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Wajd Alaidrous April 7, 2020

Effects of crowding on disease resistance in a butterfly host

by

Wajd Alaidrous

Jacobus C. de Roode
Adviser

Department of Biology

Jacobus C. de Roode

Adviser

Levi Morran

Committee Member

Hojin Kim

Committee Member

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Wajd Alaidrous

Jacobus C. de Roode

Adviser

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Abstract

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Host density is an important factor when it comes to parasite transmission and host resistance. Studies have shown that increased host density can increase contact rate between individuals and, thus, parasite transmission. However, host density can also cause physiological changes in the host, which can affect host resistance. Yet, the direction in which host density affects host resistance remains unresolved. It is also unclear whether food limitation plays a role in this effect. We investigated the effect of larval density of monarch butterflies, *Danaus plexippus*, on the resistance to their natural protozoan parasite *Ophryocystis elektroscirrha* under both unlimited and limited food conditions. We exposed monarchs to various density treatments as larvae to mimic high densities observed in sedentary populations. Data on infection probability and parasite spore load were collected as well as development time, survival, and wing morphology. Results showed that higher larval densities had minimal effects on development time and adult lifespan. Food limitation caused slightly stronger effects of density on development time, survival, and wing size and color. However, these effects were small in size, and most likely not attributed to increased infection as crowding did not show increased disease susceptibility under either food conditions. This study helps in understanding the dynamics of environmental parasite transmission in monarch populations, which can help explain the increased prevalence of parasites in sedentary monarch populations compared to migratory populations.

Key words: host-parasite interaction, host population density, larval density, environmental transmission, density-dependent transmission.

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Effects of crowding on disease resistance in a butterfly host

Wajd Alaidrous, Ania Majewska, Jacobus C. de Roode

Department of Biology, Emory University, 1510 Clifton Road, Atlanta, GA 30322, USA

Introduction

Infectious diseases pose a threat to the fitness of all organisms. The spread of parasites in a host population can be influenced by both host and parasite traits, such as host resistance and parasite growth rate and virulence (Lambrechts et al., 2006). Host-parasite interactions are also affected by environmental and ecological factors, such as seasonality (Altizer et al., 2006), resource availability (Pulkkinen and Ebert, 2004), predation (Navarro et al., 2004), and host density (Anderson and May, 1979). Host density, in particular, has long been recognized as a major driver of disease spread, by altering parasite transmission or host susceptibility. Higher host density can increase parasites transmission by increasing host contact rates (Arneberg et al., 1998), or reduce transmission by diluting infectious parasite stages (Buck and Lutterschmidt, 2017). Increasing host density could also negatively affect host susceptibility to parasitism due to food limitation or increased physiological stress (Steinhaus, 1958)– or in fact increase host resistance if hosts respond to crowding by boosting their immunity (Michel et al., 2016; Wilson and Reeson, 1998).

The effects of host density on parasite transmission are well recognized. For example, for environmentally transmitted parasites, increased host density could result in greater accumulation of infectious parasite stages in the environment (Altizer et al., 2003). For directly transmitted parasites, a commonly used model of parasite transmission in a population is density-dependent transmission,

where contact rate between individuals increases with increasing population density (Lloyd-Smith et al., 2005; McCallum et al., 2001). In contrast, when parasite transmission is dependent on the frequency of infected hosts, such as with sexually transmitted diseases (Ryder et al., 2007), increases in host density do not necessarily result in greater disease spread (Lloyd-Smith et al., 2005). However, studies have suggested that parasites in a population are more likely to be subject to density-dependent rather than frequency-dependent transmission (Ryder et al., 2005).

In contrast with the effects of host density on parasite transmission, the direction of the effect of host density on host resistance remains less clear (Michel et al., 2016). Some studies have shown that some hosts can physiologically decrease their susceptibility with increased population density following the density- dependent prophylaxis hypothesis (Michel et al., 2016). A study on sea star, *A. planci*, for example, showed that hosts phenotypically increase their immune response with increasing population density (Mills, 2012). Similarly, studies on insects including Cabbage moths (Goulson and Cory, 1995) and African worms (Reeson et al., 1998) showed that larvae reared at higher densities had higher resistance to parasites, as measured by levels of melanization. In contrast, other studies have shown that crowding increases intra-specific competition and, in turn, physiological stress (Steinhaus, 1958), supporting the crowding stress hypothesis. Intra-specific competition can also increase resource stress and induce aggression between food-limited hosts, as shown in monarch butterfly caterpillars (Collie et al., 2020). These studies suggest that higher host densities can increase host susceptibility due to increased stress (Michel et al., 2016). In fact, a study on grass carp showed that long-term crowding caused reduced immunity in hosts, increasing their susceptibility to parasites (Lin et al., 2018). Yet other studies have shown no effects of crowding on host resistance. For example, a study on crickets showed no effect on immunity with increasing host density (Adamo and Parsons, 2006).

There are many factors that can affect host susceptibility. Genetic variation in host resistance (Lefevre et al., 2011) and parasite virulence (Restif and Koella, 2003) can play a major role. Environmental factors can also impose stress that can affect host susceptibility. These factors include food limitation (McKay et al., 2016), seasonal variation (Dowell, 2001), intra-specific competition and aggression (Collie et al., 2020), and predation (Navarro et al., 2004). Crowding has been shown as a possible stress-inducing factor (Lin et al., 2018; Steinhaus, 1958). Thus, it is expected to affect host susceptibility.

Monarch butterflies, *Danaus plexippus*, and their parasites, *Ophryocystis elektroscirrha* (McLaughlin and Myers, 1970), provide an excellent system to study the effect of crowding on parasite transmission and parasite resistance. *O. elektroscirrha* is a natural parasite that infects monarchs across their range. Infection with *O. elektroscirrha* starts with the ingestion of an infectious spore by a monarch caterpillar. Spores then break open in the monarch mid-gut to release sporozoites that traverse the mid-gut wall to infect hypodermal tissues, where they replicate asexually and sexually during the larval and pupal stages. Adult butterflies then eclose covered in dormant parasite spores. Parasite spores are transferred to eggs and milkweeds (the monarch's host plant) by females during egg laying, and by male butterflies that visit milkweeds. Parasites can also be transferred from males to females during mating, after which females can transfer them to eggs and milkweed (Altizer et al., 2005; Vickerman et al., 1999). Parasite growth is detrimental to monarchs, reducing survival to adulthood, and adult fecundity, flight ability and lifespan (Bradley and Altizer, 2005; De Roode et al., 2007; De Roode et al., 2009)

Monarchs are known for their seasonal long-distance migration from eastern North America to overwintering sites in Mexico to escape freezing temperatures and deteriorating habitats (Brower, 1995; Reppert and de Roode, 2018; Urquhart and Urquhart, 1978). This migration reduces parasite prevalence (Bartel et al., 2011). Migration allows healthy monarchs to escape highly infectious areas (a process

known as migratory escape) as well as remove heavily infected monarchs from the population (a process known as migratory culling) (Altizer et al., 2011). However, recent decades have seen the formation of sedentary populations of monarchs along the Gulf of Mexico and the Atlantic Coast, (Brower et al., 2012; Satterfield et al., 2015), increasing infection risk in these populations. Previous studies showed that infection by natural parasite *O. elektroscirrha* is more prevalent in sedentary monarch populations than migratory monarch populations (Satterfield et al., 2015; Satterfield et al., 2018; Satterfield et al., 2016). This is most likely due to the fact that sedentary populations sustain higher host densities that breed all year-round and, thus, experience higher parasite transmission (Altizer et al., 2005). Loss of migration in these populations decreases escape from infectious areas compared to migrant populations. Simultaneously, the increasing host densities may also have detrimental effects on susceptibility. These non-migratory populations are characterized by very high densities of larvae per milkweed, often resulting in severe food limitation and crowding on milkweed stems. Thus, host density becomes important when exploring the infection dynamics of sedentary, non-migratory, monarchs compared to migratory monarchs.

