Consequences of Food Restriction for Immune Defense, Parasite Infection, and Fitness in Monarch Butterflies

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ABSTRACT

Organisms have a finite pool of resources to allocate toward multiple competing needs, such as development, reproduction, and enemy defense. Abundant resources can support investment in multiple traits simultaneously, but limited resources might promote trade-offs between fitness-related traits and immune defenses. We asked how food restriction at both larval and adult life stages of the monarch butterfly (*Danaus plexippus*) affected measures of immunity, fitness, and immune-fitness interactions. We experimentally infected a subset of monarchs with a specialist protozoan parasite to determine whether parasitism further affected these relationships and whether food restriction influenced the outcome of infection. Larval food restriction reduced monarch fitness measures both within the same life stage (e.g., pupal mass) as well as later in life (e.g., adult lifespan); adult food restriction further reduced adult lifespan. Larval food restriction lowered both hemocyte concentration and phenoloxidase activity at the larval stage, and the effects of larval food restriction on phenoloxidase activity persisted when immunity was sampled at the adult stage. Adult food restriction reduced only adult phenoloxidase activity but not hemocyte concentration. Parasite spore load decreased with one measure of larval immunity, but food restriction did not increase the probability of parasite infection. Across monarchs, we found a negative relationship between larval hemocyte concentration and pupal mass, and a trade-off between adult hemocyte concentration and adult life span was evident in parasitized female monarchs. Adult life span increased with phenoloxidase activity in some subsets of monarchs. Our results emphasize that food restriction can alter fitness and immunity across multiple life stages. Understanding the consequences of resource limitation for immune defense is therefore important for predicting how increasing constraints on wildlife resources will affect fitness and resistance to natural enemies.

Keywords: ecoimmunology, *Danaus plexippus*, insects, development rate, life span, resource limitation, phenoloxidase, hemocytes.

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Introduction

Growth, reproduction, and survival require energy and nutrients. Immune defenses that confer resistance to parasites and pathogens can also be energetically costly, and their expression depends on both available calories and micro- and macronutrients derived from food (Sheldon and Verhulst 1996; Lochmiller and Deerenberg 2000; Cotter et al. 2011; Povey et al. 2014). Experimentally food-restricted animals, for example, tend to have lower immune defenses than well-fed animals (Moret and Schmid-Hempel 2000; Butler and McGraw 2012; Laurentz et al. 2012; Simmons 2012), suggesting that sufficient nutrition is necessary to mount costly aspects of an immune response. Because multiple physiological pathways draw from the same pool of resources, phenotypic trade-offs often occur between immunity and other fitness-related traits, such as competitive ability (Kraaijeveld et al. 2001), growth (van der Most et al. 2011), or reproduction (Lawniczak et al. 2007; Simmons 2012). The extent or severity of these trade-offs can further depend on diet quality and composition.

Experiments addressing the effects of food limitation on immunity and immune-fitness trade-offs typically restrict diet quantity, quality, timing, or the percentages of specific macronutrients (reviewed in Ponton et al. 2011). Substantial evidence indicates that fitness components and physiological traits such as growth rate, body maintenance, and reproduction can trade-off with immunity in food-limited contexts across systems ranging from crickets (Fedorka et al. 2004) to lizards (French et al. 2007*a*), poultry (van der Most et al. 2011), and mammals (Canale and Henry 2011). Certain invertebrate immune defenses (e.g., phenoloxidase and prophenoloxidase, encapsulation) have been found to be lower in resource-poor environments (Siva-Jothy and Thompson 2002; Triggs and Knell 2012) and can trade-off with fitness measurements, such as growth, development rate (Cotter et al. 2011), and reproduction (Karl et al. 2007; Kelly and Tawes 2013; Kelly et al. 2014).

Importantly, the majority of past work focuses on food limitation at a single life stage and examines effects occurring within that same stage, even though early life nutrition can profoundly affect adult fitness and life history (Boggs and Freeman 2005; Bauerfeind et al. 2009; Boggs 2009; Dmitriew and Rowe 2011; Stoks

and Córdoba-Aguilar 2012). Further, there may be additive or synergistic effects of early- and late-life food limitation on immunity in both vertebrates (Butler et al. 2011; Butler and McGraw 2012) and invertebrates (Dmitriew et al. 2007; DeBlock and Stoks 2008; Karl et al. 2011; Jiménez-Cortés et al. 2012; Jiménez-Cortés and Córdoba-Aguilar 2013). Emerging trends from these studies in insects are that effects of early-life food restriction on fitness and immunity can persist across metamorphic boundaries and that sexes often differ in the effects of food restriction on immune and fitness traits. Further work is needed to characterize which fitness traits are expected to trade-off with which immune measures and how these relationships unfold in the contexts of food stress and parasitism.

The monarch butterfly (*Danaus plexippus*) is a well-studied insect whose natural history in temperate environments includes extreme seasonal shifts in energetic demand for reproduction and flight. Monarchs in eastern North America undergo a southward fall migration, overwinter in a nonreproductive state in Mexico for several months, and then migrate north to recolonize their breeding range during the spring and summer (Urquhart and Urquhart 1978; Malcolm et al. 1991). As larvae, monarchs feed obligately on a subset of plants in the milkweed subfamily (Asclepiadoideae), from which they sequester cardenolide toxins to use in enemy defense (Malcolm and Brower 1989; de Roode et al. 2008*a*). As adults, monarchs obtain nectar resources from flowers and convert these to lipids to fuel the fall migration and overwintering period (Alonso-Mejía et al. 1997; Brower et al. 2006). Monarchs can encounter resource limitation as larvae and adults in several ways. Caterpillar densities are generally low during the spring and summer across North America (Pleasants and Oberhauser 2012), but per plant larval densities have been shown to increase from early to late in the summer breeding season (Bartel et al. 2011) and are also high in locations where mild winter climates and the planting of exotic milkweeds allow monarchs to forego migration and breed year-round (Haeger et al. 2015; Satterfield et al. 2015). Moreover, the loss of milkweed habitat throughout the monarchs' breeding range could increase resource competition by crowding monarchs into remaining habitats (Pleasants and Oberhauser 2012). Experimental studies show that high larval density decreases monarch body size, larval survival, and adult reproductive output and increases monarch susceptibility to parasite infection (Lindsey et al. 2009; Flockhart et al. 2012). Modeling studies suggest that current monarch population declines are driven primarily by larval food limitation during the breeding season (Flockhart et al. 2015), a phenomenon exacerbated by habitat loss. Flockhart et al. (2012) further found that monarchs reared in high densities (with less larval food per animal) had reduced fecundity as adults. Thus, resource acquisition at early life stages has implications for both individual adult fitness and longer-term population viability. It has also been suggested that nectar-providing flowering plants are decreasing in availability across the United States (Goulson et al. 2015), creating resource limitation among adults (Brower et al. 2015). Adult monarchs also experience nectar resource limitation during their overwintering period, when they primarily drink water and rely on stored lipids (Alonso-Mejía et al. 1997).

