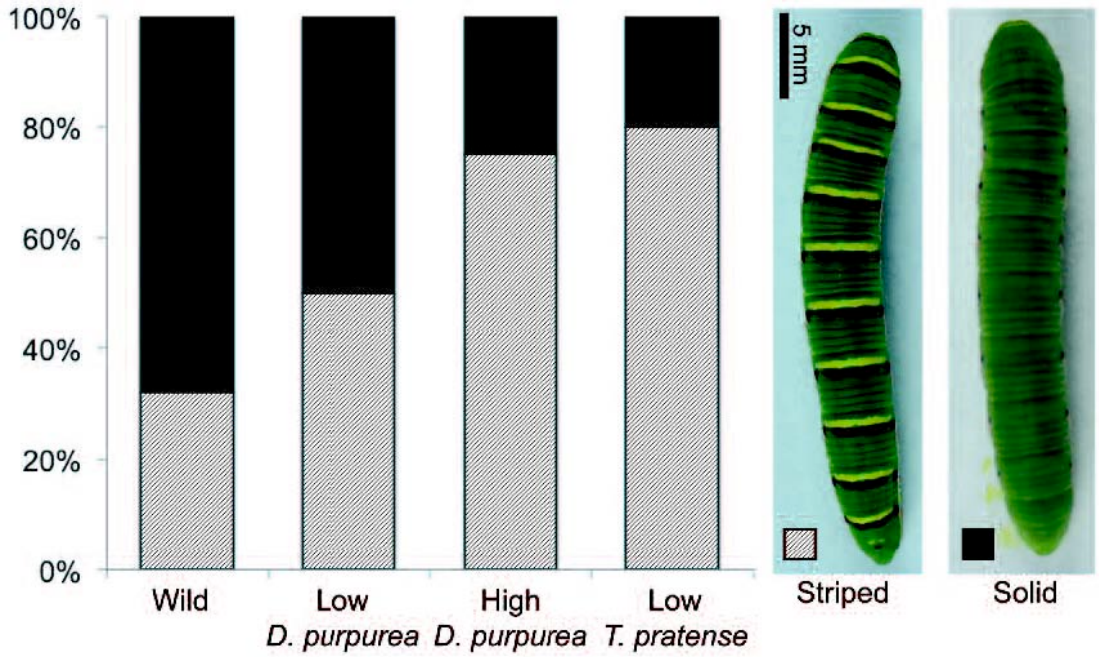

FIG. 3. Larval color patterns among wildcaught and diet treatments. Relative frequency of solid green morphs (black) and striped morphs (stripe) among samples.

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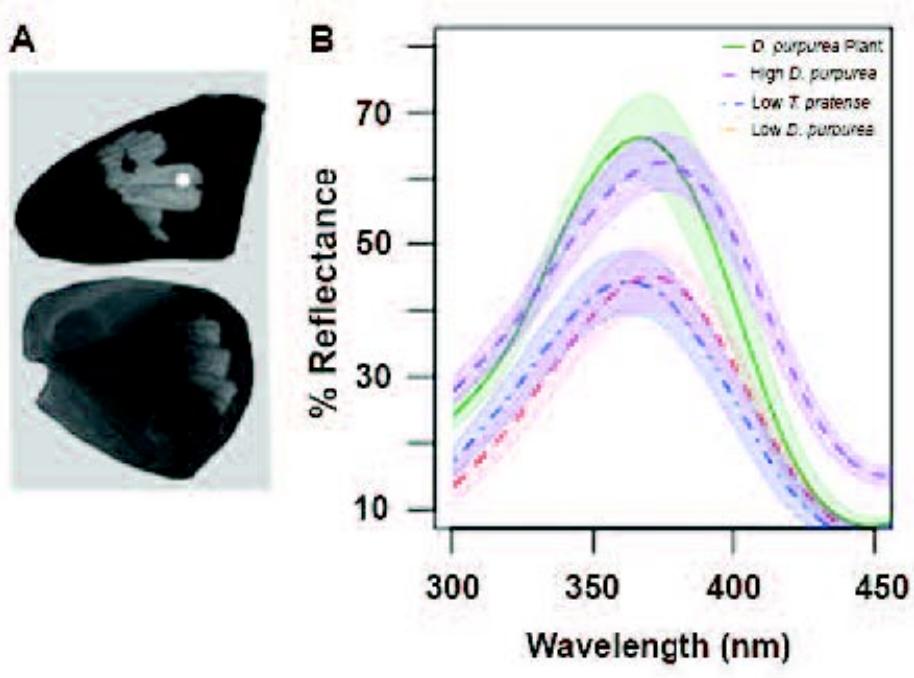
FIG. 4. Ultraviolet (UV) reflectance averages (lines) with 95% confidence intervals (shaded regions) of males across diets.



this study we not only show an optimized artificial diet for *Z. cesonia*, but also demonstrate that this diet recapitulates the natural variation observed in the wild.