The objective of this study is to determine the effect of larval density on host susceptibility to parasites in monarch butterflies (*Danaus plexippus*) in both food-unlimited and food-limited environments. Using the monarch's natural parasite *O. elektroscirrha*, we tested the effect of larval density on parasite infection and infection severity, and also analyzed the effects of crowding on survival, development time and size as proxies for monarch physiological condition. Since larvae in higher densities are more likely to experience increased levels of physiological stress, we hypothesized that higher larval density would increase susceptibility to parasites, affecting other developmental and morphological aspects of the monarchs as well.

Methods

Caterpillar source and rearing

We carried out two experiments to determine the effect of host density on disease resistance as well as monarch development and survival. Our approach used microcosms, which consisted of live potted plants in 4.5-inch pots, contained within transparent plastic tubes (4 inch diameter x 24 inch height), and capped with netting. These microcosms were used to mimic natural conditions as closely as possible, with larvae experiencing crowding on live plants with minimal interference related to animal husbandry. All the larvae and plants used in this study were reared in a greenhouse. Lab-reared monarchs, the offspring of wild-caught butterflies collected from St. Marks, Florida, mated in 0·6 m3 mesh cages. Mating pairs were moved to a separate cage. After they dissociated, the male was removed, and a tropical milkweed plant (*Asclepias curassavica*) was provided for female oviposition. This plant species was chosen specifically because it is the main species that monarchs in sedentary populations encounter (Satterfield et al., 2015; Satterfield et al., 2018; Satterfield et al., 2016). Experimental larvae were randomly picked from different lineages. They were reared on *A.curassavica* plants and randomly exposed to different larval densities (Figure 1).

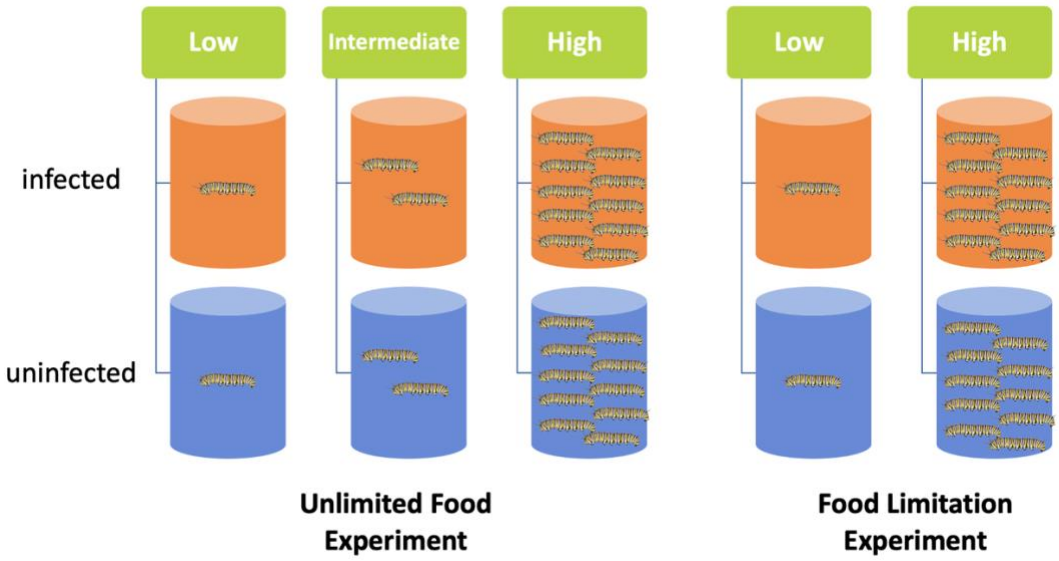

Figure 1 Experimental Method

Unlimited Food Experiment (Summer 2018). In the first experiment, we asked whether density of caterpillars per plant has an effect on survival, development, disease resistance, and size of monarchs. Two-day old larvae were reared either in low density (density=1 caterpillar/tube), intermediate density (density=2 caterpillars/tube), or high density (density=10 caterpillars/tube) on *Asclepias curassavica* enclosed in plastic tubes (Figure 1A). Because this experiment was aimed at determining the effect of density in the absence of food limitation, we provided larvae with new plants when necessary to ensure sustained food *ad libitum*. The low density treatment contained 25 infected and 25 uninfected caterpillars for a total of 50 tubes and 50 caterpillars. The intermediate density treatment contained 15 tubes of infected and 15 tubes of uninfected caterpillars with two caterpillars each for a total of 30 tubes and 60 caterpillars. The high density treatment contained 6 tubes of infected and 6 tubes of uninfected caterpillars with 10 caterpillars each for a total of 12 tubes and 120 caterpillars. Caterpillars in the infected treatment were inoculated with *O. elektroscirrha* parasites: individual caterpillars were

fed a 0.5cm^2 leaf disk of *A.curassavica* with a manually deposited dose of 10 parasites (ID E42-2) in a Petri dish. Uninfected caterpillars, which served as controls, received a leaf disk without parasite spores. Upon complete consumption of their leaf disk, caterpillars were transferred to their assigned tubes with plants. After pupation, pupa were transferred to separate 16oz Solo cups and were glued to lids using hot glue. A few hours after emergence from pupa, adult monarchs were transferred to separate glassine envelopes and held in a DigiTherm® incubator at 12°C.

Food Limitation Experiment (Fall 2020). In the second experiment, we asked how density of monarchs per plant coupled with food limitation impacts survival, development, disease resistance, and size of monarchs. The low density treatment (density=1 caterpillar/tube) contained 25 infected and 25 uninfected caterpillars for a total of 50 tubes and 50 caterpillars. The high density treatment (density=10 caterpillars/tube) contained 6 tubes of infected and 6 tubes of uninfected caterpillars with 10 caterpillars each for a total of 12 tubes and 120 caterpillars. Because the first experiment revealed minimal effect of intermediate density, and because of Covid-19-imposed research restrictions, this second experiment did not include the 2-caterpillar treatments used in the first experiment. Caterpillars in the infected treatment were inoculated with *O. elektroscirrha* parasites from (ID E42(P43)) and uninfected controls were fed parasite-free leaf disks as described the first experiment. Upon completion of their leaf disks, caterpillars were transferred to their assigned tubes.

To control for food availability, a new plant was provided once all leaves in a microcosm were consumed, which only occurred for caterpillars in the high density treatment. In contrast with the first experiment, we specifically investigated the impacts of food limitation on disease resistance. We separated larvae in the high-density treatment to into 16oz Solo cups with *A.curassavica* plant stems only on their third of fourth day of the fifth instar. This ensured high density-induced food limitation while preventing cannibalism. Stems are often consumed by monarch caterpillars once supply of leaves

is depleted and provide enough nutrition to complete the instar stage and form a pupa, while still ensuring food limitation. Stripping of leaves and subsequent feeding on stems is common in nature where larvae occur in crowded conditions. Similar to the first experiment, pupa were glued to 16oz Solo cups lids. Adult monarchs were also kept at 12°C in glassine envelopes in an incubator.