Monarchs can be infected by a variety of parasitoids, parasites, and pathogens (Altizer and de Roode 2015; Oberhauser et al. 2015), and resource limitation at both larval and adult stages might lower immunity and parasite resistance. The most widespread and best-studied monarch parasite is the specialist protozoan *Ophyrocystis elektroscirrha* (OE; McLaughlin and Myers 1970). This debilitating parasite can reach high prevalence in some monarch populations (Altizer et al. 2000; Satterfield et al. 2015). The parasite is primarily transmitted vertically from infected adults to larvae when dormant parasite spores scattered on milkweed leaves are consumed by larvae (Altizer et al. 2004). Parasites replicate internally during larval and pupal stages, and infected monarchs emerge as adults covered with millions of dormant parasite spores. OE does not replicate on adults, but the pathogenic effects are experienced at the adult stage in the form of reduced body size, adult life span, and flight performance (Bradley and Altizer 2005; de Roode et al. 2007). Further, adult parasite burdens are likely determined by larval and pupal defenses and could therefore be influenced by larval resources.

Here, we experimentally restricted food at both juvenile and adult stages of captive monarchs to examine the relationships between resources, immune defense, and fitness. Our goals were to ask how food restriction affects different immune components and to test whether effects of food restriction at both juvenile and adult stages are additive or synergistic. We also examined whether food restriction revealed negative relationships (i.e., trade-offs) between immune defense and fitness. We predicted that food restriction within a life stage would reduce immune defenses within that life stage and that larval food restriction would also have consequences for adult immunity. Because resource limitation often increases differential trait allocation, we predicted that trade-offs between immunity and fitness would be primarily evident among resource-limited monarchs. Finally, we experimentally infected a subset of monarchs with OE parasites to determine whether food restriction influenced the outcome of infection in a direction consistent with immune defense responses.

Methods

Host and Parasite Sources

Monarchs used for this experiment were the grandprogeny of adults captured from two overwintering sites of eastern North American migratory monarchs (Sierra Chincua and Cerro Pelón, Michoacán, Mexico, February 2013). We generated five distinct outcrossed family lines. Monarchs were reared in a naturally lit room with ambient light from approximately 0630 to 2030 hours and temperatures from 24°C (average nighttime low) to 29°C (average daytime high) during May–June 2013 in Athens, Georgia. After eclosion, adults were kept in individual glassine envelopes in a 25°C (daytime) to 17°C (nighttime) incubator on a 14L : 10D cycle.

We used two clones of the OE parasite originally derived from wild monarchs in eastern North America (E3, isolated from a monarch collected in July 2008 from Minnesota; E10, isolated from a monarch sampled in October 2001 from New Jersey). Clones were known to express low (E3) and high (E10) virulence from prior experiments (de Roode et al. 2008*b*; de Roode and Altizer

2010). Parasite clones had been propagated in the laboratory for multiple generations before this experiment, including a revival generation completed 6 wk the start of the study.

Larval and Adult Food Restriction Regimes

We used a factorial design to restrict food at the larval stage, adult stage, both stages, or neither stage ($N = 199–201$ monarchs per food treatment). Larvae were reared singly in 0.5-L plastic containers with mesh screen lids and fed daily with fresh cuttings of greenhouse-raised swamp milkweed (*Asclepias incarnata*). Monarch development includes five larval instars, each separated by a molt, with the fourth and fifth instars constituting the stage of the greatest absolute weight gain (Lavoie and Oberhauser 2004). Monarchs in the larva unrestricted treatment group were fed milkweed ad lib. Monarchs in the larva restricted treatment group were deprived of milkweed for 6 h between 0900 and 1600 each day during the fourth and fifth larval instars. This resulted in between 3 and 8 total days (mean ± SEM: 5.2 ± 0.04) of food restriction during larval development, depending on the time to progress from fifth instar to pupation. In the wild, this form of food restriction may occur when larvae consume an entire milkweed plant and must seek additional plants in the same or neighboring milkweed patches. Because monarchs undergo approximately 12-h molting cycles between larval instars, during which time they do not feed (A. Fritzsche McKay, personal observation), we confined the food restriction interval to 6 h so that all monarchs had the opportunity for several hours of foraging in a 24-h period.

As adults, food restriction was implemented by halving the caloric concentration in the diet of experimental subjects compared with controls. Adult unrestricted monarchs were fed with a 20% concentration (1 : 4 honey : water), and adult restricted monarchs were fed with a 10% concentration. Both food-restricted and control adults were fed to satiation every second day until 10 d posteclosion. We standardized the feeding protocol by manually unrolling each adult's proboscis into the honey water solution initially and again after feeding cessation, up to three times before terminating the feeding bout. We note, however, that this protocol did not control precisely for the volume of honey ingested by each monarch; thus, our results must be interpreted in light of the fact that monarchs could have adjusted honey intake rate in response to sugar concentration, as has been shown in other Lepidoptera (Boggs 1988). By feeding all monarchs to satiation but with different nutrient concentrations, we mimicked a naturally occurring source of food restriction. Adult monarchs, prone to dessication, will drink water from dewdrops or other sources in the absence of nectar resources (Masters et al. 1988; Brower 1999), which could dilute the ingested concentration of sugars. Additionally, the abundance of nectar flowers is increasingly patchy in the landscape, and drought conditions can reduce nectar volume and sugar concentration (Carroll et al. 2001; Nabhan 2004).

