Ecology and Evolution
Open Access

# Crowding does not affect monarch butterflies' resistance to a protozoan parasite

| | |
|---|---|
| Journal: | *Ecology and Evolution* |
| Manuscript ID | ECE-2021-11-01893.R1 |
| Wiley - Manuscript type: | Research Article |
| Date Submitted by the Author: | n/a |
| Complete List of Authors: | Alaidrous, Wajd; Emory University; King Abdullah University of Science and Technology, Division of Biological and Environmental Science and Engineering<br>Villa, Scott; Emory University<br>De Roode, Jacobus; Emory University, Biology<br>Majewska, Ania; Emory University, Biology |
| Category: | None of the above |
| Habitat: | Laboratory, Terrestrial |
| Organism: | Invertebrate |
| Approach: | Ecological Experiment |
| Abstract: | Host density is an important factor when it comes to parasite transmission and host resistance. Increased host density can increase contact rate between individuals and thus parasite transmission. 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 on 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 and parasite spore load were collected as well as development time, survival, and wing size, and melanization. Disease susceptibility under either food condition and across density treatments was similar. However, we found high larval density impacted development time, adult survival, and wing morphology when food was limited. This study aids our understanding of 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. |
| | |

Running head: crowding and disease

Title: Crowding does not affect monarch butterflies' resistance to a protozoan parasite

Wajd Alaidrous[1,2], Scott M. Villa[1], Jacobus C. de Roode[1], Ania A. Majewska[1]

Institutional affiliations:

[1] Department of Biology, Emory University, Atlanta, GA 30322, USA

[2] Division of Biological and Environmental Science and Engineering (BESE), King Abdullah University for Science and Technology, Thuwal, 23599-6900, Saudi Arabia

Corresponding author:

Ania A. Majewska

1510 Clifton Road NE

O. Wayne Rollins Research Center Rm. 1172

Department of Biology,

Emory University, Atlanta, GA 30322, USA

ania.majewska@emory.edu

**Abstract**

Host density is an important factor when it comes to parasite transmission and host resistance. Increased host density can increase contact rate between individuals and thus parasite transmission. 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 on 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 and parasite spore load were collected as well as development time, survival, and wing size, and melanization. Disease susceptibility under either food condition and across density treatments was similar. However, we found high larval density impacted development time, adult survival, and wing morphology when food was limited. This study aids our understanding of 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.

## Introduction

Host density plays an important role in host-parasite interactions. For parasites that rely on direct contact between individuals for transmission, higher host density increases transmission and infection prevalence (Arneberg et al. 1998, McCallum et al. 2001, Lloyd-Smith et al. 2005). Similarly, for parasites transmitted via the environment, increased host density can result in greater dissemination and accumulation of infectious stages in the environment and thereby increase incidence rates (Arneberg et al. 1998, Altizer et al. 2003). In parasites with complex life cycles, such as trematodes, production of infective stages is limited in time and space such that per capita host risk is diluted among all hosts (Buck and Lutterschmidt 2017), resulting in a negative relationship between density and parasitism. Other work suggests that negative-density dependent effects can occur in some host-parasite systems, particularly when hosts avoid infected individuals or areas with high transmission risk (Buck et al. 2018, Albery et al. 2020). Thus, the relationship between host density and infection risk is not always positive or straightforward.

Host density can impact susceptibility to parasitism, or the degree to which hosts are likely to become infected and experience subsequent parasite growth (Combes 2001), although the underlying mechanism and direction of the relationships are often unclear (Michel et al. 2016). Hosts can decrease their susceptibility as density increases (i.e. density-dependent prophylaxis) (Michel et al. 2016). For example, work on cabbage moths (*Mamestra brassicae*) (Goulson and Cory 1995) and African armyworms (*Spodoptera exempta*) (Reeson et al. 1998) showed that larvae reared at higher densities had greater resistance to parasites, as measured by levels of melanization, a key part of insect immune function, among other protective functions (San-Jose and Roulin 2018). In contrast, other studies have shown that crowding increases

intraspecific competition, aggression (Collie et al. 2020), and physiological stress (Steinhaus 1958), supporting the crowding stress hypothesis. Crowding as a stress-inducing factor for hosts can negatively impact host immune function (Steinhaus 1958, Michel et al. 2016, Lin et al. 2018). For instance, grass carp (*Ctenopharyngodon idella*) long-term crowding reduced immune parameters in the fish and their susceptibility to pathogens (Lin et al. 2018). Yet other work found that crowding resulted in no changes in immunity (Adamo and Parsons 2006). The complex interactions between host and parasite ecology at both the individual and community levels make predicting the influence of crowding on disease dynamics challenging.

Besides influencing transmission dynamics, crowding can also exacerbate the consequences of resource limitation and induce behavioral changes in hosts (Navarro et al. 2004). For instance, monarch butterfly caterpillars with low food quantity (milkweed leaves) were more aggressive towards conspecifics than those with higher food availability (Collie et al. 2020). More aggressive individuals likely expend more energy competing for resources, which may in turn reduce immunocompetence. However, food limitation in crowded environments also reduces food intake, which can impact host's ability to fight infection. Given that many wild animals are food-limited and experience variable density environments, it is important to better understand how crowding interacts with food availability to influence host susceptibility.

The monarch butterfly, *Danaus plexippus*, and its parasite, *Ophryocystis elektroscirrha* (McLaughlin and Myers 1970), provide a well-suited system to study the effect of crowding on host susceptibility. *O. elektroscirrha* is a natural parasite that infects monarchs across their range (McLaughlin and Myers 1970). Infection with *O. elektroscirrha* starts when a caterpillar ingests spores scattered onto eggs or plant leaves by adults (Altizer et al. 2004, de Roode et al. 2009). Transmission of the infection can occur via multiple routes. In addition to females transferring

parasites to their eggs, both infected males and females can scatter spores to milkweed. Moreover, infected males can transfer spore to females during mating, which they can then transmit to their offspring (Majewska et al. 2019). Parasites penetrate the mid-gut wall, and infect the hypodermal tissues, where they replicate asexually and sexually during the larval and pupal stages. Adults emerge covered in millions of dormant spores (McLaughlin and Myers 1970, Leong et al. 1992). Internal parasite growth is detrimental to monarchs, reducing survival to adulthood, mating success, fecundity, flight ability, and lifespan (Altizer and Oberhauser 1999, Bradley and Altizer 2005, De Roode et al. 2007, de Roode et al. 2009).

Monarchs are known for their long-distance migration from eastern North America to overwintering sites in Mexico (Urquhart and Urquhart 1978, Brower 1995, Reppert and de Roode 2018). Recent decades have seen the formation of sedentary populations of monarchs, in mild climates of the southeastern USA, along the Gulf of Mexico, as well as in California, USA, where monarchs no longer migrate and breed year-round on non-native milkweed (Brower et al. 2012, Satterfield et al. 2015, Satterfield et al. 2016). Infection by *O. elektroscirrha* is more prevalent in sedentary than migratory monarch populations (Altizer et al. 2000, Satterfield et al. 2015, Satterfield et al. 2016), which is likely due to sedentary populations sustaining high host densities that breed all year-round and, thus, experience higher parasite transmission (Altizer et al. 2004, Majewska et al. 2019). High caterpillar densities in sedentary populations have been associated with milkweed defoliation and food limitation (Fernández-Haeger et al. 2015, Satterfield et al. 2016), potentially having detrimental effects on susceptibility, highlighting the need to explore the infection dynamics under these conditions.

Here we examined the effect of larval density on host susceptibility to parasites in monarch butterflies (*Danaus plexippus*) in two experiments, one where food was unlimited and

one where food was limited. Using the monarch's natural parasite *O. elektroscirrha*, we tested the effect of larval density on susceptibility and tolerance for the different treatment groups. In addition, we examined the effects of crowding on survival and development time of immature stages, as well as lifespan, wing size, and wing melanization of adults. 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 developmental time and morphology.

## Methods

*Caterpillar sources and rearing*

We carried out two experiments to determine the effect of host density on disease susceptibility and tolerance. We used microcosms, which consisted of live potted plants, approximately 20 – 24 inches tall (50.8 – 61 cm) with two stalks, grown from seed in 4.5 inch (11.43 cm) diameter pots contained within transparent plastic tubes (4 inch diameter x 24 inch height; 10.16 cm x 61 cm) 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 were the breeding-generation offspring of wild-caught migrating North American monarch butterflies collected from St. Marks, Florida, USA (30.0737354°N, -84.1796806°W; a flyway and stopover site during the fall migration) in October 2017 and 2020 and overwintered in the laboratory. Mating and collection of eggs occurred in 0.6 $m^3$ mesh cages. Larvae were randomly picked from four non-inbred lineages for

the larval densities treatments and all larvae were reared on *A. curassavica* for the duration of the experiments. This plant species was chosen specifically because it is the main species that monarchs in sedentary populations encounter in North America (Satterfield et al. 2015, Satterfield et al. 2016, Satterfield et al. 2018). Because the parasite and monarch lineages used for the two experiments differed and a significant amount of time passed following the first experiment (unlimited food experiment), we do not directly compare the outcomes of the two experiments and instead focus on the qualitative differences in the results.

*Unlimited food experiment*

In the first experiment, caterpillars had unlimited food supply and we asked whether rearing density influenced immature monarch survival, development, susceptibility, tolerance, lifespan, as well as adult wing size and melanization. Starting on day two of larval development, larvae were reared in microcosms in one of three density treatments: singles (1 caterpillar/plant), doubles (2 caterpillars/plant), or tens (10 caterpillars/plant). We provided larvae with new plants when necessary to ensure sustained food *ad libitum*. Our design was full factorial (for sample sizes see Table 1). The singles treatment consisted of 25 replicates, doubles consisted of 15 replicates, and tens consisted of 6 replicates per inoculation treatments. Caterpillars in the inoculated treatment were individually inoculated with *O. elektroscirrha* parasites: second instar caterpillars were fed a 0.5 $cm^2$ leaf disk of *A. curassavica* with 10 manually deposited spores (stain ID: E42-2) in a Petri dish. Control caterpillars received a leaf disk without parasite spores. Upon complete consumption of their leaf disk, caterpillars were transferred to their randomly assigned microcosms. After pupation, pupae were transferred to individual 16 oz (473 mL) Solo cups and were attached to lids using hot glue. Placement of caterpillars in individual cups

assured no cannibalism occurred in the high-density treatment. Following emergence, adult monarchs were transferred to separate glassine envelopes without access to food and held in a DigiTherm® incubator at 12°C.