Survival

Several instances of premature mortality were observed in both experiments (Table 1). Most cases occurred at the pupal stage marked by an inability to emerge from the pupa properly either due to accidental physical damage or unknown physiological factors. One instance occurred at the larval stage in the Unlimited Food Experiment. A couple of instances of mortality at the larval stage occurred in the Food Limitation Experiment. In one these instances, the larva was unable to pupate properly and died while pupating. We determined the proportion of monarchs that survived from hatching to adult eclosion.

Development time

We recorded monarch larval and pupal development time. Larval development time was quantified as the number of days from egg hatching to pupation. Pupal development time was quantified as the number of days from pupation to adult eclosion from the pupa. We also calculated total development time as the sum of larval and pupal development time.

Adult lifespan, parasite load, and tolerance

To measure adult lifespan, we calculated the number of days between adult eclosion and death, measured for butterflies held in glassine envelope in the incubator without access to food, as routinely done in this experimental system (De Roode et al., 2007). The lifespans obtained in this way closely

mimic the lifespans of monarchs under more natural conditions (De Roode et al., 2009). Upon adult death, we quantified the parasite spore load of adult butterflies in the infected treatments following De Roode et al. 2007. The abdomen was removed and vortexed at maximum speed on a Vortex Genie II in 5mL of tap water for 5 minutes. Next, we counted the number of spores present in 10 µL of the 5mL suspension using a hemocytometer by averaging four chambers per sample. Parasite spore loads were $\log_{10}$-transformed for all analyses. We also used these data to quantify the proportion of infected adults in each treatment (infection probability).

Parasite spore loads provide a measure of monarch resistance, with greater spore loads indicating lower resistance (higher susceptibility). We also estimated infection tolerance by examining the relationship between adult lifespan and the square root of the spore load. Steeper reductions in adult lifespan with increasing spore load indicates lower tolerance (Lefevre et al., 2011) .

Wing size and hue

To estimate adult size, we scanned monarchs' wings with a Cannon Lide 210 scanner. Specifically, the right forewing and hindwing from each monarch were scanned on their dorsal and ventral sides. Adults with damaged and broken wings were excluded. The scans were then analyzed with ImageJ to calculate wing area. Wing area measurements for only the dorsal side were used to avoid redundancy. Wing hue and aspect ratio of length to width were also analyzed, which are two factors associated with flight performance (Davis et al., 2012). Wing hue is a measurement of wing color, where lower values indicates a color that is closer to red and higher values indicates a color that is closer to yellow. Aspect ratio is a proportion of length to width. Finally, we used wing area to obtain a size-corrected measure of parasite spore load. This was important because both parasite growth and monarch size could be affected by crowding. We did this by using the residuals of the interactions between the spore load and wing area in a general linear mixed effects model.

Statistical Analysis

Statistical analysis was performed using R 4.0.3 and R studio (version 1.4.110). The tests performed included linear mixed effects models. First, we used linear mixed effects models to test for differences in development time, adult lifespan, and spore load, with density treatment as the explanatory variable. Then, we asked if survival and infection varied with density treatment. We used binomial generalized linear mixed effects models (GLM) to compare proportions of individuals that survived to adulthood across the density treatments. Binomial GLM was also used to test for differences between the density treatments in the proportions of adults that became infected within the infection treatment. Density and infection were included as fixed effects in the analyses of development time, adult lifespan, and proportion survival, while only density was included as a fixed effect in the analyses of spore load and infection probability. Sex was also included as a fixed effect for development time. In all linear mixed effects models, the tube that the larvae were reared in was included as a random effect.

Linear mixed effects models were also used to examine the differences in tolerance between density treatments. Tolerance was measured as the interaction between adult lifespan and spore load. The square root of $\log_{10}$ spore load, density, and their interaction were included as explanatory factors in the analysis of adult lifespan.

Finally, we asked whether wing morphology varies with density treatment. Linear mixed effects models were used to compare wing area, hue and aspect ratio across the density treatments. The forewing and hindwing were analyzed separately. Only the dorsal side was analyzed for wing area, while both the ventral and dorsal sides were included in wing hue analysis, and they were analyzed separately. Density and infection were included as fixed effects. Sex was also included as a fixed effect for wing hue analysis. The tube that the larvae were reared in was included as a random effect. We also asked whether spore load is correlated with size. Linear mixed effects models were used to analyze the

interaction between $\log_{10}$ spore load and wing area to obtain residuals and calculate a size-corrected spore load measure.

Table1 Number of monarchs used in the experiment and survival to adult and infection probability

Food unlimited experiment

Infection		Density			Total
		Low	Intermediate	High	
Uninfected					
	Initial number	25	30	59	114
	Adult	25	28	53	106
	% Survival to adult	100%	93%	90%	93%
	% Infected	0	0	0	0
Infected					
	Intitial number	25	30	60	115
	Adult	22	29	58	109
	% Survival to adult	88%	97%	97%	95%
	% Infected	91%	93%	98%	95%

Food limitation experiment

Infection		Density		Total
		Low	High	
Uninfected				
	Intitial number	25	58	83
	Adult	22	49	71
	% Survival to adult	88%	84%	86%
	% Infection	0	0	0
Infected				
	Intitial number	25	59	84
	Adult	22	45	67
	% Survival to adult	88%	76%	80%
	% Infection	73%	73%	73%

Results

Unlimited Food Experiment

Survival. In the uninfected treatment, 93% monarchs survived to adulthood (Low: 100%, Intermediate: 93%, High:90%; Table 1), while in the infected treatment, survival to adulthood was 95% (Low: 88%, Intermediate: 97%, High:97%). There was no significant difference in survival probability among density treatments (Figure 2A; Table 2).

Development time. Increasing host density caused shorter larval development time (Figure 2B; Table 2) but larval development time was not affected by infection. As for pupal development time, there were no significant differences between density and infection treatments (Figure 2C; Table 2). Monarchs in the high-density treatment took a slightly but significantly shorter time to develop from egg to adult (Figure 2D; Table 2). However, infection had no effect on total development time (p=0.5). This matches previous studies that showed that parasite infection had no strong effect on development time (De Roode et al., 2007). Furthermore, males had longer larval, pupal, and development times (Table 2).

Table 2 Results of linear models investigating the effect of larval density on survival and development time in the Unlimited Food Experiment. Tube was included as a random effect in all linear models.

