Effects of Larval Rearing Density and Food Availability on Adult Size and Coloration in Monarch Butterflies (Lepidoptera: Nymphalidae)[1]

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Abstract Completing development to a reproductively mature adult is energetically expensive, and environmental stress can impose substantial fitness tradeoffs during developmental processes. We tested the prediction that high larval rearing density and food stress should compromise monarch, *Danaus plexippus* L. (Lepidoptera: Nymphalidae), larval growth and the production of melanin or other pigments in the adult wings. We reared focal larvae on caged, potted plants in control, food stress and high density treatments. Food stressed larvae experienced food shortages but no crowding (n = 1 larva per plant), whereas high density larvae were crowded (n = 5 larvae per plant) but had constant access to food. We used digital image analysis to measure the size and coloration (black and orange) of the resulting adult forewings. Larval rearing treatment affected adult size, but not wing coloration or development time. Adults emerging from the high density treatment were significantly larger than adults from the control or food stress treatments. This suggests that larval crowding may stimulate monarch larvae to increase their feeding rate, producing larger body size when there is not a subsequent food shortage caused by the crowding. Food stress did not affect adult size, wing coloration or development time, which suggests that monarch larvae can overcome moderate levels of food stress without experiencing compromised development. Sibling group affected forewing area, development time, and several aspects of wing coloration, which is consistent with previous research that has demonstrated a heritable component to size and melanism in monarch butterflies.

Key Words development time, developmental stress, heritable variation, melanism, digital image analysis

Coloration plays many roles in the animal kingdom, and often has antipredator or physiological functions (Cott 1940, Majerus 1998, Ortolani 1999). Across taxa it is used as camouflage to avoid predators (Cott 1940, Majerus 1998), or as visual communication to warn predators of toxicity (Baylis 1979, Houser 1996, Majerus 1998). Physiologically, coloration can help control heat balance [e.g., birds and mammals (Hamilton and Heppner 1967, Walsberg 1983); frogs (Osorio and Srinivasan 1991), various taxa (Majerus 1998)] and often correlates with disease resistance [e.g., mammals (Waage 1981); birds (Navara and Hill 2003); insects (Wilson et al. 2001)]. It is also correlated with life history traits that affect fitness such as production of offspring and mating success (Cott 1940, Majerus 1998).

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Intraspecific variation in wing coloration has been observed in many species of Lepidoptera (Wiernasz 1989, Ellers and Boggs 2003, 2004, Talloen et al. 2004, Davis et al. 2005, 2007). Unlike most other organisms, lepidopteran color pigments are stored in thin, flattened extenuations of specialized epithelial cells of the wing called scales (Nijhout 1991). Each scale contains a single color, so wing patterns are a mosaic of these monochrome scales. The color pattern is determined early in the final larval instar after the venation has developed, and scales develop in the first few days of the pupal stage. The cuticle of the wing is colorless. Pigments occur only within the scales and are not synthesized until 1 - 2 d prior to emergence. The color of the scales comes from chemical pigments and from the structural color of the scale, which is determined by the way it reflects UV light (Nijhout 1991). Because color pattern in adult butterflies is determined at the end of their larval stage, the environmental conditions experienced by larvae can affect adult coloration (Talloen et al. 2004, Davis et al. 2005, Gibbs and Breuker 2006). Temperature (Solensky and Larkin 2003, Davis et al. 2005), host plant quality (Talloen et al. 2004), larval density (Gibbs and Breuker 2006), and nutrient availability (Bauerfeind and Fischer 2005) have all been shown to affect lepidopteran larval development. When these environmental variables produce stressful rearing conditions, larvae can experience compromised color development, reduced growth or increased development time.

Many insects respond to changes in temperature by becoming more or less melanistic (Majerus 1998). Davis et al. (2005) found variation in larval and adult melanism due to different larval rearing temperatures. Melanin can be costly to produce (Talloen et al. 2004), and trade-offs between the amount of melanism and development time, adult size, or both have been observed in several lepidopteran species (Graham et al. 1980, Windig 1999). Talloen et al. (2004) found that melanization in the butterfly *Pararge aegeria* L. (Lepidoptera: Nymphalidae) was reduced among larvae reared on drought-stressed host plants, indicating that host plant access and quality can affect melanization because they limit nutrient availability. If larvae have less food available, they have less metabolic energy available and should produce less melanin. This allows larvae to conserve metabolic energy for metamorphosis and oogenesis (Bauerfeind and Fischer 2005).