*Food limitation experiment*

In the second experiment, we asked how density of monarchs per plant coupled with food limitation impacts immature monarch survival, development, susceptibility, tolerance, lifespan, wing size and melanization of adult monarchs. We reared caterpillars in only two density treatments: singles (1 caterpillar/plant) and tens (10 caterpillars/plant). Because the first experiment revealed minimal effect of the two-caterpillar density, and because of COVID-19-imposed research restrictions, this experiment did not include the doubles treatment. As before, our experimental design was full factorial. Second instar caterpillars in the inoculated treatment were inoculated with *O. elektroscirrha* parasites (strain ID E42 (P43)) and controls were fed parasite-free leaf disks as described in the first experiment. To limit food availability, once all leaves in a microcosm were consumed, which only occurred in the ten caterpillar treatment, we provided one new plant. Next, on the second or third day of the fifth instar stage we transferred the caterpillars from both density treatments to 16oz Solo cups with *A. curassavica* plant stems (top portions of plant) only. Stems are often consumed by monarch caterpillars once supply of leaves is depleted and provide enough nutrition to complete development to pupation, while still ensuring food limitation (SMV *unpub. data*). As in the first experiment, all pupae were transferred to new individual 16 oz Solo cups and upon emergence adult monarchs were transferred to glassine envelopes and kept at 12°C in an incubator.

*Survival, development time and adult lifespan*

We recorded death of caterpillars and pupae daily to measure immature survival. We noted larval and pupal development time by checking for pupation and eclosion once a day. Larval development time was quantified as the number of days from egg hatching to pupation, and pupal development time was quantified as the number of days from pupation to eclosion. We also calculated total development time as the sum of larval and pupal development times.

We checked the adults in the incubator daily until death, as routinely done in this experimental system (De Roode et al. 2007). We calculated lifespan as the number of days between eclosion and death. The lifespans obtained in this way closely mimic the lifespans of monarchs under more natural conditions (de Roode et al. 2009).

*Susceptibility and tolerance*

We measured host susceptibility via qualitative and quantitative resistance (Lefèvre et al. (2011). To estimate qualitative resistance, or the probability that monarchs became infected following inoculation, adult monarchs were tested for the presence or absence of parasites. We determined parasite spore load of adults in the inoculation treatment following De Roode et al. (2007). The abdomen of perished adults was removed and vortexed at maximum speed in 5 mL of tap water for 5 minutes. Next, we counted the number of spores present in 0.1 µL of the 5 mL suspension using a hemocytometer by averaging sixteen chambers per sample. Monarchs with a spore load of zero were uninfected while those with spores were infected. Parasite spore load provides a measure of quantitative resistance, or the ability to limit parasite growth once infected, where higher load indicates higher susceptibility. We performed a $\log_{10}$ transformation

on parasite spore loads for normality of error distributions and homogeneity of variance to meet model assumptions.

Finally, we estimated tolerance, the ability of the host to withstand increasing parasite load without a loss in fitness. We used adult monarch lifespan as a proxy for host fitness, which has been shown to be an important component of monarch fitness (de Roode et al. 2009). We examined the slopes of a linear relationship between adult lifespan and $\log_{10}$ parasite spore load for the three density treatments. Steeper reductions in adult lifespan with increasing parasite spore load indicate decreased tolerance (Lefèvre et al. 2011) .

*Wing size and melanization*

To estimate wing area and wing melanization, we scanned the dorsal and ventral sides of the right wing with a Canon® CanoScan LiDE 210 flatbed scanner and processed the images with ImageJ 1.52k (https://imagej.nih.gov/ij/). Briefly, we scanned wings at 300 dots per inch (dpi) to produce digital images for analysis. The scanner settings were constant for all individuals and no color correction was used. Wing analysis using scanned images has been widely used for analysing monarch wing morphology (Davis et al. 2005, Davis et al. 2007, Davis 2009, Davis et al. 2012, Hanley et al. 2013).

To process wing images, we first isolated the whole forewing and hindwing and quantified their area using the "measure" tool. Only the dorsal side of the wings were used for size to avoid redundancy. Adults with damaged wings were excluded. We then used a custom thresholding macro code to digitally separate the carotenoid-based cells from the melanin-based veins using the "thresholding" tool. Thresholding isolates the black from non-black portions of

the wings and has been used to previously analyse monarch wing colour (Davis et al. 2005, Hanley et al. 2013).

We obtained melanization scores for all four wing surfaces (i.e. dorsal and ventral forewing and hindwing). The melanization score for each wing surface ranges from 0 (pure black) to 255 (pure white) and it is a measure of "blackness", where lower values indicate more intense black coloration and greater melanin pigment in the wing. The four scores were then averaged to give an overall melanization score for each monarch. Previous work in lepidoptera suggests wing melanin pigmentation increases with immune function challenge (Freitak et al. 2005).

**Statistical Analysis**

Statistical analysis was performed using R (R Core Team 2021). We used generalized linear mixed-effects models (GLMMs) with binomial errors to test for differences in immature survival (0: perished; 1: alive) and infection status (0: uninfected; 1: infected) between density treatments. Fixed effects in the survival model included density and inoculation treatment, while in the infection model fixed effects included density and sex. We did not include sex in the analysis of immature survival because sex is unknown until adulthood. We used linear mixed-effects models (LMM) with gaussian errors to test for differences in development times (larval, pupal and total development), adult lifespan, and parasite spore load between density treatments. To assess whether parasite spore load differed between density treatment we used a LMM with fixed effects as before: density, inoculation, a density-by-inoculation interaction, and sex. In all models, the unique microcosm that the larvae were reared in was included as a random effect.

To examine the differences in tolerance between density and inoculation treatments we employed a LMM with adult lifespan as the response variable and sex, $\log_{10}$ spore load, density, and the interaction between $\log_{10}$ spore load and density as explanatory factors.

Finally, we asked whether wing morphology varies with density and inoculation treatments. LMMs were used to compare wing areas and wing melanization across the density treatments. Fixed effects included density, inoculation, the interaction between density and inoculation treatments and sex. The microcosm that the larvae were reared in was included as a random effect as before.

## Results

### Unlimited food experiment

*Survival, development time, and adult lifespan*

Immature survival probabilities tended to be high (above 90%, Table 1) and did not significantly differ among density and inoculation treatments ($p>0.05$, Table 2, Fig. 1A). We found no impact of inoculation, density treatment or their interaction on larval, pupal, or total development times ($p>0.05$; Fig. 1B). Sex significantly impacted development: males had longer larval, pupal, and total development times than females (larval: $t = 1.99$, $p = 0.05$; pupal: $t = 9.01$, $p <0.001$; total: $t = 7.32$, $p <0.001$).

Density treatment significantly impacted adult lifespan: monarchs in the ten caterpillar treatment had longer lifespan compared to those in singles and doubles densities ($t = 2.84$, $p = 0.01$). Inoculation treatment had a strong impact on lifespan: compared to inoculated monarchs, control monarchs lived about twice as long ($t = -7.67$, $p<0.001$; Fig. 1C). Sex also impacted lifespan: males lived significantly less time than females ($t = -3.04$, $p <0.01$; Table 2). Finally,

we found a significant interaction between density and inoculation treatments: monarchs in the ten caterpillar inoculated treatment combination showed significantly shorter (nearly half as long) adult lifespan compared to other treatment combinations ($t = -2.00$, $p = 0.05$; Fig. 1C).

*Wing size and melanization*

We found no effect of density, inoculation treatments, or their interaction on wing area when food was unlimited ($p>0.05$, Table 2; Fig. 1E-1F). Sex significantly impacted hindwing size: males had slightly larger hindwings than females ($t = 2.14$, $p =0.04$; Table 2). Melanin score was significantly impacted by the interaction between inoculation and density (Fig. 1D) as well as sex: adults in the double inoculated treatment had somewhat higher melanin scores (i.e. less black density; $t = 2.14$, $p = 0.03$) while males showed slightly lower melanin scores (i.e. greater black density; $t= -2.49$, $p = 0.01$).

*Susceptibility and tolerance*

We found that 95% of the adults in the inoculation treatment became infected (singles: 91%, doubles: 93%, tens: 98%; Table 1). Several caterpillars and pupae died prior to end of both experiments due to observer error (e.g. accidental physical damage) and unknown causes. Probability of infection (qualitative resistance) did not significantly differ across the density treatments ($p > 0.05$; Table 2; Fig. 2A). Analysis of the infected adults only showed no effect of density on parasite spore load (quantitative resistance; $p > 0.05$; Table 2; Fig. 2B). Adult lifespan) was negatively affected by parasite spore load ($t = -3.45$, $p <0.001$), but not by density ($p >0.05$; Table 2; Fig. 2C). We found no significant interaction between spore load and density

on lifespan ($p > 0.05$), indicating no overall differences in tolerance between density treatments. For full model outputs, see Appendix Tables S1-S3.

**Food limitation experiment**

*Survival, development time and adult lifespan*

When food was limited, survival to adulthood tended to decrease among inoculation treatments but this difference was not statistically significant ($p > 0.05$; Table 2); we also found no significant difference in survival between the singles and tens density treatments ($p > 0.05$; Fig. 3A).

Density but not inoculation affected larval and total development times: caterpillars in the high density treatment (tens) took significantly longer to develop than those in the singles treatment (larval: $t = 3.4$, $p = 0.001$; total: $t = 2.70$, $p = 0.01$; Fig. 3B). Inoculation and density treatments did not impact pupal development time ($p > 0.05$). We found that sex affected development times, with males showing longer larval ($t = 3.32$, $p = 0.001$), pupal ($t = 4.78$, $p < 0.001$), and total development ($t = 4.17$, $p < 0.001$) times compared to females (Table 2). We found no effect of the interaction between inoculation and density treatment on development times ($p > 0.05$).

Adult lifespan was significantly affected by density and inoculation treatments when food was limited for caterpillars. Monarchs in higher density (tens) had slightly shorter lifespan ($t = -3.13$, $p < 0.01$) than those in single densities and those in inoculated treatment lived shorter than controls ($t = -4.05$, $p < 0.001$; Fig. 3C). We also found a significant interaction between density and inoculation treatments: monarchs in the ten caterpillar inoculated treatment combination

showed significantly shorter adult lifespan compared to other density-inoculation treatment combinations ($t = 2.08$, $p = 0.04$; Fig. 3C).

*Wing size and melanization*

Density but not inoculation impacted wing size when food was limited: both forewing and hindwing areas were significantly smaller in the tens density treatment (forewing: $t = -8.95$, $p < 0.001$; hindwing: $t = -9.07$, $p < 0.001$; Fig. 3E-3F). We found no effect of the interaction between inoculation and density treatment on wing areas ($p > 0.05$). Sex impacted hindwing but not forewing area: males had significantly larger hindwings compared to females ($t = 2.09$, $p = 0.04$). Melanin score was impacted by density and inoculation treatments but not sex (Table 2). Monarchs in the tens density treatment had higher melanin scores compared to singles treatments ($t=5.30$, $p < 0.001$). Similarly, inoculated monarchs had higher melanin scores compared to controls ($t = 5.80$, $p < 0.001$; Fig. 3D). We found no effect of the interaction between inoculation and density treatment on the melanin score ($p > 0.05$).