Survivorship is increased with the addition of the preferred host plant. In general, increased survivorship was associated with increased access to *D. purpurea*. Poor survivorship of larvae given the 'no plant' diet treatment most often resulted in larvae that never initiated feeding and subsequently died. This was not the case for *D. purpurea* and *T. pratense* diet treatments, where most individuals feed readily after egg hatching. These findings suggest the host plants provide some 'stimulant' that allows the larvae to recognize the artificial diet as food. Although both *D. purpurea* and *T. pratense* appear to have this stimulant as larvae did frequently initiate feeding, *T. pratense* appears to perhaps be toxic at higher concentrations.

Recently, *D. purpurea* was shown to be a preferred host plant for Mississippi populations of *Z. cesonia* (Fenner et al. 2018). The poor survivorship in the diets lacking *D. purpurea* suggests that individuals may require host plant material as a feeding cue. Host plant generalist butterflies such as *Pieris rapae* and *Colias eurytheme* can both be reared on artificial diets that do not require a feeding stimulant (Taylor et al. 1981, Troetschler et al. 1985). Lepidoptera that have stronger host plant specializations may require a stimulant in their artificial diet. For example, Phaon Crescent butterflies could only be successfully reared on an artificial diet that contained 10% of their host plant (Genc & Nation 2004). *Ostrinia palustralis* larvae can be started on a diet that contains the preferred host in order to induce feeding and then be switched after 14 days to a diet without the expensive host plant (Fukuzawa et al. 2004). This two dietswitching schema allows for larvae to be reared at a reduced cost. It is possible that *Z. cesonia* could be fed on a similar schema with *D. purpurea* diets followed by a *T. pratense* diet, but given the poor survivorship of the 'No Plant' diet the presence of plant material may play a vital role in larval feeding.

It is important to note there is a high rate of carnivory among 2nd–3rd instar larvae based on observations in the laboratory. It is unclear how far larvae may disperse in the wild, and how much carnivory typically occurs. Based on these results, we recommend for greatest success that *Z. cesonia* larvae be reared in separate or lowdensity environments on an artificial diet that contains *D. purpurea*.

Artificial diet**s** captur**e** th**e** wil**d** natura**l** variation**.** Artificial diets have been developed and tested for few Pierid butterflies. Pieri*s* rapa*e* and Colia*s* eurytheme are two Pierids that have had artificial diets developed and tested, but in neither of these studies was it demonstrated if these diets could recapitulate natural variation in traits of research interest (Taylor et al. 1981, Troetschler et al. 1985). In this study, we show that both larval and adult coloration of laboratoryreared individuals are similar to those observed among wildcaught individuals. Larval coloration was not influenced by diet and the variation in the frequency of striped individuals was similar to the frequency in the wild populations. Larvae in all diets and in the wild were either solid or striped in coloration and given the frequency of these patterns, and the lack of dietary influence, this coloration is likely a genetically controlled polymorphic trait in Z. cesonia.

For adult coloration, UV pattern and brightness were similar among the treatments and recapitulated the variation seen in natural populations (Fenner et al. 2019). UV coloration is a maleonly trait and is a sexual ornament in *C. eurytheme* (Silberglied & Taylor 1978). UV brightness is the number one indicator of male mating success in *C. eurytheme* and is an honest indicator of male quality since nutrient stress can decrease overall UV brightness (Kemp et al. 2006, Papke et al. 2007). UV reflectance can serve as an important metric for male quality and thus it is important to ensure that a laboratory artificial diet can recapitulate the reflectance spectra of an individual reared on its preferred host plant. A shift in the UV spectra was observed from the *D. purpurea* artificial diets (Rmax: 360 to 370 nm) that was not observed in the plant fed or *T. pratense* diet. We are unclear as to the cause of this shift in UV spectra. UV brightness, varied greatly within and among the treatments, but it also consistently increased with the amount of *D. purpurea* available to the larvae, which suggests UV brightness could serve as an honest signal of larval resource availability in *Zerene*, as it does in *Colias*.

Collectively, the results of this study allow us to present an artificial diet optimized for rearing *Z. cesonia* that can allow for not just success in overall survivorship but will yield individuals that accurately recapitulate the natural color variation. A diet 'high' in *D. purpurea* produces the highest survivorship, while maintaining coloration similar to what is observed in the wild. We also show that an alternative potential host, *T. pratense*, is not an efficient substitute for the preferred *D. purpurea* host plant in the artificial diet. In conclusion, if possible, ample supplies of preferred host plants should be used for rearing. However, artificial diets with supplements of the preferred host will suffice to maintain laboratory colonies of *Z. cesonia* suitable for the study of color pattern variation.

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