Parasite Infection

We inoculated half of the monarchs in each food restriction treatment with the protozoan parasite OE. Second-instar larvae were fed a small square of milkweed dosed with 10 parasite spores, following de Roode et al. (2007). Control animals consumed milkweed squares with no spores. We monitored the progression of OE by visually assessing spore development in pupae, following de Roode et al. (2008*b*). No control animals showed signs of infection, and 95.3% of inoculated monarchs were found to be infected as emerging adults. To quantify adult spore load as an index of within-host parasite replication, after an infected monarch died we vortexed the abdomen in 5 mL of distilled water on high speed for 5 min to dislodge spores. A 10-μL aliquot was loaded into a hemocytometer, and spores were counted at × 400 magnification. Average spore counts per 0.1 μL (from five replicate grids) were multiplied by 5×10^4 to estimate the total number of spores per monarch abdomen. Because not all inoculated monarchs became infected, we use the predictor variable infection status in all statistical analyses except the analysis of survival to adulthood, in which case we used inoculation treatment because true infection status was not assessed until adulthood.

Immune Assays

We collected hemolymph to measure immunity at two time points, toward the end of the larval fifth instar and 9 d after adult eclosion. Hemolymph was collected from larvae by clipping a front tubercle at the base and from adults by puncturing the cuticle of an intersegmental vein on the dorsal side of the abdomen. All larvae were weighed to the nearest 0.001 g at the time of hemolymph collection to account for potential relationships between body size and immune measures.

We quantified two immune measures using well-described assays. First, we conducted hemocyte counts to quantify the concentration of immune cells in the blood. Hemocytes have various functions, including phagocytosis, encapsulation, and production of humoral immune effector molecules, such as antimicrobial peptides (Lavine and Strand 2002; Strand 2008). The total concentration of hemocytes at the larval stage has been found to be mildly protective against OE in adult monarchs (S. Altizer, unpublished data). Immediately after collection, 2 μL of hemolymph was rapidly diluted to 1 : 10 in sterile Pringle's saline (1 × in 1 L dD H_20: 9.0 g NaCl, 0.2 g KCl, 0.2 g CaCl, 4.0 g dextrose) and loaded onto Kova glasstic hemocytometer slides. We counted hemocytes under phase contrast microscopy at × 400 in two replicate chambers per sample and calculated the average number of hemocytes per microliter.

Second, we measured the propensity of monarch blood to melanize in response to a bacterial elicitor, through the activity of the enzyme phenoloxidase (PO). PO activity involves the production of melanin pigment, which is deposited onto foreign bodies to suppress growth (Söderhäll and Cerenius 1998). A 6-μL sample of hemolymph was mixed 1 : 1 with ice cold Pringle's saline in an Eppendorf tube. A total of 10 μL of diluted sample was loaded into a well of a 96-well plate with 190 μL of assay buffer (in dD H_20: 50 mM Na_2PO_4 monobasic monohydrate adjusted to 6.5 pH, 2 mM dopamine, and heat-killed *Micrococcus luteus* elicitor at 3% total volume). We mea-

sured absorbance at 490 nm every 24 s at 30°C for 300 measures (total time: 01:59:36), using a Biotek microplate reader. We calculated the slope of the kinetic curve (absorbance per hour) during the linear phase of the reaction to estimate the rate of melanization (Hall et al. 1995; Barnes and Siva-Jothy 2000).

All bled larvae yielded enough hemolymph for both the hemocyte and PO assays, but in many adult monarchs, the volume of hemolymph collected was insufficient for both assays to be implemented. In these cases, we prioritized quantifying hemocyte concentration over PO activity. Of the 195 adults selected for hemolymph sampling, 105 bled a sufficient volume for both immune assays, 77 bled enough for hemocyte counts only, three bled enough for PO activity only, and 10 adults bled an insufficient volume for either immune measure. Finally, because prior work showed that wounding monarchs to draw hemolymph affected OE infection outcome (S. Altizer, unpublished data), we examined the effect of bleeding on infection status and other response variables, using a factorial design. Some monarchs were bled at both of the time points ($N = 86$), some only as larvae ($N = 112$), and some only as adults ($N = 109$), and a subset remained unbled ($N = 94$).

Fitness Metrics

To examine effects of food restriction on monarch development and fitness proxies, we recorded body mass (to the nearest milligram) on day 5 postpupation and on day 10 following adult eclosion. Pupal mass reflects the total resources that each individual amassed during the larval stage and thus reflects the effect of larval food restriction, while adult mass (measured after 10 d of feeding) reflects the cumulative effects of both larval and adult food restriction. We calculated monarch development as either larval development rate (1/days from hatching to pupation) or total development rate (1/days from hatching to adult eclosion). On day 10 after eclosion, half of the adults were placed in a 14°C refrigerator (following de Roode et al. 2007), where they remained unfed and were checked daily for mortality to quantify life span; the remaining adults were frozen. Note that this method of measuring unfed life span means that all adults in the study (in both the restricted and unrestricted adult food treatment groups) experienced a degree of food limitation late in life. During the course of the study, adult females were held in conditions that promoted egg development, although they remained unmated; logistical constraints prevented us from quantifying the number of mature eggs in females.

Data Analyses

We compared survival to adulthood across larval food restriction and OE inoculation treatments, using binary logistic regression. We used two-way ANOVAs to test the main and interactive effects of food restriction and parasite infection status (fixed factors) on continuous fitness variables, including body size (pupal and adult mass), larval and total development rates, and adult life span. A series of focused tests examined the influence of drawing hemolymph on fitness variables. In cases where a fitness measure was sensitive to hemolymph collection, we retained bleed treatment in the final model for the two-way ANOVAs. The only variable determined to be sensitive to larval bleed was pupal mass, and the only variable determined to be sensitive to adult bleed was life span.

To explore how food restriction influenced immunity and parasite infection, we tested effects of larval food restriction on larval immune measures (one-way ANOVA) and of both larval and adult food restriction on adult immune measures (two-way ANOVA). As another measure of susceptibility to disease, we also used a logistic regression to examine the effects of larval food restriction on the outcome of infection (infected or not infected) among larvae that were inoculated with OE. Body mass could be an important predictor of immune defenses and was strongly influenced by food restriction in our experiment (effect of larval food restriction on larval body mass at the time of immune sampling, ANOVA: $F_{1,196} = 37.51$, $P < 0.005$; effect of adult food restriction on adult body mass 1 d after emergence: $F_{1,369} = 48.00$, $P < 0.005$). As such, we incorporated mass into our preliminary analyses in two ways. First, we tested the effects of food treatment on immune measures uncorrected for body size. Next, we reran the models including size as a covariate (ANCOVA) to explore the degree to which the effects of food were mediated by size.