Behavioral stress such as crowding can also affect larval metabolism (Gibbs and Breuker 2006). Crowding produces darker larvae in several lepidopteran species (Drooz 1966, Hodjat 1970, Johnson et al. 1985, Goulson and Cory 1995, Gotthard et al. 2009), but its effect on adult coloration has not been reported. Increased density can have positive or negative effects on larval growth (Smits 2002), although these effects seem to vary greatly among species. Two studies of solitary species reported that larval growth rate was higher and developmental time was shorter in larvae reared at higher densities, but the resulting pupal (Smits 2002) or adult (Smits 2002, Bauerfeind and Fischer 2005) mass was not affected. Larvae may develop faster in response to the risk of food loss before metamorphosis (Bauerfeind and Fischer 2005). Sillanpaa (2008) reported a positive effect of crowding on growth rate of *Epirrita autumnata* Borkhausen (Lepidoptera: Geometridae) larvae fed a high quality diet, but no effect on growth rate among larvae fed a poor quality diet. Other studies report negative effects of larval crowding, including decreased adult size, increased developmental time, and decreased survival in two solitary lepidopteran species (Goulson and Cory 1995, Gibbs et al. 2004). Increased development time can impose costs by extending larval exposure to predators and parasitoids (Gibbs et al. 2004). Hodjat (1970) and Ruohomaki et al. (2003) found that crowding decreased adult size but did not affect development

time. These results were observed in the gregarious species *Spodoptera littoralis* Boisduval (Lepidopatera: Noctuidae) (Hodjat 1970) and the solitary species *Epirrita autumnata* Borkhausen (Lepidoptera: Geometridae) (Ruohomaki et al. 2003). Decreased adult size can impose costs by decreasing reproductive fitness (Gibbs et al. 2004).

Monarch butterfly, (*Danaus plexippus* L. (Lepidoptera: Nymphalidae)), orange wing coloration is correlated with male mating frequency (Davis et al. 2007), but the source of variation in orange wing coloration remains unclear. Larval rearing temperatures affect black wing coloration (Davis et al. 2005) but do not explain all of the variation observed in captive populations. We investigated the effect of larval density and food availability on adult wing coloration and larval development in monarch butterflies. If the two environmental stressors imposed in this study affect adult monarch wing coloration, they should produce lighter coloration in adults because they limit the energy available for melanin production (Talloen et al. 2004).

It is difficult to predict the effect that larval rearing density will have on development time or size in monarchs because it appears to vary greatly among lepidopteran species and has not been studied independent of food shortage (Hodjat 1970, Goulson and Cory 1995, Smits 2002, Ruohomaki et al. 2003, Gibbs et al. 2004, Bauerfeind and Fischer 2005). Reduced food availability (in noncrowded situations) should lengthen development or reduce growth (Fischer and Fiedler 2001, Bauerfeind and Fischer 2005).

Materials and Methods

Study organisms. We collected monarch butterfly eggs and larvae from 20 milkweed patches, each at least 1.6 km apart, in Wayne and Holmes counties, OH, from June through July 2006. The eggs and larvae were reared to adults, which were checked for infection by *Ophryocystis elektroscirrha* McLauglin and Myers (Apicomplexa: Neogregarinida) (Altizer et al. 2000). We allowed 10 pairs of healthy, unrelated males and females to mate. We defined males and females as unrelated if they were collected at sites at least 1.6 km apart, which makes it unlikely that their eggs were laid by the same female. Mated females were released individually into mesh cages with a potted plant of tropical milkweed (*Asclepias curassavica* L.) for oviposition. Plants used in this experiment were potted *A. curassavica* grown from seeds in a greenhouse under a 16:8 h light:dark cycle at 28°C. Seven females laid a sufficiently large number of eggs (≥ 60) to produce at least 7 replicates within each larval rearing treatment group; we refer to these as sibling groups.