*Susceptibility and tolerance*

A total of 73% of the adults in the inoculation treatment became infected (singles: 73%, tens: 73%; Table 1). Infection probability (qualitative resistance) was not impacted by density treatment ($p > 0.05$; Fig. 4A). Analysis of the infected adults only showed that monarchs in the ten caterpillar treatment had lower parasite spore loads compared to singles (quantitative resistance; $t = -2.10$, $p = 0.05$; Fig 4B). Because both parasite growth and monarch size could be affected by crowding, and given the smaller size of infected monarchs (see above), we followed up with an analysis of parasite spore load corrected for wing size (residuals of a simple linear regression between wing area and spore load). Examination of the corrected spore load in

relation to density showed no significant differences across the density treatments ($p>0.05$; Table 2). Neither spore load nor density nor the interaction of the two influenced adult lifespan, indicating that density did not alter tolerance of infection ($p>0.05$; Fig. 4C). For full model outputs, see Appendix Tables S4-S7.

## Discussion

In this study, we examined the effect of crowding and food availability at larval stages on disease susceptibility in monarch butterflies. When food was unlimited, high density had no effect on infection probability (qualitative resistance), parasite load (quantitative resistance), or tolerance. Under food limited conditions, crowding also did not impact the probability of infection, yet monarchs reared in the highest density (ten caterpillar treatment) had a lower parasite load than those reared at the lowest density (single caterpillar treatment), suggesting that high rearing density lowers caterpillar parasite susceptibility. On the other hand, lower parasite load among the hosts held at high density might be a consequence of the starvation and small host size (Pulkkinen and Ebert 2004). Indeed, accounting for wing size, we found no significant differences in spore load between density treatments. It is also important to consider that the food type (leaves vs. stem) that caterpillars consumed under high density conditions might have impacted the parasite load and experiments examining this possibility are needed.

Interestingly, we found that in both experiments, infected monarchs showed less dense wing melanization (i.e. higher scores). Since melanin is costly to produce, these results suggest that the energetic costs of *O. elektroscirrha* reduce a monarch's "blackness." Moreover, less melanin production might also suggest a lack of resources to mount an effective immune defense

(Freitak et al. 2005). Since melanization is considered a signal of immunocompetence in insects (Wilson et al. 2001, Nakhleh et al. 2017), the differential wing melanization among infected individuals might be an honest signal of monarch health and quality. We also found an effect of food availability on wing melanin. When food was unlimited, there was no significant difference within infection treatments among singles, doubles, and tens. However, when food was limited, wing melanization was less dense for both infected and uninfected monarchs when raised in the tens treatment compared to the singles treatment. This suggests that less food also restricts a monarch's ability to produce melanin. Thus, both food availability and parasites can additively influence monarch melanization. Further, consumption of milkweed stems only at high densities might affect melanization, although this was not tested in this study. Interestingly, the darkest monarchs in our experiments were uninfected singles with unlimited food, and the least melanized ones were infected tens with limited food. Future work should assess immune parameters in monarchs under varying densities, food availability and type (stem vs. leaves), and infection status to better understand the relationships between wing melanization and immunity in this species.

Our results are in contrast with a previous study that suggested that crowding caused increased infection probability in monarchs (Lindsey et al. 2009). However, differences in methodology and milkweed species used between our study and the Lindsey et al (2009) experiment make direct comparisons of findings difficult. In particular, Lindsey et al (2009) raised caterpillars on cuttings of *A. incarnata* rather than live plants of *A. curassavica*. The quick deterioration of milkweed cuttings combined with the buildup of frass on plant material necessitated frequent handling of the caterpillars which likely increased the stress of the

caterpillars in the high density treatment compared to the study we described here. Further, caterpillars in Lindsey et al (2009) study experienced other stressors, including an unidentified viral or bacterial disease that caused high mortality and might have influenced the outcomes.

The finding that crowding in our experiments did not increase monarch susceptibility to infection does not mean that higher density will lessen disease pressure in natural monarch populations. Instead, we expect the effects of crowding to affect parasite transmission. Theory suggests that diseases that spread via density-dependent transmission show increased parasite prevalence with crowding due to increased contact rates between hosts (McCallum et al. 2001, Rader et al. 2020). Moreover, higher densities can result in greater buildup of infectious parasite stages in the environment, and thereby result in greater infection rates (Arneberg et al. 1998, Majewska et al. 2019). Both of these factors are highly relevant to monarch butterflies, some of which are foregoing migration to form sedentary populations to breed year-round (Satterfield et al. 2015, Satterfield et al. 2016). The high densities characterized by sedentary populations have been associated with increased parasite prevalence, most likely because of greater exchange of parasites between adults and greater deposition of spores onto milkweed foliage (Satterfield et al. 2015, Majewska et al. 2019). Given our results, it is unlikely that the patterns observed in the field are driven by increased susceptibility, but instead driven by greater transmission rates. 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 enhances our understanding of the infection transmission dynamics in monarch populations and possible causes for the increase in parasite prevalence in sedentary monarchs.

Food is rarely unlimited in nature and crowding is likely to increase intraspecific competition and, in turn, physiological and resource stress, all of which can negatively impact life history traits (Boggs 2009). Not surprisingly, when food was limited, fewer monarchs survived to adulthood compared to when food was unlimited. Further, crowded and food limited monarch caterpillars developed more slowly into adults and experienced shorter adult lifespans than monarchs raised singly. Crowding coupled with food limitation also caused reductions in wing size, and less dense melanin (i.e. less "blackness") in the wings. All effects observed here are consistent with numerous other studies examining the influence of crowding on life history traits in insects (Scheiring et al. 1984, Banks and Thompson 1987, Gibbs et al. 2004, Baldal et al. 2005, Alto et al. 2012).

The impact of food limitation on monarchs is particularly noticeable when comparing the results of our unlimited and limited food experiments: when food was unlimited, crowding had no effect on developmental rate or wing size, yet food limitation led to longer developmental times and smaller wing size. These findings are consistent with previous work in monarchs (e.g. Johnson et al. 2014). In another study on the effects of larval rearing density in monarchs, larvae showed similar developmental times in high density and constant food supply (Atterholt and Solensky 2010). Yet, in our study, the highest density treatment had a higher number of individuals (n=10 caterpillars), which suggests that starvation and high levels of crowding have a strong effect on development time. Atterholt and Solensky (2010) found no effect of starvation on monarch size, or development time when monarchs were raised singly. However, Atterholt and Solensky (2010) 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, some studies have shown that survival to adulthood decreased with increasing egg per plant

density (Nail et al. 2015). Thus, crowding at very high densities can have more pronounced effects on survival in nature, where additional factors such as the presence of predators are likely impacting survival.

In conclusion, our experiments revealed that monarch butterfly susceptibility and tolerance to a protozoan parasite tends to be similar across varying caterpillar densities and we found no evidence for the crowding stress hypothesis or density-dependent prophylaxis hypothesis in this system. Nonetheless, we note that under certain ecological scenarios, crowding can strongly impact other key traits, including development time, adult lifespan, and wing melanization, all of which might have consequences for the persistence of healthy monarch populations. The biggest impact of crowding may be found in altering transmission rates in monarchs, and future work should directly test this prediction.

## Literature cited

Adamo, S., and N. Parsons. 2006. The emergency life-history stage and immunity in the cricket, *Gryllus texensis*. Animal behaviour **72**:235-244.

Albery, G. F., C. Newman, J. B. Ross, D. W. MacDonald, S. Bansal, and C. Buesching. 2020. Negative density-dependent parasitism in a group-living carnivore. Proceedings of the Royal Society B **287**:20202655.

Altizer, S., C. L. Nunn, P. H. Thrall, J. L. Gittleman, J. Antonovics, A. A. Cunningham, A. P. Dobson, V. Ezenwa, K. E. Jones, and A. B. Pedersen. 2003. Social organization and parasite risk in mammals: integrating theory and empirical studies. Annual Review of Ecology, Evolution, and Systematics **34**:517-547.

Altizer, S., and K. S. Oberhauser. 1999. Effects of the protozoan parasite *Ophryocystis elektroscirrha* on the fitness of monarch butterflies (*Danaus plexippus*). Journal of Invertebrate Pathology **74**:76-88.

Altizer, S., K. S. Oberhauser, and L. P. Brower. 2000. Associations between host migration and the prevalence of a protozoan parasite in natural populations of adult monarch butterflies. Ecological Entomology **25**:125-139.

Altizer, S., K. S. Oberhauser, and K. A. Geurts. 2004. Transmission of the protozoan parasite, *Ophryocystis elektroscirrha*, in monarch butterfly populations: implications for prevalence and population-level impacts. Pages 203-218 *in* K. S. Oberhauser and M. Solensky, editors. The monarch butterfly: biology and conservation. Cornell University Press, Ithaca, NY.

Alto, B., E. Muturi, and R. Lampman. 2012. Effects of nutrition and density in *Culex pipiens*. Medical and veterinary entomology **26**:396-406.

Arneberg, P., A. Skorping, B. Grenfell, and A. F. Read. 1998. Host densities as determinants of abundance in parasite communities. Proceedings of the Royal Society B **265**:1283-1289.

Atterholt, A. L., and M. J. Solensky. 2010. Effects of larval rearing density and food availability on adult size and coloration in monarch butterflies (Lepidoptera: Nymphalidae). Journal of Entomological Science **45**:366-377.

Baldal, E., K. van der Linde, J. Van Alphen, P. Brakefield, and B. Zwaan. 2005. The effects of larval density on adult life-history traits in three species of Drosophila. Mechanisms of ageing and development **126**:407-416.

Banks, M. J., and D. J. Thompson. 1987. Regulation of damseifly populations: the effects of larval density on larval urvival, development rate and size in the field. Freshwater Biology **17**:357-365.

Boggs, C. L. 2009. Understanding insect life histories and senescence through a resource allocation lens. Functional Ecology **23**:27-37.

Bradley, C. A., and S. Altizer. 2005. Parasites hinder monarch butterfly flight: Implications for disease spread in migratory hosts. Ecology Letters **8**:290-300.

Brower, L. P. 1995. Understanding and misunderstanding the migration of the monarch butterfly (Nymphalidae) in North America: 1857-1995. Journal of the Lepidopterists Society **49**:304-385.

Brower, L. P., O. R. Taylor, E. H. Williams, D. A. Slayback, R. R. Zubieta, and M. I. Ramirez. 2012. Decline of monarch butterflies overwintering in Mexico: Is the migratory phenomenon at risk? Insect Conservation and Diversity **5**:95-100.