We ran ANOVAs on linear models (anova(lm()) in R) to test whether relationships between immunity and fitness were affected by our design variables. Two larval fitness metrics—development rate and pupal mass—were used as response variables against predictors of larval immunity (either hemocyte concentration or PO activity), larval food treatment, larval mass, sex, and two-way interactions between immune measures and design variables (food treatment and sex; table 1). Because only a subset of the animals sampled for hemocytes could be sampled for PO, we included the two immune measures in separate models to maximize statistical power. Infection status, parasite clone, and monarch family line were included in initial models but were not significant in models of either larval fitness measure and were removed from final reported models. For adult life span, we tested effects of adult immunity (hemocyte concentration or PO activity in separate models), OE infection status, larval food treatment, adult food treatment, adult mass, sex, and select two-way and three-way interactions between immune measures and design variables (table 2). Because OE infection had a pronounced negative effect on adult life span, generating a bimodal distribution in the data, we conducted model diagnostic procedures following Venables and Ripley (year), and we verified that model residuals were normally distributed.

Response variables for all immune measures (larval and adult hemocyte concentrations and PO activity) were $\log_{10}$ transformed to normalize the error variance. Adult life span data distributions were improved ($W = 0.979$, $P = 0.003$) by a power transformation using a λ of 0.3, as determined by Box-Cox procedure in R; model residuals were also approximately normal for development rate (larval: $W = 0.957$, $P < 0.005$; total: $W = 0.975$, $P < 0.005$). Before analyses, we removed five outliers (greater than ± 2 SD from mean) for

Table 1: Response of juvenile fitness measures (development rate and pupal mass) to immune measures, food restriction, and sex

	Larval log(hemocyte concentration)				Larval log(PO activity)			
	F	df	*P*	Direction	*F*	df	*P*	Direction
Larval development rate:								
Immunity covariate	36.84	1	***	+	9.49	1	***	+
Larval food restriction	4.65	1	*	R < U	.00	1		
Larval mass	14.60	1	***	+	6.36	1	*	+
Sex	2.37	1			.40	1		
Immunity covariate × larval food	.48	1			.29	1		
Immunity covariate × sex	3.65	1			2.15	1		
Error		151				98		
Pupal mass:								
Immunity covariate	7.62	1	**	−	.15	1		
Larval food restriction	72.64	1	***	R < U	62.31	1	***	R < U
Larval mass	.18	1			.40	1		
Sex	40.21	1	***	F < M	24.26	1	***	F < M
Immunity covariate × larval food	.00	1			.54	1		
Immunity covariate × sex	2.20	1			2.77	1		
Error		150				97		

Note. The order of explanatory terms reflects the order they appeared in the models; terms were sequentially assessed by type I sum of squares. Plus and minus signs indicate the direction of effects for continuous mass and immunity covariates. R, restricted; U, unrestricted; F, female; M, male; PO, phenoloxidase.

* $0.01 < P < 0.05$.

** $0.005 < P < 0.01$.

*** $P < 0.005$.

pupal mass, one outlier for adult body mass, and one outlier for PO activity that were biologically unrealistic and deemed to be the result of mechanical or observer error. All statistical models used type I (sequential) sum of squares. One-way and two-way ANOVAs were implemented in SPSS (ver. 22.0; 2013), while linear models were implemented in R (R Development Core Team 2015).

Results

Food Restriction and Infection Effects on Survival and Fitness Measures

Larval food restriction did not affect the likelihood of survival to adulthood (94% for restricted [$N = 199$] and 95.5% for unrestricted [$N = 201$]; $\chi^2_1 = 0.81$, $P = 0.37$). Likewise, survival to adulthood did not depend on OE inoculation (94.5% and 95% for control [$N = 201$] and inoculated [$N = 199$] monarchs, respectively; $\chi^2_1 = 0.001$, $P = 0.98$).

Larval and total development rates responded similarly to design variables, so we report results only for larval development rate (1/days from hatch to pupation). Food-restricted larvae developed more slowly than those fed ad lib. (fig. 1*A*; $F_{1,378} = 41.92$, $P < 0.005$), but the rate was unaffected by parasite infection ($F_{1,378} = 0.86$, $P = 0.355$). Development rate varied among monarch lineages ($F_{4,376} = 7.28$, $P = 0.002$), but lineages responded in the same way to food restriction (no interaction between food treatment and lineage; $F_{4,376} = 0.43$, $P = 0.789$). Because pupal mass was lower in monarchs sampled for hemolymph as larvae ($F_{1,379} = 6.34$, $P = 0.012$), the effect of hemolymph sampling was included as a covariate in models investigating the effects of larval food restriction and infection. Pupal mass was significantly lower in food-restricted larvae (fig. 1*B*; $F_{1,371} = 93.56$, $P < 0.005$) but was unaffected by parasite infection ($F_{1,371} = 0.88$, $P = 0.417$) and the interaction between food treatment and infection ($F_{1,371} = 0.48$, $P = 0.487$).

Adult mass was significantly reduced by both larval and adult food restriction (fig. 2*A*; larval restriction: $F_{1,364} = 69.04$, $P < 0.005$; adult restriction: $F_{1,364} = 55.77$, $P < 0.005$), but there was no interactive effect of the two food treatments ($F_{1,364} = 0.21$, $P = 0.644$), indicating that the effect of food restriction at multiple life stages is additive but not synergistic. Additionally, adult mass was lower in infected than uninfected monarchs ($F_{1,364} = 4.26$, $P = 0.040$). Because adult life span was lower in adults sampled for hemolymph ($F_{1,204} = 4.92$, $P = 0.028$), the effect of hemolymph sampling was included in subsequent models for this response variable. Adult life span was significantly shorter in monarchs that were restricted as larvae (fig. 2*B*; $F_{1,195} = 6.76$, $P = 0.01$) and as adults ($F_{1,195} = 93.15$, $P < 0.005$), but there was no significant interaction between the two food restriction treatments ($F_{1,195} = 0.05$, $P = 0.817$). Infected adults had shorter life spans ($F_{1,195} = 174.22$, $P < 0.005$), and we found an interaction between adult food restriction and infection ($F_{1,195} = 10.23$, $P = 0.002$), such that monarchs that were both restricted as adults and infected had shorter life spans than expected by additive effects of these variables alone (fig. 2*B*).