Larval rearing treatments. We used a paintbrush to transfer first-instar larvae ($n = 420$) within 24 h of hatching onto a plant, and randomly assigned each plant to 1 of 3 treatments (control, food stress, or high density). Each plant supported larvae from just one sibling group, and offspring from each female (sibling groups) were distributed evenly among the treatment groups ($n = 8$ or 9 sibling group replicates per treatment group).

After transferring each larva onto a plant, we enclosed each plant with a cage consisting of 4 bamboo stakes (60 cm length) covered with a mesh bag closed by a rubber band around the pot. The caged plants were kept in a greenhouse (16:8 h light:dark cycle at 28°C), and plants in the 3 treatments were evenly distributed throughout the greenhouse to control for any effects of temperature variation within the greenhouse (Bauerfeind and Fischer 2005). Larvae in all treatments were fed potted *A. curassavica*.

Larvae in the control treatment had constant access to potted *A. curassavica* (no food stress) and were reared individually on plants (low density). Larvae in the food stress treatment were reared individually on plants (low density), but were removed from their host plant for three 4-h periods following the 2nd, 3rd, and 4th molt. We removed each larva in this treatment group from its plant on the first morning on which the larva started eating following each of these molts, and put it in a plastic container with a mesh lid. After 4 h, we returned the larvae to the same plant. Larvae in the high density treatment had constant access to potted *A. curassavica* (no food stress), but were reared in groups of 5 larvae on 1 potted *A. curassavica* plant. We gave new plants to all treatment groups as needed (less than 2 leaves remaining) with each plant being replaced at least twice during the experiment. Because we defined the high density and control treatments as experiencing no food stress, any larvae in these 2 treatments that experienced a food shortage were eliminated from the experiment. Larvae were checked daily at 0800 h, and each molt was recorded. If the larva on the control or food stress plants died, we removed the plant from the experiment. Plants in the high density treatment remained in the experiment as long as they had at least 4 larvae. Larvae were provided with a piece of styrofoam fixed to the top of each cage during the late 5th instar for pupation.

Adult size and color measurements. We recorded the date of emergence and sex of each adult. We calculated development time as the number of days lapsed between egg hatch to adult emergence. We checked adults for infection by *O. elektroscirrha* (Altizer et al. 2000), and found only 1 individual that was infected and, therefore, removed from the study. Adults were placed in glassine envelopes 4 - 8 h after emergence, kept at room temperature (~23°C), and fed 20% honey water ad libitum every 3 d.

We scanned the wings of all the adults on a Hewlett-Packard Scan Jet 3970 scanner on the day after emergence. The same resolution, light, and exposure settings were used for all the scans, and the adults were scanned in standard pinning position. Before scanning, each adult was chilled on ice for 10 min to minimize movement. The wing images were imported into Adobe Photoshop (Adobe Systems, Mountain View, CA) and analyzed using the Fovea Pro 4.0 Image Analysis Plug-in (Reindeer Graphics, Inc., Asheville, NC) following methods described by Davis et al. (2005, 2007). We cropped the right and left forewing from the image of each male and saved it as a separate image. We ran a Fovea Pro measurement routine on each image to measure the total area of the forewing (mm^2), percent black and density of black on each forewing. Percent black is the proportion of the wing that contains black coloration. The values for the density of black range from 0 - 255, with 0 being completely black and 255 being completely white. To measure orange color, we ran a Fovea Pro measurement routine to measure the average hue, saturation, and luminance values for all the pixels within the middle cell of each forewing. Hue relates to "color" (i.e., the distinction between red, orange, yellow, green, etc.) and is measured in degrees of the color wheel from 0 - 360. However, no circular statistics were necessary in this study because the hues only ranged from 31 - 42 degrees. Within this range, low hue values indicate orange color that is closer to red; whereas, higher values indicate orange color that is closer to yellow. Saturation is the intensity of color (i.e., the difference between pink and red) and is measured on a scale of 1 - 255 with lower values indicating duller, more gray color. Luminance is the overall darkness or brightness of the color (i.e., if it were a black and white image) and is also on a scale from 1 - 255 with 1 being completely black and 255 being completely white (Davis et al. 2007). The

average of the left and right forewing measurements for each size and color variable was used for data analysis, resulting in 1 size variable (average forewing area) and 5 color variables measuring both melanism (average forewing percent black and black density) and orange color (average hue, saturation and luminance of the middle forewing cell).