Buck, J., S. Weinstein, and H. Young. 2018. Ecological and evolutionary consequences of parasite avoidance. Trends in ecology & evolution **33**:619-632.

Buck, J. C., and W. I. Lutterschmidt. 2017. Parasite abundance decreases with host density: evidence of the encounter-dilution effect for a parasite with a complex life cycle. Hydrobiologia **784**:201-210.

Collie, J., O. Granela, E. B. Brown, and A. C. Keene. 2020. Aggression is induced by resource limitation in the monarch caterpillar. Iscience **23**:101791.

Combes, C. 2001. Parasitism: the ecology and evolution of intimate interactions. University of Chicago Press, Chicago, IL, USA.

Davis, A. K. 2009. Wing color of monarch butterflies (Danaus plexippus) in eastern North America across life stages: migrants are "redder" than breeding and overwintering stages. Psyche **2009**.

Davis, A. K., J. Chi, C. Bradley, and S. Altizer. 2012. The redder the better: wing color predicts flight performance in monarch butterflies. PloS one **7**:e41323.

Davis, A. K., N. Cope, A. Smith, and M. J. Solensky. 2007. Wing color predicts future mating success in male monarch butterflies. Annals of the Entomological Society of America **100**:339-344.

Davis, A. K., B. D. Farrey, and S. Altizer. 2005. Variation in thermally induced melanism in monarch butterflies (Lepidoptera: Nymphalidae) from three North American populations. Journal of Thermal Biology **30**:410-421.

De Roode, J., L. Gold, and S. Altizer. 2007. Virulence determinants in a natural butterfly-parasite system. Parasitology **134**:657-668.

de Roode, J. C., J. Chi, R. M. Rarick, and S. Altizer. 2009. Strength in numbers: High parasite burdens increase transmission of a protozoan parasite of monarch butterflies (*Danaus plexippus*). Oecologia **161**:67-75.

Fernández-Haeger, J., D. Jordano, and M. P. Zalucki. 2015. Monarchs across the Atlantic Ocean. Pages 247-256 *in* K. S. Oberhauser, K. R. Nail, and S. Altizer, editors. Monarchs in a chaning world. Biology and conservation of an iconic butterfly. Cornell University Press, Ithica, NY.

Freitak, D., A. Vanatoa, I. Ots, and M. J. Rantala. 2005. Formation of melanin-based wing patterns is influenced by condition and immune challenge in *Pieris brassicae*. Entomologia Experimentalis et Applicata **116**:237-243.

Gibbs, M., L. A. Lace, M. J. Jones, and A. J. Moore. 2004. Intraspecific competition in the speckled wood butterfly *Pararge aegeria*: effect of rearing density and gender on larval life history. Journal of Insect Science **4**:4-16.

Goulson, D., and J. S. Cory. 1995. Responses of *Mamestra brassicae* (Lepidoptera: Noctuidae) to crowding: interactions with disease resistance, colour phase and growth. Oecologia **104**:416-423.

Hanley, D., N. G. Miller, D. T. Flockhart, and D. R. Norris. 2013. Forewing pigmentation predicts migration distance in wild-caught migratory monarch butterflies. Behavioral Ecology **24**:1108-1113.

Johnson, H., M. J. Solensky, D. A. Satterfield, and A. K. Davis. 2014. Does skipping a meal matter to a butterfly's appearance? Effects of larval food stress on wing morphology and color in monarch butterflies. PloS one **9**:e93492.

Lefèvre, T., A. J. Williams, and J. C. de Roode. 2011. Genetic variation in resistance, but not tolerance, to a protozoan parasite in the monarch butterfly. Proceedings of the Royal Society B **278**:751-759.

Leong, K. L., H. Kaya, M. A. Yoshimura, and D. F. Frey. 1992. The occurrence and effect of a protozoan parasite, *Ophryocystis elektroscirrha* (Neogregarinida: Ophryocystidae) on overwintering monarch butterflies, *Danaus plexippus* (Lepidoptera: Danaidae) from two California winter sites. Ecological Entomology **17**:338.

Lin, W., L. Li, J. Chen, D. Li, J. Hou, H. Guo, and J. Shen. 2018. Long-term crowding stress causes compromised nonspecific immunity and increases apoptosis of spleen in grass carp (*Ctenopharyngodon idella*). Fish & shellfish immunology **80**:540-545.

Lindsey, E., M. Mehta, V. Dhulipala, K. Oberhauser, and S. Altizer. 2009. Crowding and disease: effects of host density on response to infection in a butterfly–parasite interaction. Ecological Entomology **34**:551-561.

Lloyd-Smith, J. O., P. C. Cross, C. J. Briggs, M. Daugherty, W. M. Getz, J. Latto, M. S. Sanchez, A. B. Smith, and A. Swei. 2005. Should we expect population thresholds for wildlife disease? Trends in ecology & evolution **20**:511-519.

Majewska, A. A., S. Sims, A. Schneider, S. Altizer, and R. J. Hall. 2019. Multiple transmission routes sustain high prevalence of a virulent parasite in a butterfly host. Proceedings of the Royal Society B **286**:20191630.

McCallum, H., N. Barlow, and J. Hone. 2001. How should pathogen transmission be modelled? Trends in ecology & evolution **16**:295-300.

McLaughlin, R., and J. Myers. 1970. *Ophryocystis elektroscirrha* sp. n., a Neogregarine Pathogen of the Monarch Butterfly *Danaus plexippus* (L.) and the Florida Queen Butterfly *D. gilippus berenice* Cramer 1. The Journal of Protozoology **17**:300-305.

Michel, J., D. Ebert, and M. D. Hall. 2016. The trans-generational impact of population density signals on host-parasite interactions. BMC evolutionary biology **16**:1-12.

Nail, K. R., C. Stenoien, and K. S. Oberhauser. 2015. Immature monarch survival: effects of site characteristics, density, and time. Annals of the Entomological Society of America **108**:680-690.

Nakhleh, J., L. El Moussawi, and M. A. Osta. 2017. The melanization response in insect immunity. Advances in Insect Physiology **52**:83-109.

Navarro, C., F. De Lope, A. Marzal, and A. Møller. 2004. Predation risk, host immune response, and parasitism. Behavioral Ecology **15**:629-635.

Pulkkinen, K., and D. Ebert. 2004. Host starvation decreases parasite load and mean host size in experimental populations. Ecology **85**:823-833.

R Core Team. 2021. R: A language and environment for statistical computing. R foundation for statistical computing. Version 4.0.4.

Rader, B., S. V. Scarpino, A. Nande, A. L. Hill, B. Adlam, R. C. Reiner, D. M. Pigott, B. Gutierrez, A. E. Zarebski, and M. Shrestha. 2020. Crowding and the shape of COVID-19 epidemics. Nature medicine **26**:1829-1834.

Reeson, A. F., K. Wilson, A. Gunn, R. S. Hails, and D. Goulson. 1998. Baculovirus resistance in the noctuid *Spodoptera exempta* is phenotypically plastic and responds to population density. Proceedings of the Royal Society B **265**:1787-1791.

Reppert, S. M., and J. C. de Roode. 2018. Demystifying monarch butterfly migration. Current Biology **28**:R1009-R1022.

San-Jose, L. M., and A. Roulin. 2018. Toward understanding the repeated occurrence of associations between melanin-based coloration and multiple phenotypes. The American Naturalist **192**:111-130.

Satterfield, D. A., J. C. Maerz, and S. Altizer. 2015. Loss of migratory behaviour increases infection risk for a butterfly host. Proceedings of the Royal Society B **282**:20141734.

Satterfield, D. A., J. C. Maerz, M. D. Hunter, D. T. Flockhart, K. A. Hobson, D. R. Norris, H. Streit, J. C. de Roode, and S. Altizer. 2018. Migratory monarchs that encounter resident monarchs show life-history differences and higher rates of parasite infection. Ecology Letters **21**:1670-1680.

Satterfield, D. A., F. X. Villablanca, J. C. Maerz, and S. Altizer. 2016. Migratory monarchs wintering in California experience low infection risk compared to monarchs breeding year-round on non-native milkweed. Integrative and comparative biology **56**:343-352.

Scheiring, J., D. Davis, A. Ranasinghe, and C. Teare. 1984. Effects of larval crowding on life history parameters in *Drosophila melanogaster* Meigen (Diptera: Drosophilidae). Annals of the Entomological Society of America **77**:329-332.

Steinhaus, E. A. 1958. Crowding as a possible stress factor in insect disease. Ecology **39**:503-514.

Urquhart, F., and N. Urquhart. 1978. Autumnal migration routes of the eastern population of the monarch butterfly (*Danaus p. plexippus* L.; Danaidae; Lepidoptera) in North America to the overwintering site in the Neovolcanic Plateau of Mexico. Canadian Journal of Zoology **56**:1759-1764.

Wilson, K., S. C. Cotter, A. F. Reeson, and J. K. Pell. 2001. Melanism and disease resistance in insects. Ecology letters **4**:637-649.

590 **Table 1.** Number of monarchs used in each experiment along with percent of individuals surviving to adulthood and percent of
591 infected adults in each treatment.

| Food unlimited | | | | | |
|---|---|---|---|---|---|
| **Inoculation treatment** | | **Density treatment** | | | |
| | | **Singles** | **Doubles** | **Tens** | **Total** |
| **Control** | **Initial number of caterpillars** | 25 | 30 | 59 | 114 |
| | **Number emerged** | 25 | 28 | 53 | 106 |
| | **% surviving to adulthood** | 100 | 93 | 90 | 93 |
| | **% infected** | 0 | 0 | 0 | 0 |
| **Inoculated** | **Initial number of caterpillars** | 25 | 30 | 60 | 115 |

| Food limited | | | | |
|---|---|---|---|---|
| **Inoculation treatment** | | **Density treatment** | | |
| | | **Singles** | **Tens** | **Total** |
| **Control** | **Initial number of caterpillars** | 25 | 58 | 83 |
| | **Number emerged** | 22 | 49 | 71 |
| | **% surviving to adulthood** | 88 | 84 | 86 |
| | **% infected** | 0 | 0 | 0 |
| **Inoculated** | **Initial number of caterpillars** | 25 | 59 | 84 |

| | | | | | |
|---|---|---|---|---|---|
| | **Number emerged** | 22 | 29 | 58 | 109 |
| | **% surviving to adulthood** | 88 | 97 | 97 | 95 |
| | **% infected** | 91 | 93 | 98 | 95 |

| | | | | |
|---|---|---|---|---|
| | **Number emerged** | 22 | 45 | 67 |
| | **% surviving to adulthood** | 88 | 76 | 80 |
| | **% infected** | 73 | 73 | 73 |