Food Restriction Effects on Immune Defense and Infection

Larval food restriction decreased larval immune defenses. Uncorrected for body size, both PO activity ($F_{1,103} = 5.97$, $P = 0.016$)

Table 2: Response of adult life span to immune measures, food restriction, adult body mass, sex, and several two-way and three-way interactions

	Adult log(hemocyte concentration)			Adult log(PO activity)		
Explanatory variables	*F*	df	*P*	*F*	df	*P*
OE infection status	78.57	1	***	48.05	1	***
Immunity covariate	.65	1		17.10	1	***
Larval food restriction	9.10	1	**	.02	1	
Adult food restriction	73.14	1	***	27.24	1	***
Adult mass	6.66	1	*	2.07	1	
Sex	25.76	1	***	14.96	1	***
OE infection status × immunity covariate	.10	1		9.48	1	**
OE infection status × larval food restriction	.31	1		.70	1	
OE infection status × adult food restriction	4.47	1	*	3.43	1	
OE infection status × sex	1.20	1		3.46	1	
Immunity covariate × larval food	2.68	1		.01	1	
Immunity covariate × adult food	.93	1		1.79	1	
Immunity covariate × sex	3.15	1		.20	1	
Larval food restriction × adult food restriction	.14	1		2.32	1	
OE infection status × immunity covariate × sex	**4.18**	1	*	**4.12**	1	*
OE infection status × immunity covariate × larval food	.22	1		.37	1	
OE infection status × immunity covariate × adult food	.61	1		.52	1	
Error		77			40	

Note. The order of explanatory terms reflects the order they appeared in the models; terms were sequentially assessed by type I sum of squares. OE, *Ophyrocystis elektroscirrha*; PO, phenoloxidase.

* $0.01 < P < 0.05$.

** $0.005 < P < 0.01$.

*** $P < 0.005$.

and hemocyte concentration ($F_{1,160} = 4.35, P = 0.039$) were lower in larvae fed restricted diets (fig. 3*A*). When larval mass was included as a covariate in the models of immune measures as a function of larval food restriction, the effect of the food restriction disappeared (hemocyte concentration: $F_{1,155} = 0.39$, $P = 0.534$; PO activity: $F_{1,101} = 1.47, P = 0.299$), suggesting that the effect of larval food restriction on immunity was mediated by changes in body size.

Adult PO activity was reduced by both larval and adult food restriction (fig. 3; larval food: $F_{1,110} = 5.03, P = 0.027$; adult food: $F_{1,110} = 7.25, P = 0.008$) but not by the interaction between the two treatments ($F_{1,110} = 1.39, P = 0.241$). Adult PO activity increased with adult body mass ($r^2 = 0.05, P = 0.013$), and when we included adult mass as a covariate in the above model, the effect of both larval and adult food restriction disappeared (larval food: $F_{1,105} = 0.17$, $P = 0.682$; adult food: $F_{1,110} = 0.06, P = 0.808$). Adult hemocyte concentration was unaffected by food restriction at either stage or the interaction between the two restriction regimes (fig. 3; larval food: $F_{1,186} = 0.32, P = 0.574$; adult food: $F_{1,186} = 0.27, P = 0.61$; interaction: $F_{1,186} = 0.01, P = 0.934$).

There was no effect of food restriction on infection probability by the OE parasite among inoculated monarchs ($\chi^2_1 = 0.08, P = 0.78$). Among a subset of infected monarchs, the final spore load differed by OE clone ($F_{1,175} = 13.14, P < 0.005$) and was higher for clone E10 (known to be more virulent on the basis of prior work) than clone E3. In the initial model of OE spore load (as a function of parasite clone and larval food restriction), spore load was higher in monarchs fed ad lib. as larvae ($F_{1,175} = 4.37, P = 0.038$); however, when pupal mass was included as a covariate in this model, the effect of larval food restriction disappeared, suggesting that OE growth is limited by host resources amassed as larvae. Finally, we found a weak but significant negative relationship between larval hemocyte concentration and OE spore load (fig. A1).

Across all monarchs, larval hemocyte concentration was positively correlated with larval PO activity ($r^2 = 0.15, P < 0.005$). The two immune measures were not correlated in adults ($r^2 = 0.01, P = 0.19$), and neither larval hemocytes nor larval PO activity predicted levels of hemocytes or PO activity in adults (hemocyte concentration: $r^2 = -0.001, P = 0.35$; PO activity: $r^2 = -0.03, P = 0.94$).

Food Restriction Effects on Immunity-Fitness Relationships

Larvae that developed faster to the pupal stage had greater larval hemocyte concentration and greater larval PO activity, counter to our expectations (table 1). Larval development rate was reduced by larval food restriction but was not affected by sex or the two-way interaction between any pair of variables (table 1). Food-restricted and female monarchs formed smaller pupae

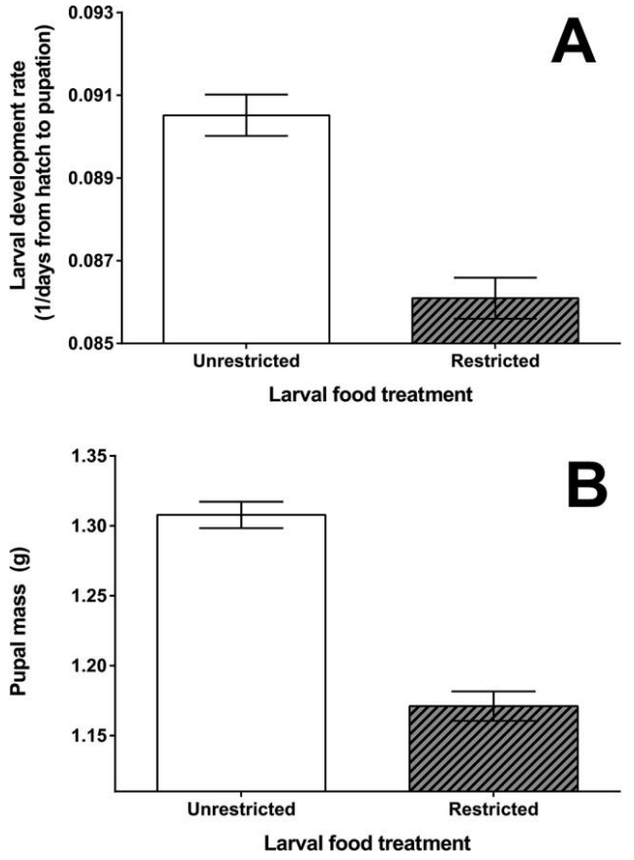

Figure 1. Larval food restriction reduces two measures of juvenile fitness: larval development rate (1/days from hatch to pupation; *A*) and pupal mass (g; *B*). Infection by a protozoan parasite did not influence these two variables. Data show means ± 1 SE.

than unrestricted and male monarchs; across all monarchs, having higher hemocyte concentration was associated with forming smaller pupae (table 1). Pupal mass was not associated with PO activity or interactions between PO activity and design variables.