We obtained wing scans from a total of 298 adults, but used only 1 randomly selected adult from each of the high density replicates to maintain independence of observations (total n = 149: 25 males + 30 females from the control treatment, 29 males + 25 females from the food stress treatment, and 22 males + 18 females from the high density treatment).

Data analysis. Monarchs exhibit sexual dimorphism in wing coloration, so we included sex as a fixed independent variable, in addition to larval rearing treatment, in the statistical analyses. Sibling groups were included as a random independent variable. Forewing area and all 5 color variables were normally distributed for both males and females in all three treatment groups (Kolmogorov-Smirnov test: $P > 0.05$). Development time was also normally distributed within the high density and food stress treatments (Kolmogorov-Smirnov test: $P > 0.05$), and deviated only marginally from a normal distribution in the control treatment (Kolmogorov-Smirnov test: $P = 0.02$).

We used analysis of variance (SPSS 2004) to assess the effects of larval rearing treatment, sex and sibling group on forewing area, development time, and all 5 color variables. Because there were 7 separate analyses for dependent variables related to different aspects of wing size and color, we applied a Bonferroni adjustment and concluded statistical significance when $P < 0.007$ (=0.05/7) to maintain an experimentwise error rate of $P < 0.05$ (Sokal and Rohlf 1995). We began each analysis with the full model (dependent variable = treatment + sex + sibling + treatment*sex + treatment*sibling + sex*sibling + treatment*sex*sibling) and simplified each model by sequentially removing each nonsignificant interaction term and comparing Akaike information criteria (AIC) between the full model and the model with k-1 terms (Crawley 2002). We excluded interaction terms from the simplified model if the associated probability was greater than 0.15 or if their removal resulted in a reduction or only a small increase (<4) of the AIC value. We further simplified each model by removing nonsignificant main effect terms if they were not present in any remaining interaction term and if the associated P value was greater than 0.15 or if their removal resulted in a reduction or only a small increase (<4) of the AIC value. We report these final simplified models, with comparisons of means where appropriate using Tukey's post hoc tests at the 0.05 level. All models were checked for equality of error variances.

Results

Development time. Development time was not affected by larval rearing treatments, but did vary among sibling groups (Table 1). Males and females required a similar number of days to complete development from egg to adult emergence.

Adult size. Adult wing area was the only dependent variable affected by larval rearing treatment (Table 1). The high density treatment produced larger adults than either the food stress or control treatments (Tukey HSD: $P < 0.001$), which were not significantly different from each other (Tukey HSD: $P = 0.15$; Fig. 1). Sibling group had a significant effect on adult wing area, and a marginally significant effect on a butterfly's size response to larval rearing conditions (Table 1, Fig. 1). Males were slightly larger than females (Table 1).

Table 1. Results of simplified analysis of variance models describing the effects of larval rearing treatment (Trt), sex and sibling group on development time and adult wing area and coloration of monarch butterflies.

Dependent	Independent	df	F	p	Partial eta^2
Wing Area*	Trt	2	8.18	0.006	0.57
	Sex	1	5.34	0.023	0.04
	Sibling	6	6.26	0.003	0.76
	Trt * Sibling	12	2.20	0.015	0.17
	Error	127			
% Black†	Trt	2	0.25	0.786	0.04
	Sex	1	400.01	<0.001	0.99
	Sibling	6	0.91	0.530	0.40
	Trt * Sibling	12	2.05	0.025	0.17
	Sex * Sibling	6	1.64	0.143	0.08
	Error	121			
Black Density†	Sex	1	254.31	<0.001	0.64
	Sibling	6	5.03	<0.001	0.18
	Error	141			
Hue†	Trt	2	2.81	0.064	0.04
	Sex	1	6.39	0.013	0.04
	Sibling	6	3.15	0.006	0.12
	Error	139			
Saturation†	Sex	1	242.55	<0.001	0.98
	Sibling	6	1.74	0.258	0.64
	Sex * Sibling	6	4.18	<0.001	0.16
	Error	135			
Luminance†	Sex	1	457.71	<0.001	0.76
	Sibling	6	5.71	<0.001	0.20
	Error	141			
Development Time‡	Trt	2	1.92	0.150	0.03
	Sibling	6	3.72	0.002	0.14
	Error	140			

*Wing area was measured in mm^2.