**Table 2.** Summary of the variables included in the two experiments. Fixed effects were density, inoculation treatment, interaction between density and inoculation treatment, and sex. Microcosm identification was included as a random effect in all models. Each row summarizes a model for a different response variable. 'ns' represents a non-significant term, and '//' indicates that the variable was not included in the model. Asterisks denote the p-value, *p < 0.05, **p < 0.01; ***p < 0.001. For full model results, see Appendix Tables S1-7.

| | | **Fixed effect** | | | | |
|---|---|---|---|---|---|---|
| **Response variable** | | **Density (Single/Doubles/Tens)** | **Inoculation (Inoculated/Control)** | **Density x Inoculation** | **Sex (M/F)** | **Spore load** |
| Unlimited food | Immature survival (0/1) | *ns* | *ns* | // | // | // |
| | Larval development time | *ns* | *ns* | *ns* | M * | // |
| | Pupal development time | *ns* | *ns* | *ns* | M *** | // |
| | Total development time | *ns* | *ns* | *ns* | M *** | // |
| | Adult lifespan | Tens ** | Inoculated *** | Tens x Inoculated ** | M ** | // |
| | Forewing area | *ns* | *ns* | *ns* | *ns* | // |
| | Hindwing area | *ns* | *ns* | *ns* | M * | // |

| | | | | | | |
|---|---|---|---|---|---|---|
| | Melanin score | *ns* | *ns* | Doubles x Inoculated ** | M * | // |
| | Infection (0/1) | *ns* | // | // | *ns* | // |
| | Spore load | *ns* | // | // | *ns* | // |
| | Tolerance | *ns* | *ns* | *ns* | M *** | *** |
| | **Response variable** | **Density (Single/Tens)** | **Inoculation (Inoculated/Control)** | **Density x Inoculation** | **Sex (M/F)** | **Spore load** |
| Food limited | Immature survival (0/1) | *ns* | *ns* | // | // | // |
| | Larval development time | Tens *** | *ns* | *ns* | M *** | // |
| | Pupal development time | *ns* | *ns* | *ns* | M *** | // |
| | Total development time | Tens *** | *ns* | *ns* | M *** | // |
| | Adult lifespan | Tens *** | Inoculated *** | Tens x Inoculated * | *ns* | // |
| | Forewing area | Tens *** | *ns* | *ns* | *ns* | // |
| | Hindwing area | Tens *** | *ns* | *ns* | M * | // |
| | Melanin score | Tens *** | Inoculated *** | *ns* | *ns* | // |
| | Infection (0/1) | *ns* | *ns* | // | // | // |

| | | | | | | |
|---|---|---|---|---|---|---|
| | Spore load | Tens * | *ns* | *ns* | *ns* | // |
| | Size-corrected spore load | *ns* | *ns* | *ns* | *ns* | // |
| | Tolerance | *ns* | *ns* | *ns* | *ns* | *ns* |

598

599

**Figure Legends**

**Figure 1.** Density and inoculation treatment in relation to (A) proportion of surviving immature monarchs to adulthood, (B) total development time, (C) adult lifespan, (D) wing melanin score, (E) forewing, and (F) hindwing area in the unlimited food experiment. Bars represent means, color of bars represent treatment (blue: control; orange: inoculated), and error bars represent standard errors of the mean. Box plots show median values (thick black middle lines) with first and third quartiles (boxes), maximum and minimum values (whiskers), and outliers (black points). Different letters above box plots indicate significant differences (Table S7-S12).

**Figure 2.** Effect of density treatment (single, double, ten) in relation to (A) proportion of monarchs that became infected in the inoculated treatment, and (B) $\log_{10}$ parasite spore load and (C) tolerance (the slope of the relationship between adult lifespan and parasite spore load) in the unlimited food experiment. Bars represent means, and error bars represent standard errors of the mean. Color of bars, points and lines represent density treatment (yellow: singles; orange: doubles; dark orange: tens). Box plots show median values (thick black middle lines) with first and third quartiles (boxes), maximum and minimum values (whiskers), and outliers (black points). Different letters above box plots indicate significant differences (Table S13-S14).

**Figure 3.** Density and inoculation treatment in relation to (A) proportion of surviving immature monarchs to adulthood, (B) total development time, (C) adult lifespan, (D) wing melanin score , (E) forewing, and (F) hindwing area in the food limitation experiment. Bars represent means, color of bars represent treatment (blue: control; orange: inoculated), and error bars represent standard errors of the mean. Different letters above box plots indicate significant differences (Table S15-S20).

**Figure 4**. Density in relation to (A) proportion of monarchs that became infected in the inoculated treatment, (B) $\log_{10}$ parasite spore load, and (C) tolerance (the slope of the relationship between adult lifespan and parasite spore load) in the food limitation experiment. Bars represent means, and error bars represent standard errors of the mean. Color of bars, points and lines represent density treatment (yellow: singles; dark orange: tens). Different letters above box plots indicate significant differences (Table S21-S22).



**Data Accessibility Statement**: Data used in this study will be available on Figshare after manuscript acceptance.

**Competing Interests Statement**: We declare no competing interests.

**Author Contributions:**

**Wajd Alaidrous**: Conceptualization (lead); Data curation (lead); Formal analysis (supporting); Investigation (lead); Methodology (lead); Project administration (lead); Validation (supporting); Visualization (supporting); Writing-original draft (lead); Writing-review & editing (supporting).

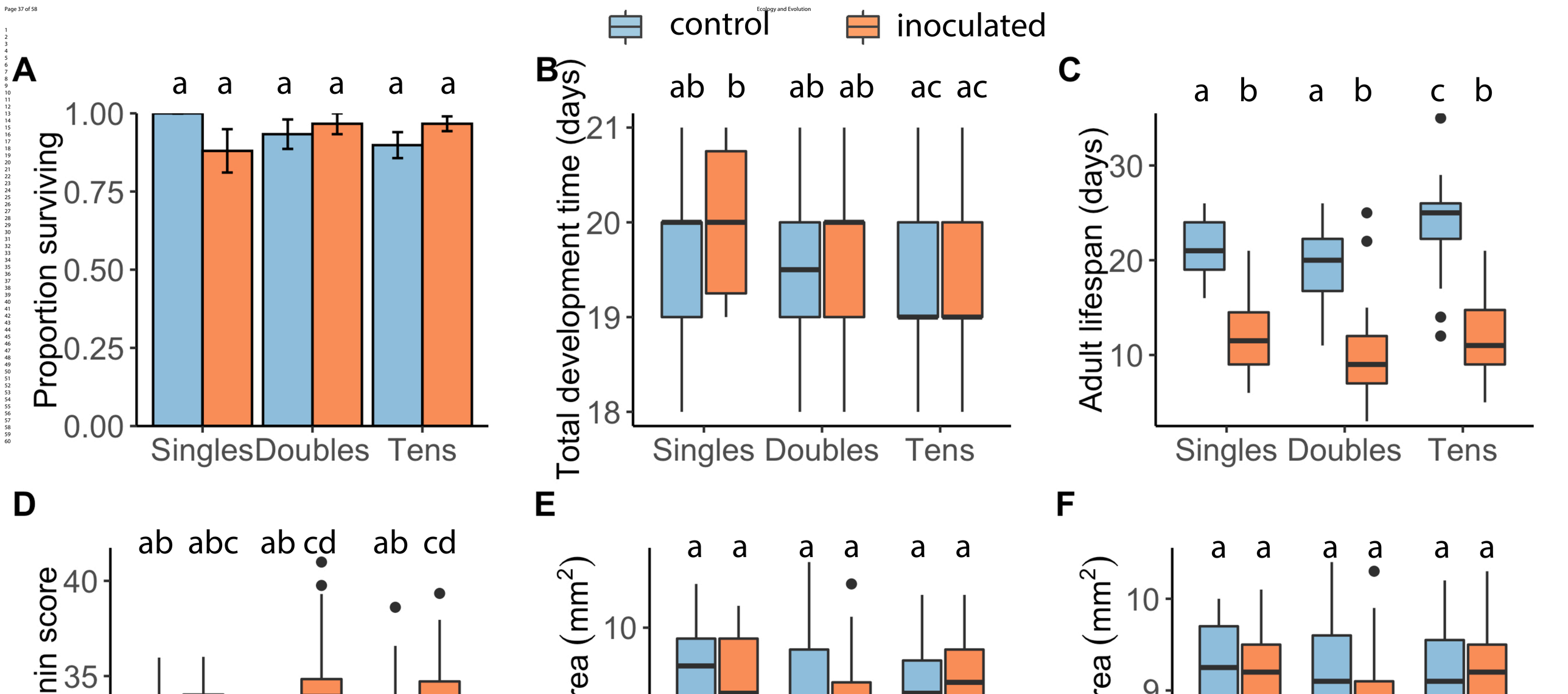
**Ania A. Majewska**: Data curation (supporting); Formal analysis (lead); Software (supporting); Supervision (supporting); Validation (lead); Visualization (supporting); Writing-original draft (supporting); Writing-review & editing (lead).

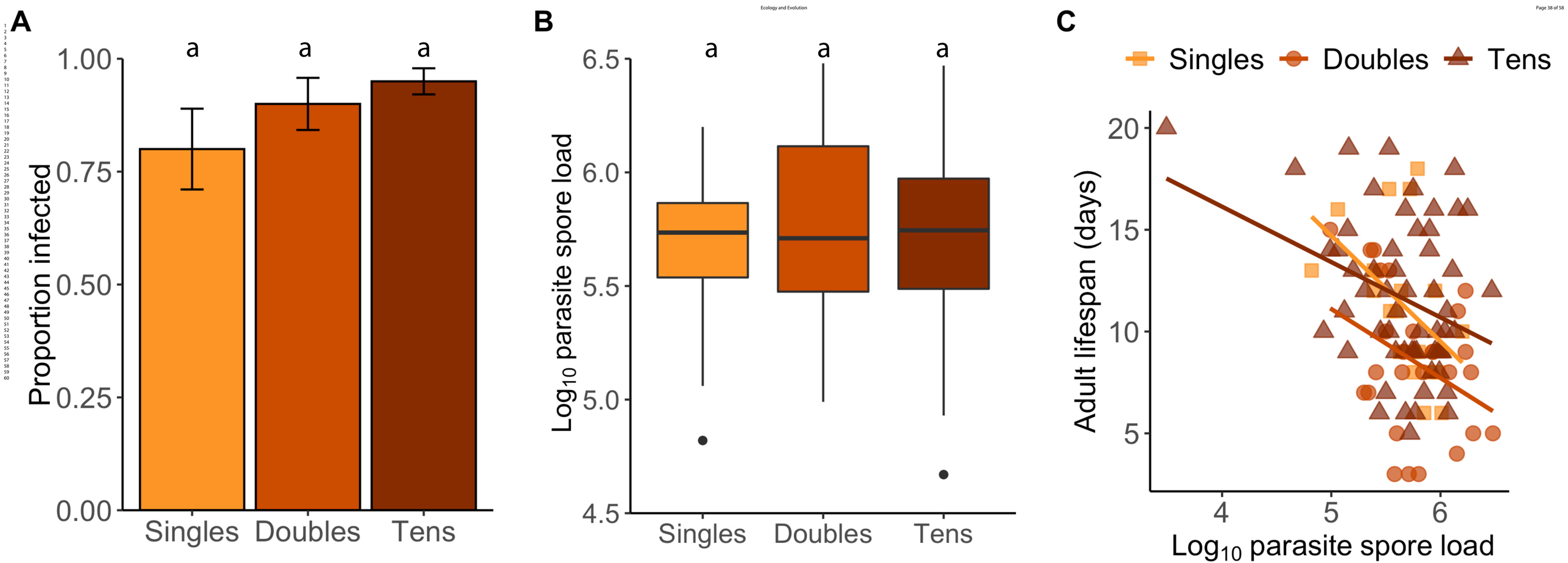
**Scott M. Villa**: Data curation (supporting); Formal analysis (supporting); Methodology (supporting); Software (supporting); Writing-review & editing (supporting).