Our models of adult life span showed significant three-way interactions between OE infection status, sex, and each measure of immunity, which we interpret in lieu of significant two-way interactive or main effects including these terms (table 2). When monarchs were not infected by OE, life span increased with hemocyte concentration in both sexes (fig. 1*A*); when monarchs were infected by OE, life span decreased with hemocyte concentration among female monarchs only (fig. 1*B*). In other words, infected females with higher adult hemocyte concentrations died more quickly, whereas there was no effect of hemocyte concentration on life span in infected males. Adult life span was also affected by a three-way interaction between OE infection status, sex, and PO activity. In males, life span increased with PO activity equally as strongly in OE-infected and uninfected monarchs; in females, life span increased with PO activity more strongly in OE-infected than in uninfected monarchs (fig. 4*C*, 4*D*).

Discussion

Our study showed that food restriction lowered fitness measures and components of immunity on short timescales and that food restriction early in life affected a subset of traits at later life stages. Food restriction in larval monarchs lowered both measures of immunity and reduced larval growth. Larval food restriction also lowered adult immunity and fitness (mass and life span), and these effects were additive—not interactive—with adult food restriction. Adult food restriction reduced adult mass, life span, and phenoloxidase activity but not hemocyte concentration. We also found evidence for trade-offs between immune defense and fitness measures for a subset of monarchs. Across all monarchs, there was a negative relationship between larval hemocytes and pupal mass, and in parasite-infected females, there was a negative relationship between adult hemocytes and life span. These immune

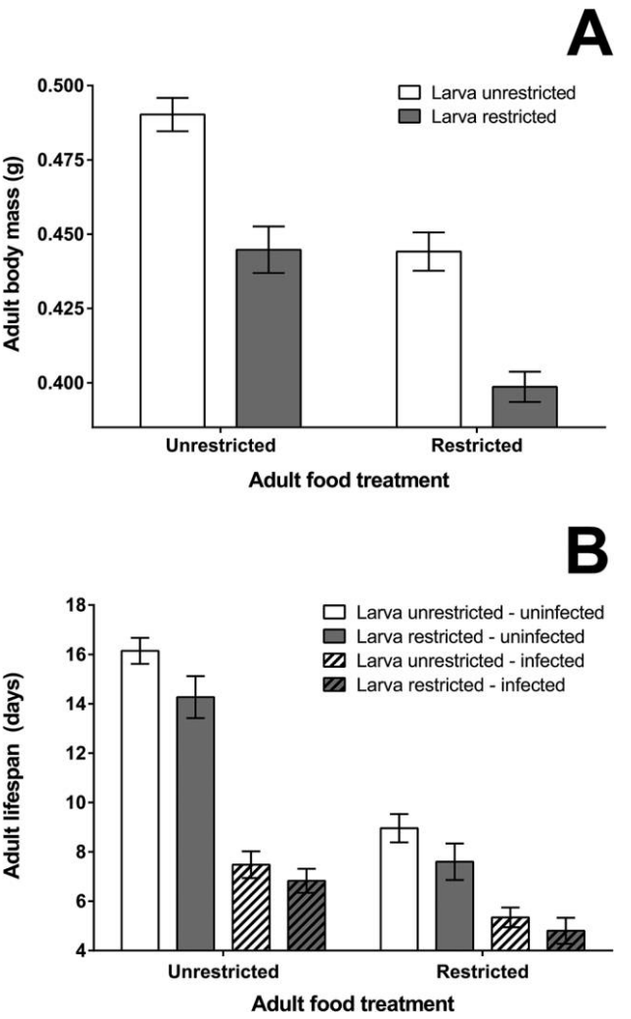

Figure 2. Larval and adult food restriction reduces two measures of adult fitness: body mass (g; *A*) and adult life span (d; *B*). Adult life span and mass were significantly reduced by food restriction at both life stages. Life span was also significantly reduced by protozoan parasite infection and the interaction between adult food restriction and parasite infection. Data show means ± 1 SE.