Response Variable	**Fixed Effect**	**Estimate (SE)**	**z value**	**P-value**
Survival probability	Density: Intermediate	0.20 (0.86)	0.233	0.816
	Density: High	-0.23 (0.72)	-0.318	0.750
	Infection: Infected	0.36 (0.65)	0.548	0.584
Response Variable	**Fixed Effect**	**Estimate (SE)**	**t value**	**P-value**
Larval Development Time	**Density: Intermediate**	**-0.23 (0.11)**	**-2.191**	**0.030 ****
	Density: High	**-0.33 (0.10)**	**-3.298**	**0.002 ****
	Infection: Infected	0.15 (0.08)	1.863	0.071 .
	Sex: Male	**0.15 (0.07)**	**2.074**	**0.039 ***
Pupal Development Time	Density: Intermediate	-0.01 (0.08)	-0.111	0.912
	Density: High	-0.13 (0.08)	-1.748	0.093 .
	Infection: Infected	-0.04 (0.06)	-0.736	0.475
	Sex: Male	**0.53 (0.06)**	**9.101**	**<2e-16 *****
Total Development Time	Density: Intermediate	-0.24 (0.13)	-1.819	0.070 .
	Density: High	**-0.47 (0.12)**	**-3.855**	**<0.001 *****
	Infection: Infected	0.10 (0.10)	1.016	0.317
	Sex: Male	**0.69 (0.09)**	**7.441**	**<0.001 *****

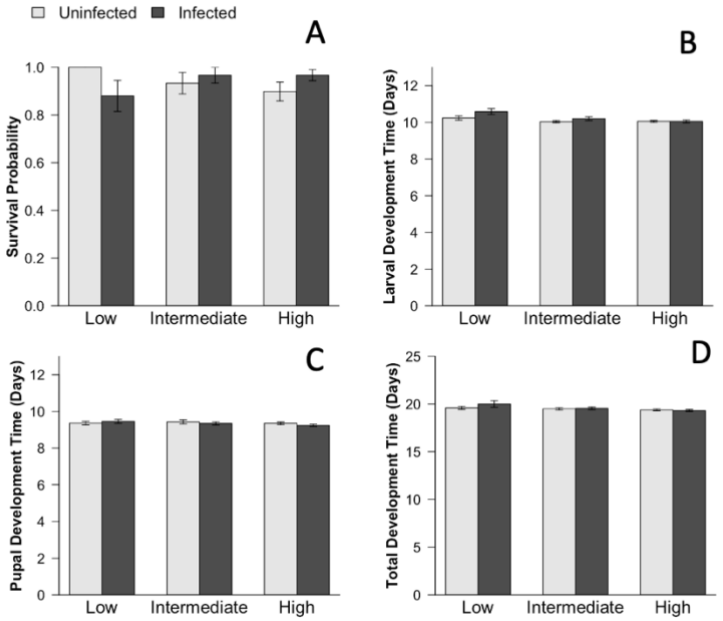

Figure 2 Effects of density and infection on survival probability (A), larval (B), pupal (C), and total (D) development times in the Unlimited Food Experiment. Bars represent means and error bars represent standard errors of the mean.

Wing morphology. Wing data collected included forewing and hindwing area, ventral and dorsal hue, and aspect ratio. Results showed that crowding had no effect on wing area when food was unlimited (Figure 3A-B; Table 3). However, males had slightly larger hindwings than females (Table 3). Males also had higher hue on dorsal forewing and hindwing, but lower hue on the ventral sides compared to females (Table 3). There was no significant difference between the different treatments in the forewing hue (Figure 3C-D; Table 3). In the hindwing, however, the intermediate density treatment had slightly significantly higher hue on the dorsal hindwing (Figure 3E; Table 3). Also, infection slightly decreased hue on the ventral hindwing (Figure 3F; Table). There was also no significant difference in the aspect ratio between density and infection treatments (Figure 3G; Table 3).

Table 3 Results of linear models investigating the effect of larval density on wing area , hue, and aspect ratio in the Unlimited Food Experiment. Tube was included as a random effect in all linear models.

Response Variable	Fixed Effect	Estimate (SE)	t value	P-value
Wing Area				
Forewing Area	Density: Intermediate	-0.09 (0.12)	-0.785	0.434
	Density: High	-0.04 (0.12)	-0.333	0.741
	Infection: Infected	-0.07 (0.10)	-0.718	0.476
	Sex: Male	0.10 (0.08)	1.287	0.200
Hindwing Area	Density: Intermediate	-0.15 (0.13)	-1.148	0.253
	Density: High	-0.07 (0.13)	-0.560	0.578
	Infection: Infected	-0.20 (0.10)	-1.953	0.055 .
	Sex: Male	**0.17 (0.08)**	**2.100**	**0.037 ***
Wing Hue				
Dorsal Forewing Hue	Density: Intermediate	0.29 (0.38)	0.769	0.582
	Density: High	0.15 (0.33)	0.467	0.491
	Infection: Infected	0.50 (0.26)	1.913	0.356
	Sex: Male	**2.10 (0.26)**	**11.482**	**<0.001 *** **
Ventral Forewing Hue	Density: Intermediate	0.14 (0.15)	0.910	0.368
	Density: High	-0.02 (0.14)	-0.134	0.803
	Infection: Infected	-0.14 (0.12)	-1.253	0.287
	Sex: Male	**-0.38 (0.10)**	**-3.664**	**<0.001 *** **
Dorsal Hindwing Hue	**Density: Intermediate**	**0.44 (0.17)**	**2.528**	**0.014 ***
	Density: High	0.16 (0.16)	1.007	0.289
	Infection: Infected	0.18 (0.13)	1.429	0.204
	Sex: Male	**0.28 (0.12)**	**2.377**	**0.018 ***
Ventral Hindwing Hue	Density: Intermediate	-0.26 (0.22)	-1.169	0.275
	Density: High	-0.19 (0.20)	-0.984	0.277
	Infection: Infected	**-0.85 (0.16)**	**-5.364**	**<0.001 *** **
	Sex: Male	**-0.69 (0.15)**	**-4.492**	**<0.001 *** **
Forewing Aspect Ratio				
	Density: Intermediate	4.62e-03 (1.41e-02)	0.328	0.743
	Density: High	1.83e-04 (1.24e-02)	0.015	0.988
	Infection: Infected	1.68e-02 (9.89e-03)	1.695	0.101