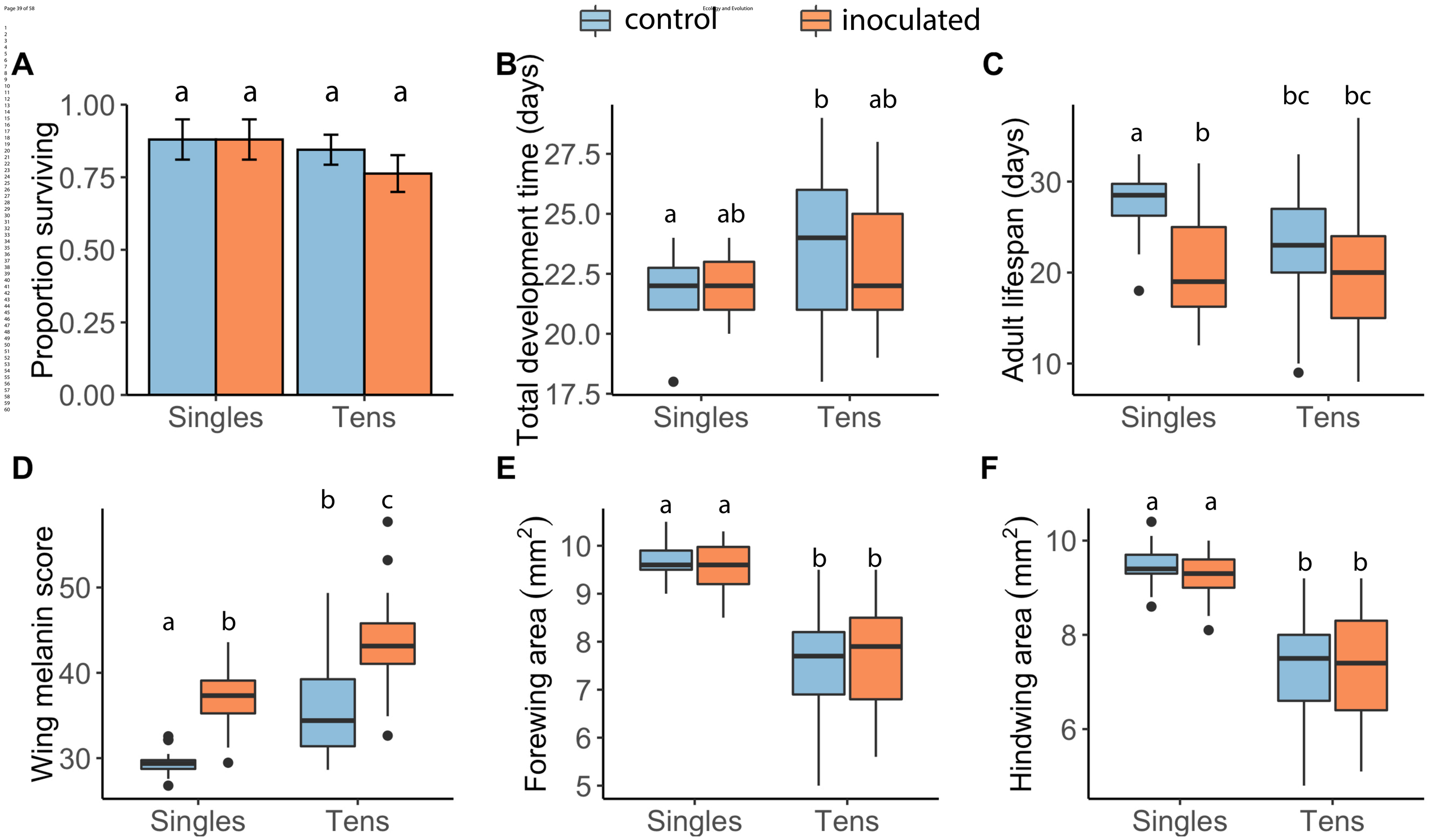
**Jacobus C. de Roode**: Conceptualization (supporting); Data curation (supporting); Formal analysis (supporting); Funding acquisition (lead); Investigation (supporting); Methodology (supporting); Project administration (supporting); Resources (lead); Supervision (lead); Writing-original draft (supporting); Writing-review & editing (supporting).

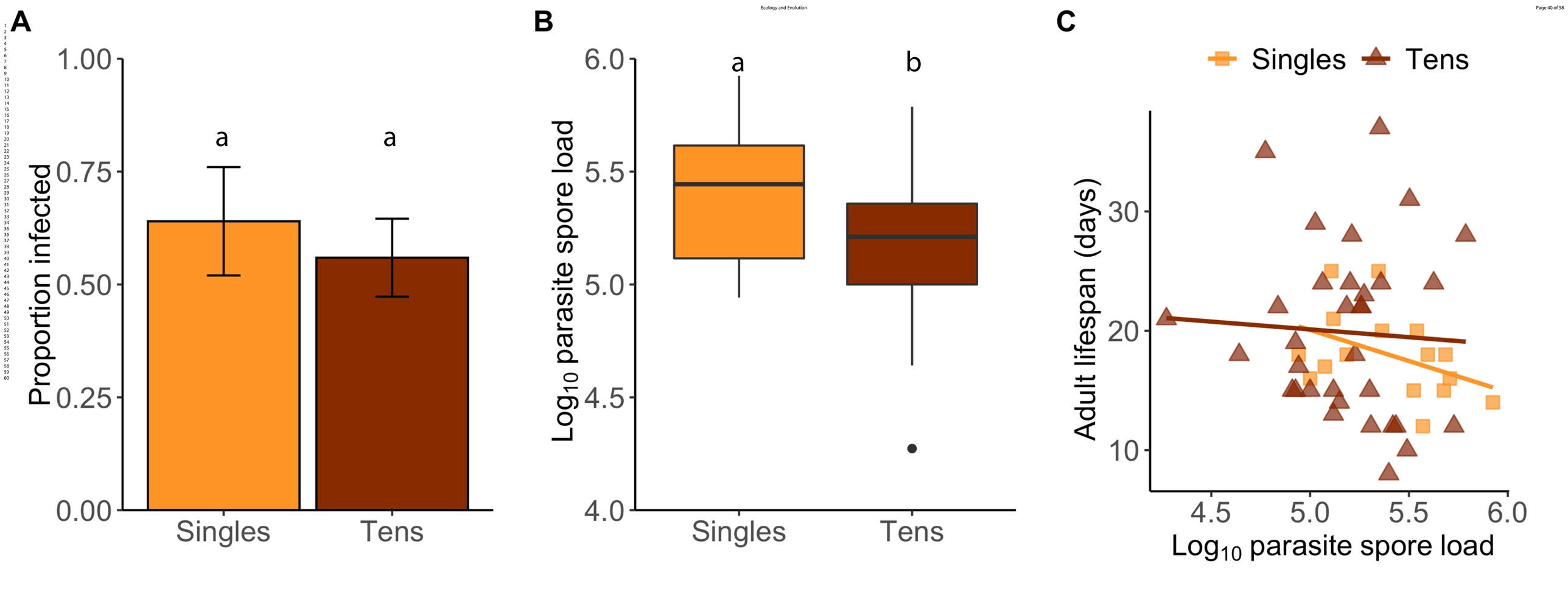
**Acknowledgments**

We would like to thank Isabella Cantor, Joselyne Chavez, Erik Edwards and Elizabeth Sun for their assistance with the experiments. AAM and SMV were supported by NIH/NIGMS K12 Postdoctoral Fellowship at Emory University (5K12GM000680-19). This work was supported by NSF grant DEB-1754431 to JCdR.


A
B
C
control
inoculated
Proportion surviving
1.00
0.75
0.50
0.25
0.00
a a a a a a
Singles Doubles Tens
Total development time (days)
21
20
19
18
ab b ab ab ac ac
Adult lifespan (days)
30
20
10
a b a b c b
D
E
F
Wing melanin score
40
35
30
ab abc ab cd ab cd
Forewing area (mm2)
10
9
8
a a a a a a
Hindwing area (mm2)
10
9
8
a a a a a a


A
a
a
a
1.00
0.75
0.50
0.25
0.00
Proportion infected
Singles
Doubles
Tens
B
a
a
a
6.5
6.0
5.5
5.0
4.5
Log10 parasite spore load
Singles
Doubles
Tens
C
Singles
Doubles
Tens
20
15
10
5
Adult lifespan (days)
4
5
6
Log10 parasite spore load


control
inoculated
A
Proportion surviving
1.00
0.75
0.50
0.25
0.00
a a a a
Singles
Tens
B
Total development time (days)
27.5
25.0
22.5
20.0
17.5
a ab b ab
Singles
Tens
C
Adult lifespan (days)
30
20
10
a b bc bc
Singles
Tens
D
Wing melanin score
50
40
30
a b b c
Singles
Tens
E
Forewing area (mm2)
10
9
8
7
6
5
a a b b
Singles
Tens
F
Hindwing area (mm2)
10
8
6
a a b b
Singles
Tens


A
1.00
0.75
0.50
0.25
0.00
Proportion infected
a
a
Singles
Tens
B
6.0
5.5
5.0
4.5
4.0
Log10 parasite spore load
a
b
Singles
Tens
C
Singles
Tens
30
20
10
Adult lifespan (days)
4.5
5.0
5.5
6.0
Log10 parasite spore load

**Appendix**

To accompany **Crowding does not affect monarch butterflies' resistance to a protozoan parasite**
by Wajd Alaidrous, Scott M. Villa, Jacobus C. de Roode, Ania A. Majewska