†Wing color variables include the percent of the right and left forewings that contained black pigmentation (% Black), the intensity or opacity of black pigmentation (Black Density), and the average hue, saturation and luminance of the orange color on the middle cells of the right and left forewings.

‡Development time was recorded as the number of days lapsed from egg hatching to adult emergence.

**Because there were seven separate analyses, we applied a Bonferroni adjustment and concluded statistical significance when $P < 0.007 = 0.05/7$) to maintain an experiment-wise error rate of $P < 0.05$.

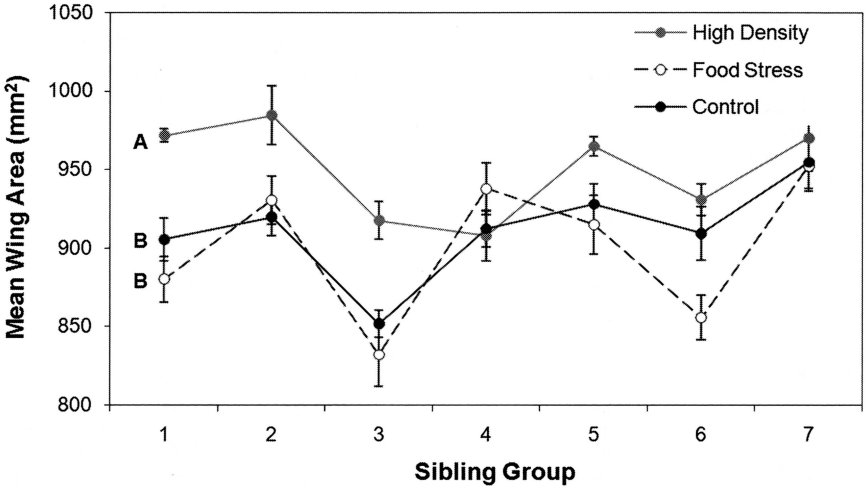

Fig. 1. Monarch larvae reared at high densities (5 larvae/plant) emerged as significantly larger adult butterflies than larvae in either the food stress or control treatments (both with 1 larva/plant; $P < 0.001$; different letters indicate significant differences between larval rearing treatments). Adult wing area differed among sibling groups ($P = 0.003$). Because sex did not affect wing area, males and females are pooled. Error bars show ± one standard error. Lines connecting mean values are shown only to enhance the overview.

Adult coloration. Larval rearing treatment did not affect any wing color trait (Table 1). Sex affected all 5 color variables, although the effect on orange hue was only marginally significant (Table 1). Females have more black coloration on their forewings than males, but the black is less dense, or intense (Fig. 2). The orange on female forewings is duller (lower saturation and luminance values) than the orange on male wings. Sibling groups affected all five color variables ($P < 0.007$), except the percent of black on the right and left forewings ($P = 0.530$). There was no main effect of sibling group on the saturation of the orange color on the middle cells of the right and left forewings ($P = 0.258$), but there was an interaction between sex and sibling ($P < 0.001$) such that male saturation was fairly consistent across sibling groups, whereas female orange saturation varied between sibling groups (Fig. 2).

Discussion

Adult size and development time. Adult monarchs from the high density larval rearing treatment were significantly larger than those from the control or food stress treatments, yet there was no difference in the time required for development (Table 1, Fig. 1). Previous studies of other lepidopteran species reported that larvae grown at high densities developed more slowly, which resulted in smaller size (Goulson and Cory 1995, Gibbs et al. 2004), or had an increased growth rate and shorter development time that did not affect overall size (Smits 2002, Bauerfeind and Fischer 2005).