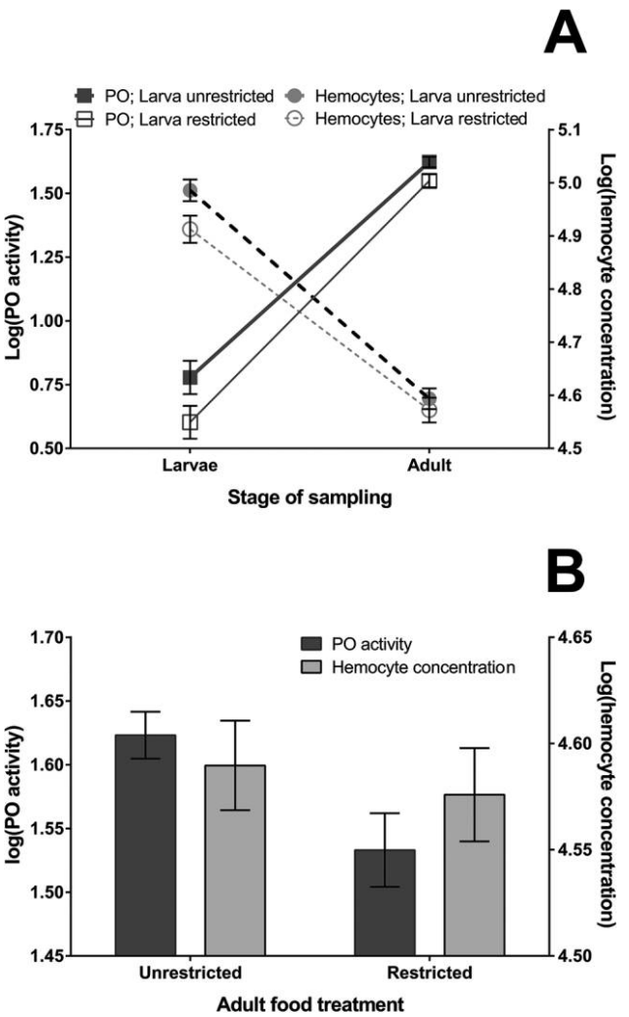

Figure 3. *A*, Effects of larval food restriction on larval and adult immune measures. Monarchs unrestricted as larvae had higher average phenoloxidase (PO) activity (red lines) than food-restricted larvae when assayed at both the larval and the adult stages. Hemocyte concentration (blue lines) was higher in unrestricted larvae when measured at the larval stage, but there was no difference in adult hemocyte concentration. *B*, Effects of adult food restriction on adult immune measures. PO activity—but not hemocyte concentration—was significantly lower in food-restricted relative to unrestricted adult monarchs. Data show means ± 1 SE. A color version of this figure is available online.

trade-offs occurred only with hemocyte concentration, because phenoloxidase activity was actually associated with longer adult life span, especially in infected females.

Individuals acquiring fewer resources might sacrifice immune defense in favor of other fitness traits, and abundant resources might eliminate such trade-offs and lead to higher investment in immunity. Although substantial past work has shown that food limitation can reveal trade-offs (Moret and Schmid-Hempel 2000; Alonso-Alvarez and Tella 2001; French et al. 2007*b*, Boots 2011; Simmons 2012; Kelly et al. 2014), most experiments explore resource-dependent trade-offs only within the same life stage in which the food restriction occurred. Our study also asked how food restriction at both juvenile and adult stages affect immune traits and fitness both within the life stage of food restriction as well as later in life. These ontogenetic effects are particularly important in holometabolous insects, whose acquisition and allocation of resources as larvae constrain the resources available as adults (Boggs 2009). We found that larval food restriction influenced adult immune and fitness measures and had some limited consequences for the relationships between immune and fitness traits.

Like food restriction, parasite infection could reveal trade-offs between traits by depleting the hosts' available resources. In this study, we found a negative relationship between adult life span and adult hemocyte concentration, which was only significant in female monarchs infected with the OE parasite. Monarchs with high concentrations of hemocytes as larvae had lower OE spore loads as adults (fig. A1), suggesting that investing more in larval hemocytes suppresses parasite growth. Because the OE parasite replicates internally before the host's adult life stage, defenses earlier in life are more likely to limit parasite development. Although average larval and adult hemocyte concentrations did not differ between parasitized and unparasitized monarchs, OE infection lowered adult body size and life span substantially, suggesting that the parasite depleted host resources or damaged hosts in a way that limited their survival. We also see evidence that having higher phenoloxidase activity as an adult corresponded to increased longevity, an effect that was stronger in infected than in uninfected monarchs. This result suggests a protective quality of phenoloxidase activity against OE pathology, yet previous work has shown that larval phenoloxidase does not protect OE during its critical establishment period during the hosts' larval stage (S. Altizer, unpublished data).

The trade-off we observed between adult hemocyte concentration and adult life span was most evident in infected female monarchs. Sex differences in immunity—with males typically displaying reduced immune function relative to females—are well documented in vertebrates (Nunn et al. 2009) but remain less well characterized in invertebrates. In particular, the direction of sex biases in immunity is inconsistent in invertebrate studies: in some cases, females have been found to have higher phenoloxidase activity than males, especially at reproductively mature stages (Adamo et al. 2001), but in other cases females were more poorly defended against pathogens than males (Rantala and Roff 2007; Stoehr 2007). In monarchs, past work showed that females tend to have higher hemocyte concentrations than males in the absence of OE infection but fewer than males in the presence of infection (Lindsey and Altizer 2009). Further, there is little evidence and theory predicting why the sexes may differ in the relationships between immunity and life-history traits and the response of these traits to food stress (Rolff 2002). Females—whose fitness is linked to lifetime egg production—should invest more strongly in immunity to survive pathogen damage for a longer time but might sacrifice immunity under food limitation because resources limit egg production (McKean and Nunney 2005; Kelly and Jennions 2009; Kelly 2011). An experiment by McKean and

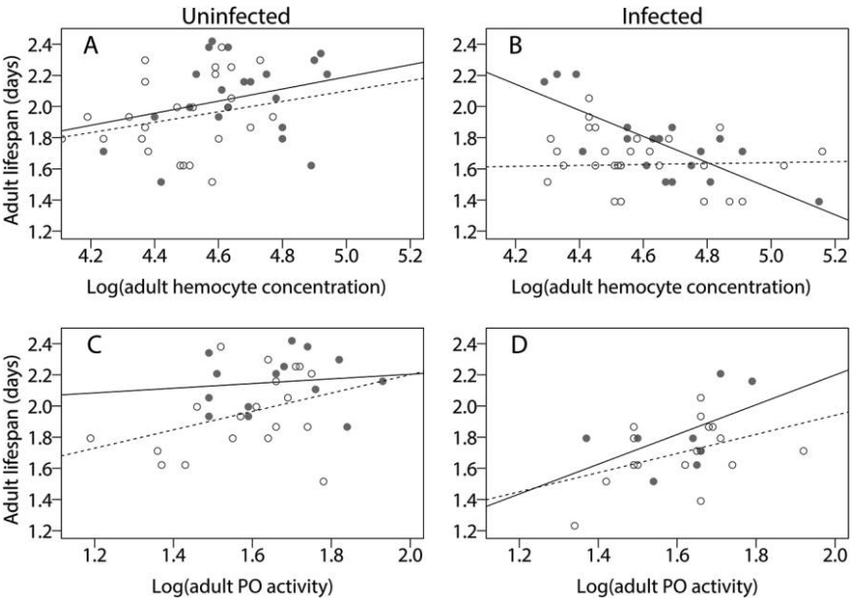

Figure 4. Relationships between adult life span and immune measures differ by *Ophyrocystis elektroscirrha* (OE) infection status and sex. Hemocyte concentration (*A*, *B*) and phenoloxidase (PO) activity (*C*, *D*) in uninfected (*A*, *C*) and infected (*B*, *D*) monarchs. Life span decreased with hemocyte concentration in uninfected monarchs (*A*) but increased with hemocyte concentration in infected female monarchs (*B*). Life span generally increased with PO activity, especially in infected monarchs (*D*). Filled circles and solid lines, females; open circles and dashed lines, males. A color version of this figure is available online.