**Table S1**. Results of models investigating the effect of larval density on (a) survival, (b) development time, and lifespan in the unlimited food experiment. Microcosm was included as a random effect in all linear models. Significant effect sizes (P-value ≤ 0.05) appear in bold.

a)

| Response Variable | Fixed Effect | Estimate (SE) | z value | P-value |
|---|---|---|---|---|
| Immature survival (0/1) | Density: Doubles | 0.20 (0.86) | 0.23 | 0.82 |
| | Density: Tens | -0.23 (0.72) | -0.32 | 0.75 |
| | Inoculation: Inoculated | 0.36 (0.65) | 0.55 | 0.58 |

b)

| Response Variable | Fixed Effect | Estimate (SE) | t value | d.f. | P-value |
|---|---|---|---|---|---|
| Larval development time | Density: Doubles | -0.17 (0.15) | -1.14 | 128.32 | 0.26 |
| | Density: Tens | -0.22 (0.14) | -1.51 | 30.79 | 0.14 |
| | Inoculation: Inoculated | 0.30 (0.15) | 1.93 | 180.99 | 0.06 |
| | **Sex: Male** | **0.14 (0.07)** | **1.99** | **199.50** | **0.05** |
| | Density: Doubles x Inoculation: Inoculated | -0.14 (0.21) | -0.66 | 131.77 | 0.51 |
| | Density: Tens x Inoculation: Inoculated | -0.25 (0.21) | -1.22 | 32.66 | 0.23 |

| Response Variable | Fixed Effect | Estimate (SE) | t value | d.f. | P-value |
|---|---|---|---|---|---|
| Pupal development time | Density: Doubles | 0.06 (0.12) | 0.47 | 121.88 | 0.64 |
| | Density: Tens | -0.07 (0.11) | -0.61 | 19.14 | 0.55 |
| | Inoculation: Inoculated | 0.06 (0.12) | 0.48 | 187.12 | 0.63 |
| | **Sex: Male** | **0.53 (0.06)** | **9.01** | **199.93** | **<0.001** |
| | Density: Doubles x Inoculation: Inoculated | -0.14 (0.17) | -0.81 | 125.95 | 0.42 |
| | Density: Tens x Inoculation: Inoculated | -0.14 (0.16) | -0.89 | 20.55 | 0.38 |
| Total development time | Density: Doubles | -0.11 (0.18) | -0.62 | 170.86 | 0.54 |
| | Density: Tens | -0.28 (0.17) | -1.68 | 52.00 | 0.10 |
| | Inoculation: Inoculated | 0.36 (0.19) | 1.83 | 198.23 | 0.07 |
| | **Sex: Male** | **0.68 (0.09)** | **7.32** | **201.58** | **<0.001** |
| | Density: Doubles x Inoculation: Inoculated | -0.28 (0.27) | -1.03 | 173.10 | 0.30 |
| | Density: Tens x Inoculation: Inoculated | -0.39 (0.24) | -1.58 | 55.19 | 0.12 |
| Adult lifespan | Density: Doubles | -1.61 (1.13) | -1.42 | 130.76 | 0.16 |
| | **Density: Tens** | **3.10 (1.09)** | **2.84** | **31.77** | **0.01** |
| | **Inoculation: Inoculated** | **-9.03 (1.18)** | **-7.67** | **181.82** | **<0.001** |
| | **Sex: Male** | **-1.68 (0.55)** | **-3.04** | **197.53** | **<0.01** |

| Response Variable | Fixed Effect | Estimate (SE) | t value | d.f. | P-value |
|---|---|---|---|---|---|
| | Density: Doubles x Inoculation: Inoculated | -0.50 (1.64) | -0.31 | 134.17 | 0.76 |
| | **Density: Tens x Inoculation: Inoculated** | **-3.14 (1.57)** | **-2.00** | **33.44** | **0.05** |

13

**Table S2.** Results of models investigating the effect of larval density on wing areas (forewing and hindwing), and melanin score in the unlimited food experiment. Microcosm was included as a random effect in all linear models. Significant effect sizes (P-value ≤ 0.05) appear in bold.

| Response Variable | Fixed Effect | Estimate (SE) | t value | d.f. | P-value |
|---|---|---|---|---|---|
| Forewing area | Density: Doubles | -0.10 (0.17) | -0.60 | 120.67 | 0.55 |
| | Density: Tens | -0.18 (0.17) | -1.05 | 40.81 | 0.30 |
| | Inoculation: Inoculated | -0.16 (0.17) | -0.97 | 163.11 | 0.33 |
| | Sex: Male | 0.10 (0.08) | 1.37 | 187.93 | 0.17 |
| | Density: Doubles x Inoculation: Inoculated | 0.01 (0.24) | 0.06 | 123.85 | 0.95 |
| | Density: Tens x Inoculation: Inoculated | 0.28 (0.24) | 1.14 | 42.01 | 0.26 |
| Hindwing area | Density: Doubles | -0.08 (0.18) | -0.43 | 129.49 | 0.66 |
| | Density: Tens | -0.13 (0.18) | -0.71 | 46.79 | 0.48 |
| | Inoculation: Inoculated | -0.20 (0.18) | -1.06 | 168.39 | 0.29 |
| | **Sex: Male** | **0.18 (0.08)** | **2.14** | **189.21** | **0.04** |
| | Density: Doubles x Inoculation: Inoculated | -0.14 (0.26) | -0.56 | 132.51 | 0.57 |
| | Density: Tens x Inoculation: Inoculated | 0.11 (0.26) | 0.43 | 48.13 | 0.67 |
| Melanin score | Density: Doubles | -0.66 (0.73) | -0.90 | 124.38 | 0.37 |
| | Density: Tens | 0.66 (0.74) | 0.89 | 40.47 | 0.38 |
| | Inoculation: Inoculated | 1.38 (0.76) | 1.81 | 166.27 | 0.07 |

| Response Variable | Fixed Effect | Estimate (SE) | t value | d.f. | P-value |
|---|---|---|---|---|---|
| | **Sex: Male** | **-0.86 (0.35)** | **-2.49** | **187.10** | **0.01** |
| | **Density: Doubles x Inoculation: Inoculated** | **2.28 (1.07)** | **2.14** | **128.01** | **0.03** |
| | Density: Tens x Inoculation: Inoculated | 0.40 (1.07) | 0.37 | 42.49 | 0.71 |

**Table S3.** Results of models investigating the effect of larval density on infection status, spore load, and tolerance in the unlimited food experiment. Microcosm was included as a random effect in all linear models. Significant effect sizes (P-value ≤ 0.05) appear in bold.

a)

| Response Variable | Fixed Effect | Estimate (SE) | z value | P-value |
|---|---|---|---|---|
| Infection (0/1) | Density: Doubles | 0.30 (1.04) | 0.29 | 0.77 |
| | Density: Tens | 1.74 (1.25) | 1.39 | 0.16 |
| | Sex: Male | -1.43 (1.15) | -1.25 | 0.21 |

b)

| Response Variable | Fixed Effect | Estimate (SE) | t value | d.f. | P-value |
|---|---|---|---|---|---|
| Spore load | Density: Doubles | 0.11 (0.13) | 0.80 | 84.02 | 0.43 |
| | Density: Tens | -0.03 (0.12) | -0.22 | 39.00 | 0.83 |
| | Sex: Male | -0.14 (0.09) | -1.66 | 92.01 | 0.10 |
| Tolerance (adult lifespan) | **Spore load** | **-7.79 (2.61)** | **-3.45** | **83.70** | **<0.001** |
| | Density: Doubles | -17.83 (15.95) | -1.12 | 80.72 | 0.26 |
| | Density: Tens | -25.32 (13.73) | -1.84 | 86.38 | 0.07 |
| | Spore load x Density: Doubles | 2.76 (2.79) | 0.99 | 80.82 | 0.33 |
| | Spore load x Density: Tens | 4.51 (2.42) | 1.87 | 86.56 | 0.07 |
| | **Sex: Male** | **-3.31 (0.64)** | **-5.16** | **86.63** | **<0.001** |

**Table S4.** Results of models investigating the effect of larval density on (a) survival, (b) development time, and lifespan in the food limitation experiment. Microcosm was included as a random effect in all linear models. Significant effect sizes (P-value ≤ 0.05) appear in bold.

a)

| Response Variable | Fixed Effect | Estimate (SE) | z value | P-value |
|---|---|---|---|---|
| Immature survival (0/1) | Density: Tens | -0.61 (0.54) | -1.13 | 0.26 |
| | Inoculation: Inoculated | -0.37 (0.49) | -0.76 | 0.45 |

b)

| Response Variable | Fixed Effect | Estimate (SE) | t value | d.f. | P-value |
|---|---|---|---|---|---|
| Larval development time | **Density: Tens** | **1.83 (0.54)** | **3.40** | **61.77** | **0.001** |
| | Inoculation: Inoculated | 0.51 (0.59) | 0.87 | 130.02 | 0.38 |
| | **Sex: Male** | **1.04 (0.33)** | **3.32** | **132.96** | **0.001** |
| | Density: Tens x Inoculation: Inoculated | -1.46 (0.77) | -1.90 | 62.67 | 0.06 |
| Pupal development time | Density: Tens | -0.08 (0.18) | -0.47 | 51.17 | 0.64 |
| | Inoculation: Inoculated | -0.15 (0.20) | -0.78 | 129.81 | 0.44 |
| | **Sex: Male** | **0.53 (0.11)** | **4.78** | **133.00** | **<0.001** |
| | Density: Tens x Inoculation: Inoculated | 0.18 (0.25) | 0.69 | 51.89 | 0.49 |
| Total development time | **Density: Tens** | **1.75 (0.65)** | **2.70** | **59.47** | **0.01** |
| | Inoculation: Inoculated | -0.36 (0.70) | -0.52 | 128.85 | 0.61 |
| | **Sex: Male** | **1.63 (0.39)** | **4.17** | **132.83** | **<0.001** |

| Response Variable | Fixed Effect | Estimate (SE) | t value | d.f. | P-value |
|---|---|---|---|---|---|
| | Density: Tens x Inoculation: Inoculated | -1.28 (0.93) | -1.38 | 60.42 | 0.17 |
| Adult lifespan | **Density: Tens** | **-4.67 (1.50)** | **-3.13** | **133.00** | **<0.01** |
| | **Inoculation: Inoculated** | **-7.11 (1.76)** | **-4.05** | **133.00** | **<0.001** |
| | Sex: Male | -0.49 (1.00) | -0.49 | 133.00 | 0.63 |
| | **Density: Tens x Inoculation: Inoculated** | **4.44 (2.13)** | **2.08** | **133.00** | **0.04** |

32

**Table S5.** Results of linear models investigating the effect of larval density on wing areas (forewing and hindwing), and melanin score in the food limitation experiment. Microcosm was included as a random effect in all linear models. Significant effect sizes (P-value ≤ 0.05) appear in bold.