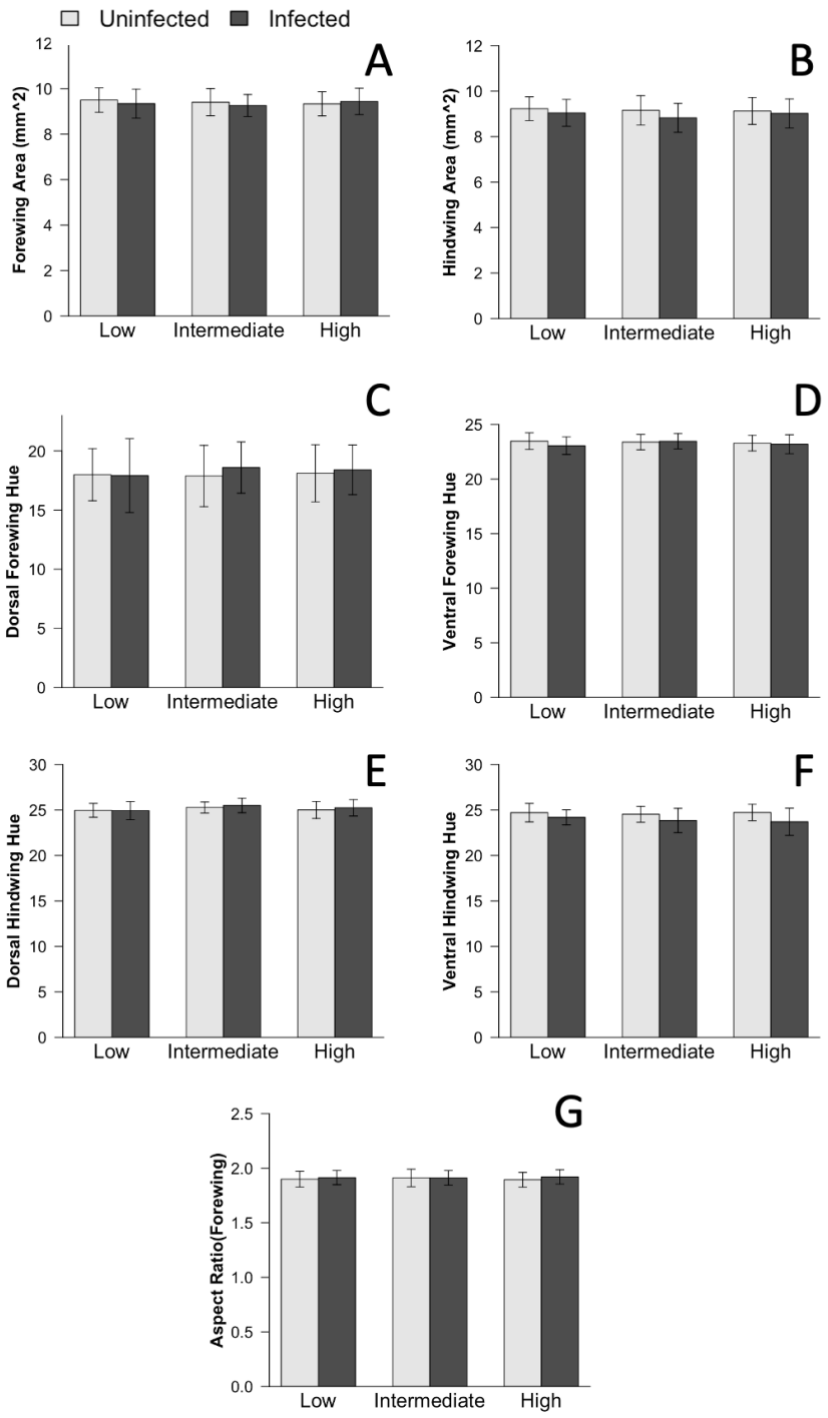

Figure 3 Effect of density and infection on wing area (A-B) , forewing hue (C-D), hindwing hue (E-F), and aspect ratio (G) in the Unlimited Food Experiment. Bars represent means and error bars represent standard errors of mean.

Infection, spore load, adult lifespan, and tolerance. To quantify host resistance to parasite, we looked at the infection probability. We found that 95% of the adults in the infected treatment became infected (Low: 91%, Intermediate: 93%, High: 98%; Table 1). Infection probability did not differ across the density treatments (Figure 4A; Table 4). We then analyzed the spore load counts for the infected adults. Results did not show any differences in the spore load across the different density treatments (Figure 4B; Table 4). Looking at adult lifespan, we found that intermediate density treatment lived shorter as adults (Figure 4C; Table 4). Infected monarchs also lived shorter. Finally, we looked at the tolerance to parasite. Adult life span was strongly affected by spore load, but not density (Figure 5; Table 4). There was no significant interaction between spore load and density, suggesting that there was no variation in tolerance across the different densities.

Table 4 Results of linear models investigating the effect of larval density on infection, adult lifespan, spore load, and tolerance in the Unlimited Food Experiment. Tube was included as a random effect in all linear models.

Response Variable	**Fixed Effect**	**Estimate (SE)**	**z value**	**P-value**
Infection probability	Density: Intermediate	0.30 (1.04)	0.288	0.773
	Density: High	1.74 (1.25)	1.39	0.164
Response Variable	**Fixed Effect**	**Estimate (SE)**	**t value**	**P-value**
Adult Lifespan	**Density: Intermediate**	**-1.92 (0.83)**	**-2.324**	**0.021***
	Density: High	1.44 (0.80)	1.811	0.078 .
	Infection: Infected	**-10.50 (0.65)**	**-16.173**	**<2e-16 *** **
Spore load	Density: Intermediate	0.10 (0.13)	0.774	0.441
	Density: High	-0.02 (0.12)	-0.155	0.878
Tolerance	**Spore load**	**-24.20 (11.52)**	**-2.100**	**0.0383 ***
	Density: Intermediate	-21.50 (34.50)	-0.623	0.5346
	Density: High	-25.63 (29.42)	-0.871	0.3858
	Sporeload: DensityIntermediate	8.03 (14.45)	0.556	0.5797
	Sporeload: DensityHigh	10.91 (12.36	0.883	0.3795

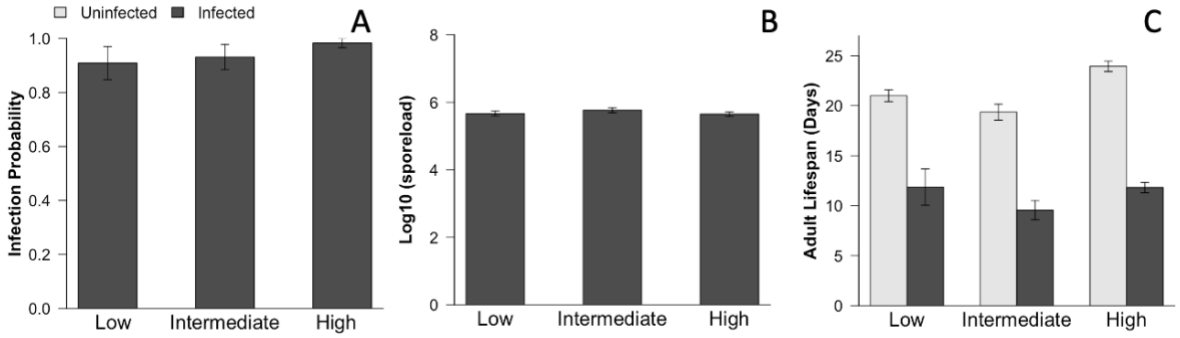

Figure 4 Effect of density and infection on infection probability (A) , Log_{10} Sporeload (B), and adult lifespan (C) in the Unlimited Food Experiment. Bars represent means and error bars represent standard errors of mean.

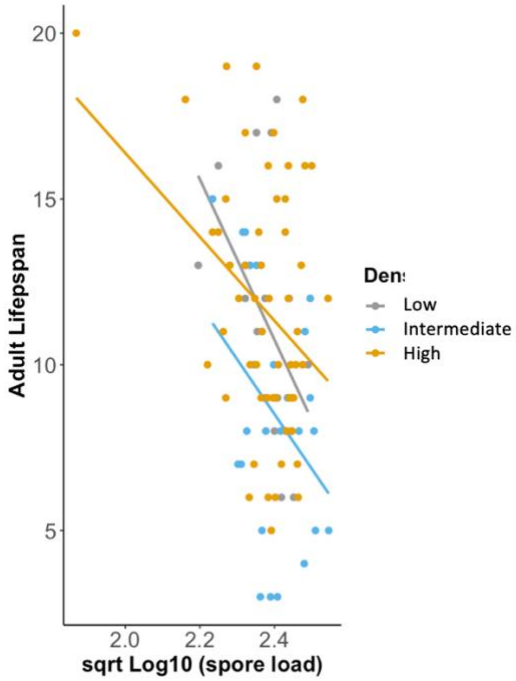

Figure 5 Effect of density and infection on tolerance. Lines represent regression lines. No significant different in tolerance was found between the density treatments.

Food Limitation Experiment

Survival. When food was limited, 86% monarchs survived to adulthood in the uninfected treatment (Low: 88%, High:84%; Table 1), while survival decreased to 80% in the infected treatment (Low: 88%, High:76%). However, these survival probabilities of infected and uninfected monarchs were not statistically different. There was no significant difference in survival probability across the different density treatments (Figure 6A; Table 5). Higher mortality was observed in the Food Limitation Experiment compared to the Unlimited Food Experiment (Table 1). In the high density treatment, this could be a result of increased physiological stress due to the imposed lack of resources.