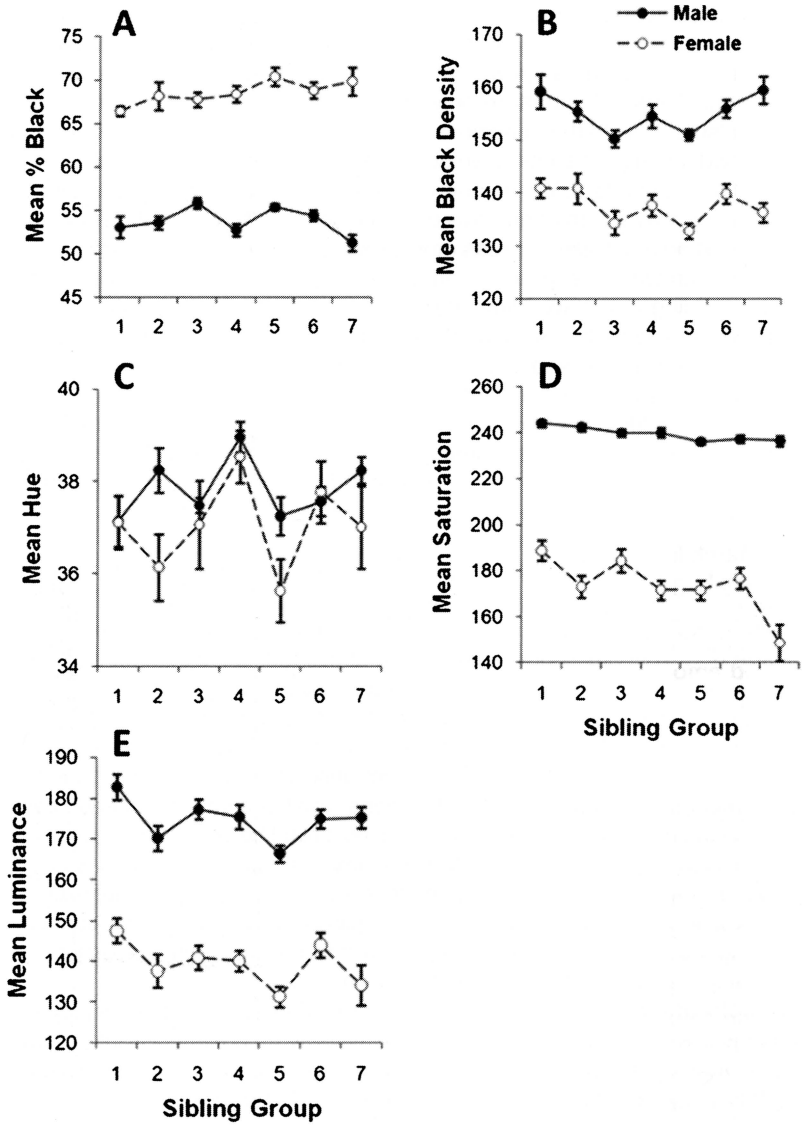

Fig. 2. Adult monarch butterfly wing coloration was affected by sex and sibling group. Female wings had (A) a significantly higher percent of their wings comprised of black pigmentation ($P < 0.001$), but (B) a lower black forewing density than males ($P < 0.001$). The orange areas of female wings were (C) more reddish (i.e., lower hue values; $P = 0.013$) and (D, E) duller than male wings (i.e., lower saturation, $P < 0.001$, and luminance, $P < 0.001$). Solid circles and lines show male coloration; open circles and dashed lines show female coloration. Error bars show ± one standard error. Lines connecting mean values are shown only to enhance the overview.

Others found a decrease in size, but no effect on development time (Hodjat 1970, Ruohomaki et al. 2003). One other study has reported a pattern similar to what we observed: an increased larval growth rate accompanied by no change in development time (Sillanpaa 2008). This pattern only occurred in response to a high quality diet, which was also similar to the conditions created in our study. This variation in effects of larval crowding suggests that developmental response to crowding may be highly variable in the Lepidoptera. We measured the effects of density independent of food shortage, which may be an alternative explanation for why this study revealed an increase in resulting adult size whereas most previous studies have reported reductions or no effect of crowding on pupal or adult size (*but see* Sillanpaa 2008). In this experiment, crowding may have stimulated the larvae in the high density treatment to eat more quickly due to the associated risk of food shortage. However, because they never experienced a food shortage, larvae in the high density treatment may have actually eaten or assimilated more plant material and grown larger than larvae in the other treatments. Additionally, because they never exhausted the food supply, they did not have to complete development prematurely, which could explain why development time was not affected by larval density. These data suggest that monarch larvae are capable of perceiving and responding to larval density, either through cues derived from the larvae or the host plant.