Nunney (2005) with *Drosophila* showed that females—but not males—exhibited immunosuppression under food-limited conditions, and males experienced immunosuppression only under high mating demand. Experiments similar to ours by Karl et al. (2011) in tropical butterflies and by Kelly and Tawes (2013) in field crickets have shown that relationships between food restriction and immune defenses depended on sex. For example, female tropical butterflies showed a greater reduction in phenoloxidase activity (but not hemocyte concentration) than males under food stress (Karl et al. 2011). The differences between males and females in the severity of immune trade-offs under food restriction may derive from difference in the costs of reproductive tissue (Kelly 2011). In our study, both females and males remained unmated virgins, but females did most likely develop mature eggs. This egg development could represent another outlet for resource allocation faced by females but not males. Our study provides further evidence that the cost of immunity for fitness traits is context dependent, with food availability, reproductive activity, and parasitism each potentially driving sex differences in the optimal investment in immunity.

This study explored immune defenses over time and across life stages, with repeated measures for individuals. There are relatively few examples of the ontogeny of insect immune profiles (but see Doums et al. 2002; Schmid et al. 2008; Wilson-Rich et al. 2008; Laughton et al. 2011; Urbański et al. 2014), and our experiment further explores the degree to which lepidopteran insects shift their investment in different defenses across life stages, and the extent to which these changes depend on resources. We found that investment in two immune defenses changed across monarch ontogeny, with larvae investing more strongly in immune cell (hemocyte) production and adults investing more strongly in melanization (PO activity). Further, the two immune measures responded differently to food restriction at both life stages. Larval food restriction lowered both hemocyte concentration and PO activity at the larval stage, and the effects of larval food restriction on PO activity persisted when immunity was sampled at the adult stage. Adult food restriction reduced only adult phenoloxidase activity but not hemocyte concentration. Karl et al. (2011), working with tropical butterflies, also found that PO activity but not hemocyte concentration was reduced by larval food restriction, but they found that both measures were reduced by adult food restriction. The similarity in results suggests a degree of generality in the responses of these invertebrate immune measures to stress and life-history changes. Although in our study hemocytes were relatively insensitive to food restriction, the trade-offs we observed with fitness traits (both at larval and adult stages) involved hemocytes, not PO activity. On one hand, this could be driven by our lower sample size for PO activity and poorer statistical power to detect effects. On the other hand, hemocytes and PO activity represent different components of defense that might respond differently to resource limitation.

Although lab experiments cannot perfectly replicate natural conditions, our selected modes of food restriction paralleled natural sources of resource limitation in wild monarchs. Monarch larval densities in the wild are typically low, but during

phases of high larval density (peak summer breeding season and winter breeding in mild climates), larvae can deplete entire plants or patches of milkweed. In such cases, larvae have been observed wandering on the ground in search of new milkweed plants (D. A. Satterfield, A. Fritzsche McKay, and S. Altizer, personal observation). Milkweed densities vary tremendously across habitat types, with the lowest density now in herbicide-tolerant corn and soy monoculture plots, in the areas that once represented a crucial component of the breeding range (Hartzler and Buhler 2000; Oberhauser et al. 2001; Pleasants and Oberhauser 2012). In our study, we also reduced the caloric content of food provided to adults (mimicking reduced nectar availability while ensuring adequate hydration) and found significant negative effects on monarch mass, life span, and the phenoloxidase immune defense. Throughout their migration, monarchs forage on nectar and convert these resources to stored lipids, which fuel the butterflies through the remainder of their migration and overwintering period (Alonso-Mejía et al. 1997; Brower et al. 2006). After extensive studies of the nectaring behavior of overwintering monarch butterflies in Mexico, Brower (1999) found that in some years monarchs forage from flowers nearly devoid of nectar and may actually expend more energy and lose more water than they gain by foraging. Of growing importance under climate change, drought stress can reduce flower nectar volume and sugar concentration (Halpern et al. 2010; Brower et al. 2015), reducing the nutritional benefit to foraging butterflies during their migration. While the potential drivers of monarch decline are numerous (Brower et al. 2012; Pleasants and Oberhauser 2012; Flockhart et al. 2015), our work supports other suggestions that conserving food resources is of prime importance for monarchs and identifies potential physiological and fitness consequences of resource loss.

Natural environments are not benign but stressful, and predictions about individual and population health should be made assuming such limits to fitness and defense against natural enemies. In this study, we showed that food restriction at multiple life stages affects monarch fitness and immunity and that both food stress and parasite infection can reveal trade-offs whereby organisms sacrifice immune defense in favor of other fitness traits. Resource limitation is an escalating concern for monarchs because milkweed and nectar flowers are diminishing in availability across a human-dominated landscape. Understanding to what extent these migratory insects suffer reduced performance as larvae and adults because of resource limitation is important to both the basic field of ecoimmunology and the long-term persistence of this iconic butterfly.

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APPENDIX

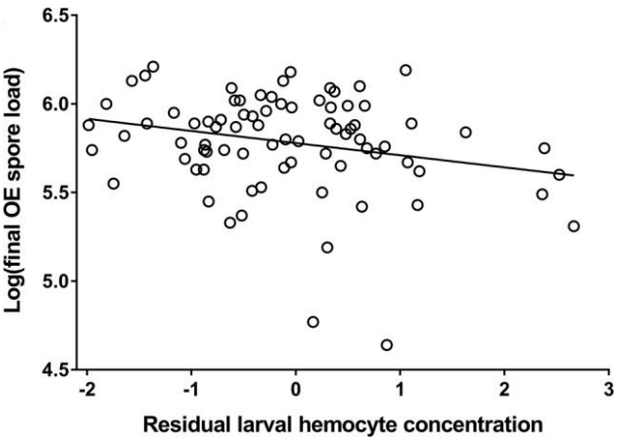

Figure A1. Relationship between residual larval hemocyte concentration and the outcome of *Ophyrocystis elektroscirrha* parasitism (log of the final spore load). This weak but significant relationship ($r^2 = 0.051$, $P = 0.029$) suggests some protective value of investing in hemocytes early in life in terms of minimizing parasite growth later in life.

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