Development time. Results showed that larvae in the high density treatment developed significantly more slowly than those in the low density treatment (Figure 6B; Table 5). Consequently, monarchs in the high-density treatment took more days to develop from egg to adult than those in the low-density treatment (Figure 6D; Table 5). However, there was no significant difference between the pupal development times across the density and infection treatments (Figure 6C; Table 5). Males also had longer larval, pupal, and total development times (Table 5).

Table 5 Results of linear models investigating the effect of larval density on survival and development time in the Food Limitation Experiment. Tube was included as a random effect in all linear models.

Response Variable	**Fixed Effect**	**Estimate (SE)**	**z value**	**P-value**
Survival proportion	Density: High	-0.61 (0.54)	-1.128	0.259
	Infection: Infected	-0.37 (0.49)	-0.755	0.450
Response Variable	**Fixed Effect**	**Estimate (SE)**	**t value**	**P-value**
Larval Dvelopment Time	**Density: High**	**1.11 (0.39)**	**3.204**	**0.006 ****
	Infection: Infected	-0.33 (0.39)	-0.847	0.402
	Sex: Male	**1.03 (0.33)**	**3.113**	**0.002 ****
Pupal Development Time	Density: High	0.02 (0.12)	0.019	0.985
	Infection: Infection	-0.05 (0.12)	-0.391	0.699
	Sex: Male	**0.53 (0.11)**	**4.892**	**<0.001 *****
Total Development Time	**Density: High**	**1.12 (0.46)**	**2.412**	**0.019 ***
	Infection: Infected	-0.36 (0.48)	-0.789	0.434
	Sex: Male	**1.58 (0.39)**	**4.038**	**<0.001 *****

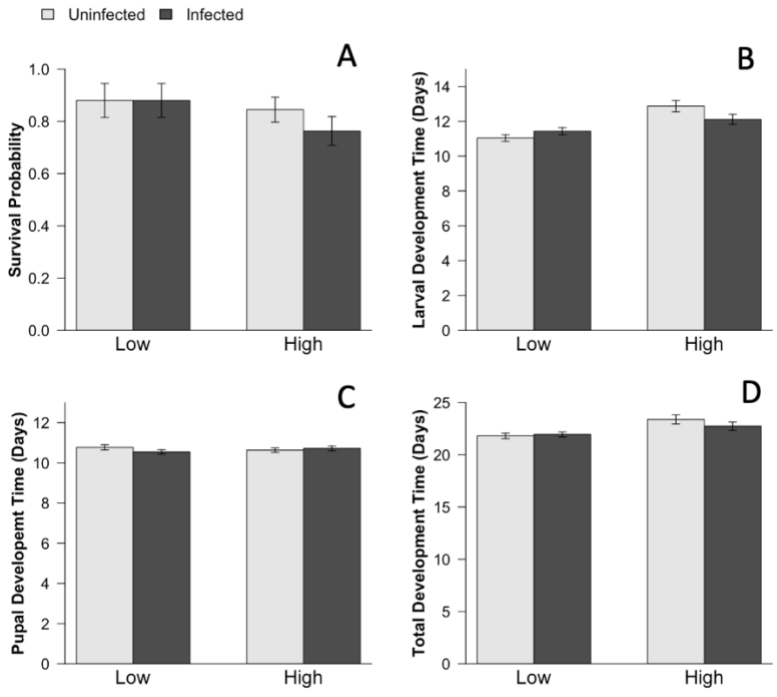

Figure 6 Effects of density and infection on survival probability (A), larval (B), pupal (C), and total (D) development times in the Food Limitation Experiment. Bars represent means and error bars represent standard errors of the mean.

Wing morphology. Results revealed that crowding decreased areas of both forewing and hindwing when food was limited (Figure 7A-B, Table 6). This is consistent with previous studies that showed that starvation leads to wing size reduction in monarchs (Johnson et al., 2014). Males had slightly larger wing area than females (Table 6). Males also had higher hue on the dorsal forewing and hindwing but a decreased hue on the ventral hindwing compared to females (Table 6). Furthermore, infection decreased forewing hue on dorsal and ventral sides (Figure 7C-D; Table 6). Crowding also decreased hue in the ventral forewing. As for the hindwing, both the dorsal and ventral sides also showed decreased hue when infected (Figure 7E-F, Table 6). Crowding also increased hue on the dorsal side, but decreased hue on ventral side. There was no significant difference in the aspect ratios across the density treatments (Figure 7G; Table 6).

Table 6: Results of linear models investigating the effect of larval density on wing area, hue, and aspect ratio in a the Food Limitation Experiment. Tube was included as a random effect in all linear models.

Response Variable	Fixed Effect	Estimate (SE)	t value	P-value
Wing Area				
Forewing Area	**Density: High**	**-2.02 (0.16)**	**-12.359**	**<2e-16 *****
	Infection: Infected	2.03e-4 (0.15)	0.001	0.999
	Sex: Male	0.27 (0.15)	1.801	0.074 .
Hindwing Area	**Density: High**	**-2.10 (0.17)**	-12.548	**<2e-16 *****
	Infection: Infected	-0.06 (0.16)	-0.396	0.694
	Sex: Male	**0.33 (0.15)**	**2.148**	**0.034 ***
Wing Hue				
Dorsal Forewing Hue	Density: High	0.14 (0.46)	0.308	0.759317
	Infection: Infected	**-1.99 (0.44)**	**-4.522**	**<0.001 *****
	Sex:Male	**4.99 (0.42)**	**11.797**	**<0.001 *****
Ventral Forewing Hue	**Density: High**	**-0.52 (0.24)**	**-2.193**	**0.032 ***
	Infection: Infected	**-1.34 (0.23)**	**-5.887**	**<0.001 *****
	Sex:Male	0.38 (0.21)	1.793	0.075 .
Dorsal Hindwing Hue	**Density: High**	**0.50 (0.22)**	**2.333**	**0.023 ***
	Infection: Infected	**-0.73 (0.20)**	**-3.603**	**0.001 ****
	Sex:Male	**1.41 (0.20)**	**7.031**	**<0.001 *****
Ventral Hindwing Hue	**Density: High**	**-0.64 (0.21)**	**-3.088**	**0.003 ****
	Infection: Infected	**-0.66 (0.20)**	**-3.319**	**0.002 ****
	Sex:Male	**-1.40 (0.19)**	**-7.521**	**<0.001 *****
Forewing Aspect Ratio				
	Density: High	0.002 (0.014)	0.164	0.870
	Infection: Infected	0.01 (0.01)	1.182	0.239