We expected the food stress treatment to produce smaller butterflies that took longer to develop than the control treatment, because less food would be available to them (Bauerfeind and Fischer 2005), but there was no difference between these 2 treatments in adult size (Fig. 1) or development time. Food stress reduces the amount of resources available, leaving less energy for growth and reproduction. However, it is possible that the larvae in the food stress treatment still allocated the usual amount of energy to growth, but reduced energy allocation to some other trait (e.g., reproductive traits). Alternatively, experimental protocol may explain why we did not observe a decrease in size in the food stress treatment. It was often difficult to determine whether a larva had begun eating yet after its molt, and if a larva was removed from its host plant before it had begun to eat after molting, the treatment would not be effective in imposing food stress. It is also possible that monarch larvae can respond to moderate levels of food stress without experiencing compromised development. Relatedness (i.e., sibling groups) had a significant effect on both forewing area and development time, which indicates that there is a genetic component to adult size and rate of development in monarchs.

Adult coloration. Treatment did not affect any of the color variables (Table 1), indicating that larval rearing density and food availability do not affect adult coloration in monarchs. Melanin is costly to produce, requiring large amounts of metabolic energy, and we had predicted that larval stress due to crowding or food deprivation would force a trade-off between melanin production and energy needed to compensate for the stress they were experiencing (Talloen et al. 2004). The treatments in this study may not have affected adult coloration because they were not stressful enough to necessitate an energy trade-off between adjusting to stress and color production. However, making the treatments more extreme would have made this study less ecologically relevant, because the treatments would have been more exaggerated than a monarch larva would be likely to face in the wild. Monarch eggs are infrequently found on milkweed plants with more than 1 - 2 eggs per plant (Drury and Dwyer 2005). It would be unusual to find even 5 individuals on 1 plant in the wild, therefore putting any more larvae on the high density plants would have been unrealistic. Monarch larvae often leave their host plant in response to plant quality, overcrowding, thermoregulation,

escape from predators, or response to plant cardenolides and latex (Vickerman and de Boer 2002). However, common milkweed plants (*Asclepias syriaca* L.), the dominant host plant of monarchs in the Eastern North American monarch population, are clonal and typically grow in dense patches (Brower 1969, Wassenaar and Hobson 1998, Hartzler and Buhler 2000) so it is unlikely that a larva would be off its plant for more than 4 h before finding a new plant nearby, making a longer period of food deprivation less ecologically relevant.

Sibling groups varied significantly in all 5 color variables except percent of black on the forewings (Table 1). This is largely consistent with data reported by Davis et al. (2005), although they found that both measures of black coloration (percent black and black density) were affected by sibling groups. This study shows that sibling groups varied significantly in all 3 measures of orange coloration (hue, saturation and intensity: Fig. 2), a pattern that has never before been reported in monarchs. These results indicate that there is a genetic component to several aspects of adult wing coloration in monarch butterflies.

Conclusion

When host plants are consistently available, larval crowding produced larger adult monarch butterflies without affecting development time, suggesting that monarch larvae detect and respond to the presence of other herbivores with increased feeding. This experiment did not test whether this response is triggered by the presence of herbivores per se or by the chemical response of the host plant to increased herbivory, nor does it reveal whether monarchs respond only to conspecific herbivores or more generally to any additional herbivore. Both would be interesting avenues for future research.

Food stress, imposed as a series of 4-h food deprivation periods, did not affect adult development time or wing area. If density and food availability have an effect on adult coloration in wild monarch butterflies, these effects should have been evident from the treatment levels used in this study. For this reason, it seems reasonable to conclude that larval density and food stress do not affect adult coloration in wild populations of monarch butterflies. The current study also has shown that sibling affects at least some aspects of adult size and wing coloration in monarchs, as well as development time, which suggests an underlying genetic component to growth and the development of wing pigmentation in monarch butterflies.

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