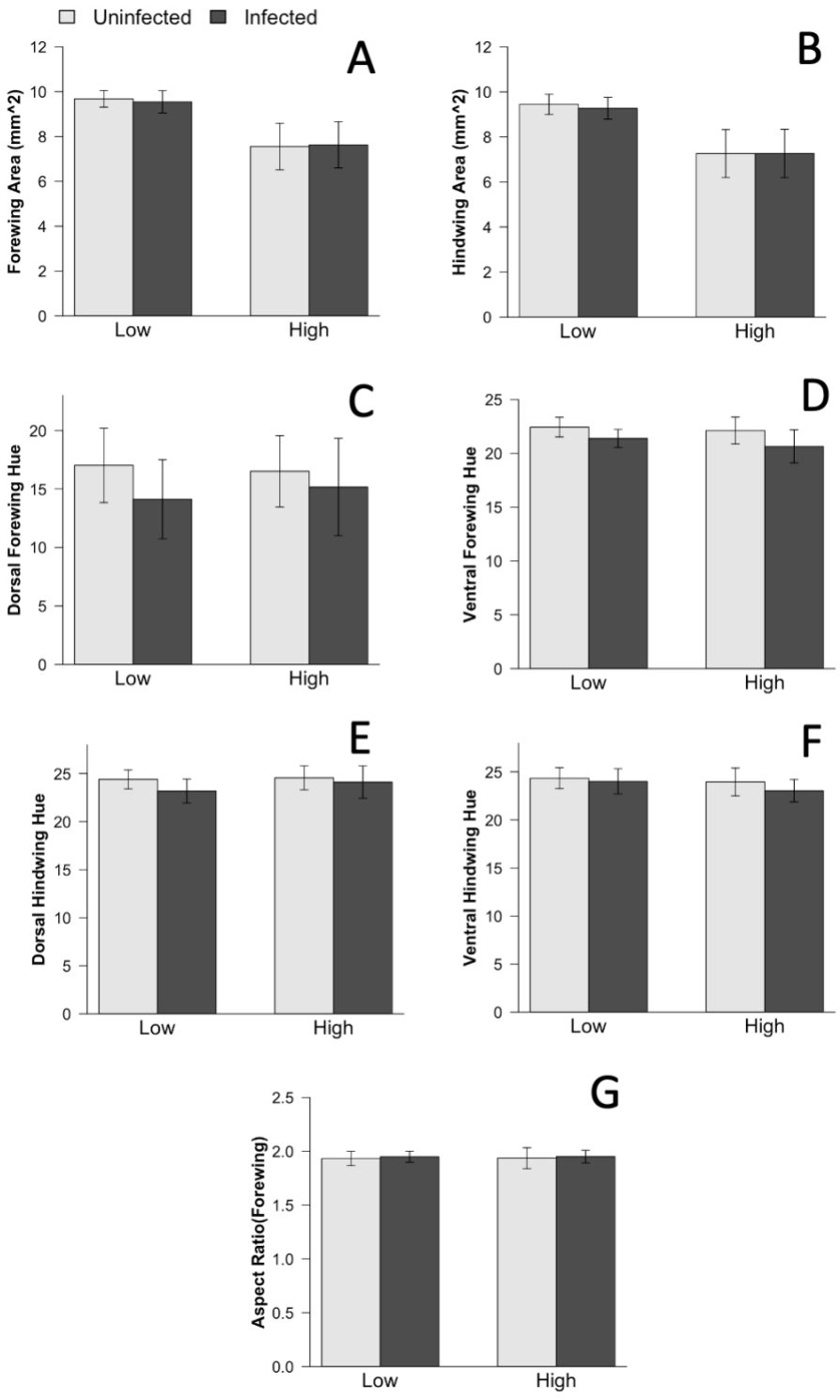

Figure 7 Effect of density and infection on wing area (A-B) , forewing hue (C-D), hindwing hue (E-), and aspect ratio (G) in the Food Limitation Experiment. Bars represent means and error bars represent standard errors of mean.

Infection, spore load, adult lifespan, and tolerance. As for infection probability, 73% of the adults in the infected treatment became infected (Low: 73%, High: 73%; Table 1). Infection probability did not differ across the density treatments (Figure 8A; Table 7). Infection probabilities were lower in the food limitation experiment compared to the unlimited food experiment. One possible explanation for decreased probability in the high density treatment is that parasites also depend on food resource. In fact, host starvation has been shown to affect parasite fitness and spread and can lead to a decline in parasite populations (Pulkkinen and Ebert, 2004). Other explanations are differences in infectivity of the parasites used (De Roode and Altizer, 2010), inherent differences in the genetic resistance of the monarchs used (De Roode and Altizer, 2010; Lefevre et al., 2011), or differences in the anti-parasitic properties of the plant individuals used in both experiments (Sternberg et al., 2012).

Table 7: Results of linear models investigating the effect of larval density on development time, survival, infection, and tolerance in the Food Limitation Experiment. Tube was included as a random effect in all linear models.

Response Variable	**Fixed Effect**	**Estimate (SE)**	**z value**	**P-value**
Infection proportion	Density: High	0.04 (0.66)	0.064	0.949
Response Variable	**Fixed Effect**	**Estimate (SE)**	**t value**	**P-value**
Adult Lifespan	**Density: High**	**-2.49 (1.07)**	**-2.328**	**0.021 ***
	Infection: Infected	**-4.10 (0.99)**	**-4.108**	**<0.001*** **
Spore load	**Density: High**	**-0.22 (0.10)**	**-2.158**	**0.046 ***
Spore load corrected for wing size	Density: High	-0.05 (0.10)	-0.504	0.623
Tolerance	Spore load	-23.94 (25.65)	-0.933	0.356
	Density: High	-39.99 (70.20)	-0.570	0.572
	Spore load: Density	17.91 (30.39)	0.589	0.559

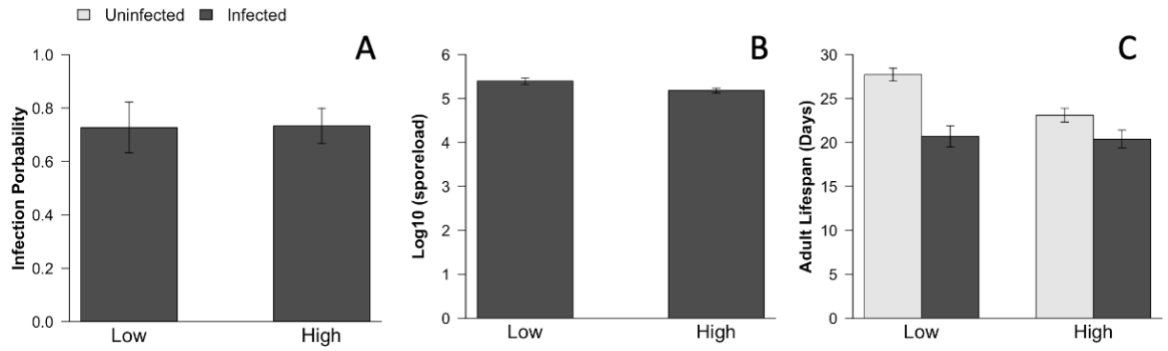

Figure 8 Effect of density and infection on infection probability (A), Log_{10} Sporeload (B), and adult lifespan (C) in the Food Limitation Experiment. Bars represent means and error bars represent standard errors of mean.

Infected monarchs in the high-density treatment had a higher spore load (Figure 8B; Table 7). However, this did not translate to longer adult lifespan. Since these monarchs were smaller, there is a possibility that they had less physical space for parasite spores, which could explain the decreased spore load in the high-density treatment. Thus, it was important to correct the spore load for wing size. Analyzing the relationship between residuals and spore load to account for wing area, we no longer observed a significant difference in spore load across the density treatments (Figure 9; Table 7). Adult lifespan results showed that crowding resulted in a shorter adult lifespan (Figure 8C; Table 7). Infection also caused a shorter adult life span. Lastly, neither spore load nor density had a strong effect on adult life span. There was also no significant interaction between spore load and density, suggesting that there was no variation in tolerance across the different densities (Figure 10; Table 7).

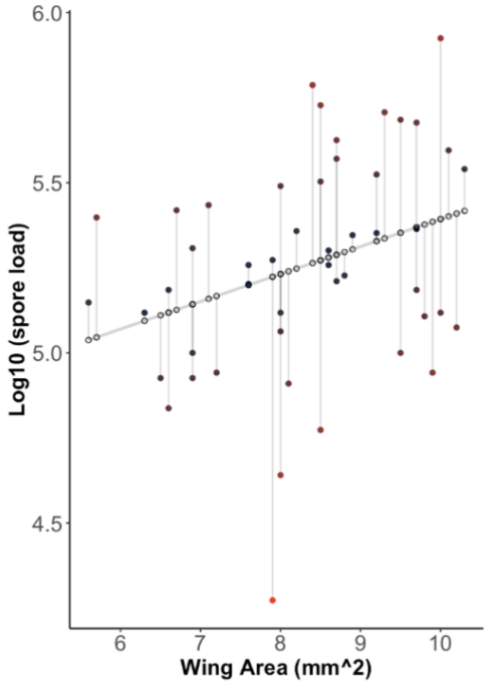

Figure 9 Effect of density and infection on spore load corrected for wing area. Line represent regression line. Colored points represent residuals. Redder dots represent larger residuals.

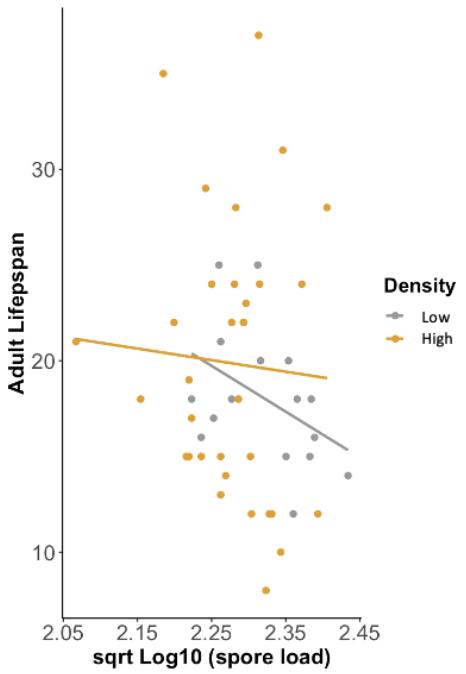

Figure 10 Effect of density and infection on tolerance. Lines represent regression lines. No significant different in tolerance was found between the density treatments.

Discussion

Host density plays an important role in host-parasite interactions. Higher host densities can increase host contact rates, increasing parasite transmission rate through density-dependent transmission (McCallum et al., 2001). Greater host density can also increase dissemination of parasites to the environment, and thereby increase transmission rates (Arneberg et al., 1998). However, host density can also affect host resistance, but the direction of this effect is still unclear. While some studies support the density-dependent prophylaxis hypothesis, where high density causes a greater investment in increasing resistance, other studies support the crowding stress hypothesis, where environmental stress

makes hosts more susceptible to diseases (Michel et al., 2016). In this study, we examined the effect of crowding at larval stages on disease resistance in monarch butterflies.

Our results showed that when food was unlimited, crowding had some effect on development, such that crowding decreased larval and, in turn, total development time. However, the effect was small, and we also found no effects on wing area or hue. Moreover, there was also no meaningful effects on survival, infection probability, spore load, adult lifespan or tolerance.

Food is rarely unlimited in nature, and crowding is likely to increase intra-specific competition and, in turn, physiological and resource stress. Thus, it was important to test the effect of food limitation and increased competition to determine how crowding affects host resistance against parasites. Not surprisingly, when food became limited, crowded monarchs developed more slowly into adults, and experienced shorter adult lifespans. Crowding also caused a reduction in monarch size and ventral wing hue. While crowding under these food limitation conditions did not increase the probability of infection, monarchs in the high-density treatment had a lower spore load than those in the low-density treatment. However, this was a direct consequence of these starved monarchs being smaller: when accounting for wing size, spore load analysis showed no significant difference between density treatments. Thus, the lower spore load in the high treatment could be attributed to their smaller sizes.

Our experiments show that the effects of density on size, development, and survival were more pronounced when the food source was limited. This could possibly be due to increased physiological stress caused by the competition. However, these effects were most likely not due to increased infection, since density levels had no effect on infection probability or spore load. Thus, these results collectively provide conclusive evidence that crowding has no effect on host susceptibility to parasites given a uniform parasite dose. These results are in contrast with one previous study on the effects of crowding on infection in monarchs, which suggested that crowding caused increased infection

probability (Lindsey et al., 2009). However, that experiment differed in significant ways from the experiments described here. First, caterpillars were raised on horizontal cuttings of *Asclepias incarnata* rather than erect live plants of *A. curassavica*. The quick deterioration of milkweed shoots, combined with the buildup of frass on those shoots necessitated much more monarch handling and stress of the high-density caterpillars than induced in the experiments described here. Second, that study experienced additional stressors, including an unidentified viral or bacterial disease that killed a majority of monarchs in that experiment, and may have confounded the results.

In another study on the effects of larval rearing density and food stress, larvae in higher densities were larger than those in lower densities when given unlimited food, suggesting that crowding stimulates larvae to increase their feeding rate (Atterholt and Solensky, 2010). However, higher density (n=5) had no effect on development. In our study, the higher density treatment had a higher density (n=10), which suggests that extremely higher levels of crowding has a stronger effect on development time. In the Atterholt and Solensky study, there was also no effect of starvation on monarch size, development time, or wing coloration in low densities. However, they imposed food stress by removing larvae from their food source at certain intervals and this method might not have been effective at imposing food stress. Furthermore, when it comes to the effect of density on survival, some studies showed that survival to adulthood decreased with increasing egg per plant density (Nail et al., 2015). This suggests that crowding at very high densities can have more pronounced effects on survival in nature.

The fact that crowding does not increase monarch susceptibility to infection does not mean that higher density will not result in greater disease pressure in natural monarch populations. However, we expect the effects of crowding to affect parasite transmission instead. Density-dependent transmission models suggest that higher densities can lead to increased parasite prevalence in organisms due to

increased contact rates (McCallum et al., 2001; Rader et al., 2020). Experimentally, studies on the insect Indian meal moth showed that parasites are subject to density-dependent transmission, where parasite transmission increased with increasing larval density (Knell et al., 1996). Moreover, higher densities can result in the greater buildup of infectious parasite stages in the environment, and thereby result in greater infection rates as well (Arneberg et al., 1998). Both of these factors are highly relevant to monarch butterflies, many of which are foregoing migration to form sedentary populations to breed year-round (Satterfield et al., 2015; Satterfield et al., 2018; Satterfield et al., 2016) . The high densities characterized by these sedentary populations have already been associated with increased parasite prevalence (Satterfield et al., 2015; Satterfield et al., 2018; Satterfield et al., 2016). Given our results here, it is unlikely that these patterns are driven by increased monarch susceptibility, and that they are instead driven by greater transmission rates.

Previous work has shown that the dose of parasites is a crucial determinant of parasite infection probability and sporeload in monarch butterflies (De Roode et al., 2007), and that differences in infection outcome are more likely driven by the effective dose of parasites that establishes an infection as opposed to anti-growth immunity following infection (De Roode et al., 2011). These results, combined with the studies conducted here, suggest that the biggest impact of crowding may be found in altering infectious doses in monarchs, and future work should directly test this prediction. As more migratory monarchs switch to sedentary lifestyles, it becomes increasingly important to study infection dynamics in sedentary populations and the role of lost migration in shaping parasite transmission. This study helps us understand the infection transmission dynamics in monarch populations and possible causes for the increase in parasite prevalence in sedentary monarchs.

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