| Response Variable | Fixed Effect | Estimate (SE) | t value | d.f. | P-value |
|---|---|---|---|---|---|
| Forewing area | **Density: Tens** | **-2.09 (0.23)** | **-8.95** | **83.17** | **<0.001** |
| | Inoculation: Inoculated | -0.09 (0.27) | -0.34 | 131.72 | 0.73 |
| | Sex: Male | 0.26 (0.15) | 1.73 | 131.44 | 0.08 |
| | Density: Tens x Inoculation: Inoculated | 0.13 (0.33) | 0.41 | 82.56 | 0.68 |
| Hindwing area | **Density: Tens** | **-2.15 (0.24)** | **-9.07** | **80.15** | **<0.001** |
| | Inoculation: Inoculated | -0.12 (0.28) | -0.47 | 132.65 | 0.64 |
| | **Sex: Male** | **0.33 (0.16)** | **2.09** | **132.54** | **0.04** |
| | Density: Tens x Inoculation: Inoculated | 0.09 (0.34) | 0.29 | 80.54 | 0.77 |
| Melanin score | **Density: Tens** | **6.00 (1.13)** | **5.30** | **64.20** | **<0.001** |
| | **Inoculation: Inoculated** | **7.32 (1.26)** | **5.80** | **126.87** | **<0.001** |
| | Sex: Male | -1.27 (0.72) | -1.80 | 129.00 | 0.07 |

| Response Variable | Fixed Effect | Estimate (SE) | t value | d.f. | P-value |
|---|---|---|---|---|---|
| | Density: Tens x Inoculation: Inoculated | 0.71 (1.63) | 0.43 | 65.12 | 0.67 |

**Table S6.** Results of linear models investigating the effect of larval density on (a) infection status, (b) spore load, size corrected spore load, and tolerance in the food limitation experiment. Microcosm was included as a random effect in all models. Significant effect sizes (P-value ≤ 0.05) appear in bold.

a)

| Response Variable | Fixed Effect | Estimate (SE) | z value | P-value |
|---|---|---|---|---|
| Infection (0/1) | Density: Tens | 0.08 (0.66) | 0.12 | 0.91 |
| | Sex: Male | -0.21 (0.60) | -0.36 | 0.72 |

b)

| Response Variable | Fixed Effect | Estimate (SE) | t value | d.f. | P-value |
|---|---|---|---|---|---|
| Spore load | **Density: Tens** | **-0.21 (0.10)** | **-2.10** | **16.83** | **0.05** |
| | Sex: Male | -0.05 (0.09) | -0.59 | 45.83 | 0.56 |
| Size-corrected spore load | Density: Tens | -0.06 (0.13) | -0.53 | 17.05 | 0.60 |
| | Sex: Male | -0.04 (0.04) | -1.02 | 188.54 | 0.31 |
| Tolerance (adult lifespan) | Spore load | -5.20 (5.63) | -0.92 | 44.00 | 0.36 |
| | Density: Tens | -19.41 (35.79) | -0.54 | 44.00 | 0.59 |
| | Spore load x Density: Tens | 3.90 (6.60) | 0.58 | 44.00 | 0.56 |
| | Sex: Male | 0.03 (1.85) | 0.02 | 44.00 | 0.99 |

**Table S7**. Post hoc pair-wise Tukey contrast results on probability of survival model (food unlimited experiment; Fig. 1A). Significant effect contrasts (P-value ≤ 0.05) appear in bold.

| Contrast | Estimate (SE) | z value | P value |
|---|---|---|---|
| Doubles Control - Singles Control == 0 | -6.67e-02 (6.51e-02) | -1.02 | 0.91 |
| Tens Control - Singles Control == 0 | -1.02e-01 (5.83e-02) | -1.75 | 0.50 |
| Singles Inoculated - Singles Control == 0 | -1.20e-01 (6.78e-02) | -1.77 | 0.48 |
| Doubles Inoculated - Singles Control == 0 | -3.33e-02 (6.51e-02) | -0.51 | 1.00 |
| Tens Inoculated - Singles Control == 0 | -3.33e-02 (5.82e-02) | -0.57 | 0.99 |
| Tens Control - Doubles Control == 0 | -3.51e-02 (5.52e-02) | -0.64 | 0.99 |
| Singles Inoculated - Doubles Control == 0 | -5.33e-02 (6.51e-02) | -0.82 | 0.96 |
| Doubles Inoculated - Doubles Control == 0 | 3.33e-02 (6.23e-02) | 0.54 | 0.99 |
| Tens Inoculated - Doubles Control == 0 | 3.33e-02 (5.50e-02) | 0.61 | 0.99 |
| Singles Inoculated - Tens Control == 0 | -1.83e-02 (5.83e-02) | -0.31 | 1.00 |
| Doubles Inoculated - Tens Control == 0 | 6.84e-02 (5.52e-02) | 1.24 | 0.81 |
| Tens Inoculated - Tens Control == 0 | 6.84e-02 (4.67e-02) | 1.46 | 0.68 |
| Doubles Inoculated - Singles Inoculated == 0 | 8.67e-02 (6.51e-02) | 1.33 | 0.77 |
| Tens Inoculated - Singles Inoculated == 0 | 8.67e-02 (5.82e-02) | 1.49 | 0.67 |
| Tens Inoculated - Doubles Inoculated == 0 | 2.57e-16 (5.50e-02) | 0.00 | 1.00 |

**Table S8**. Post hoc pair-wise Tukey contrast results on total development model (food unlimited experiment; Fig. 1B). Significant effect contrasts (P-value ≤ 0.05) appear in bold.

| Contrast | Estimate (SE) | z value | P value |
|---|---|---|---|
| Doubles Control - Singles Control == 0 | -0.10 (0.21) | -0.49 | 0.99 |
| Tens Control - Singles Control == 0 | -0.21 (0.18) | -1.14 | 0.86 |
| Singles Inoculated - Singles Control == 0 | 0.40 (0.22) | 1.83 | 0.44 |
| Doubles Inoculated - Singles Control == 0 | -0.05 (0.20) | -0.24 | 0.99 |
| Tens Inoculated - Singles Control == 0 | -0.29 (0.18) | -1.60 | 0.60 |
| Tens Control - Doubles Control == 0 | -0.11(0.18) | -0.62 | 0.99 |
| Singles Inoculated - Doubles Control == 0 | 0.50 (0.21) | 2.34 | 0.17 |
| Doubles Inoculated - Doubles Control == 0 | 0.05 (0.20) | 0.26 | 0.99 |
| Tens Inoculated - Doubles Control == 0 | -0.19 (0.18) | -1.08 | 0.89 |
| **Singles Inoculated - Tens Control == 0** | **0.61 (0.19)** | **3.18** | **0.02** |
| Doubles Inoculated - Tens Control == 0 | 0.16 (0.18) | 0.92 | 0.94 |
| Tens Inoculated - Tens Control == 0 | -0.08 (0.15) | -0.54 | 0.99 |
| Doubles Inoculated - Singles Inoculated == 0 | -0.45 (0.21) | -2.12 | 0.28 |
| **Tens Inoculated - Singles Inoculated == 0** | **-0.69 (0.19)** | **-3.63** | **<0.01** |
| Tens Inoculated - Doubles Inoculated == 0 | -0.24 (0.17) | -1.39 | 0.73 |

**Table S9.** Post hoc pair-wise Tukey contrast results on adult lifespan model (food unlimited experiment; Fig. 1C). Significant effect contrasts (P-value ≤ 0.05) appear in bold.

| Contrast | Estimate (SE) | z value | P value |
|---|---|---|---|
| Doubles Control - Singles Control == 0 | -1.61 (1.13) | -1.42 | 0.71 |
| **Tens Control - Singles Control == 0** | **3.10 (1.10)** | **2.84** | **0.05** |
| **Singles Inoculated - Singles Control == 0** | **-9.03 (1.18)** | **-7.67** | **<0.001** |
| **Doubles Inoculated - Singles Control == 0** | **-11.14 (1.15)** | **-9.69** | **<0.001** |
| **Tens Inoculated - Singles Control == 0** | **-9.07 (1.09)** | **-8.34** | **<0.001** |
| **Tens Control - Doubles Control == 0** | **4.71 (1.08)** | **4.35** | **<0.001** |
| **Singles Inoculated - Doubles Control == 0** | **-7.42 (1.17)** | **-6.34** | **<0.001** |
| **Doubles Inoculated - Doubles Control == 0** | **-9.53 (1.15)** | **-8.34** | **<0.001** |
| **Tens Inoculated - Doubles Control == 0** | **-7.46 (1.08** | **-6.91** | **<0.001** |
| **Singles Inoculated - Tens Control == 0** | **-12.13 (1.13)** | **-10.73** | **<0.001** |
| **Doubles Inoculated - Tens Control == 0** | **-14.24 (1.10)** | **-12.91** | **<0.001** |
| **Tens Inoculated - Tens Control == 0** | **-12.17 (1.04)** | **-11.72** | **<0.001** |
| Doubles Inoculated - Singles Inoculated == 0 | -2.11 (1.19) | -1.78 | 0.48 |
| Tens Inoculated - Singles Inoculated == 0 | -0.04 (1.13) | -0.03 | 1.00 |
| Tens Inoculated - Doubles Inoculated == 0 | 2.07 (1.10) | 1.88 | 0.41 |

**Table S10.** Post hoc pair-wise Tukey contrast results on wing melanin score model (food unlimited experiment; Fig. 1D). Significant effect contrasts (P-value ≤ 0.05) appear in bold.

| Contrast | Estimate (SE) | z value | P value |
|---|---|---|---|
| Doubles Control - Singles Control == 0 | -0.66 (0.73) | -0.90 | 0.95 |
| Tens Control - Singles Control == 0 | 0.66 (0.74) | 0.89 | 0.95 |
| Singles Inoculated - Singles Control == 0 | 1.38 (0.76) | 1.81 | 0.46 |
| **Doubles Inoculated - Singles Control == 0** | **3.00 (0.74)** | **4.04** | **<0.001** |
| **Tens Inoculated - Singles Control == 0** | **2.44 (0.74)** | **3.31** | **0.01** |
| Tens Control - Doubles Control == 0 | 1.32 (0.74) | 1.77 | 0.46 |
| Singles Inoculated - Doubles Control == 0 | 2.04 (0.77) | 2.66 | 0.08 |
| **Doubles Inoculated - Doubles Control == 0** | **3.66 (0.75)** | **4.91** | **<0.001** |
| **Tens Inoculated - Doubles Control == 0** | **3.10 (0.74)** | **4.19** | **<0.001** |
| Singles Inoculated - Tens Control == 0 | 0.72 (0.78) | 0.93 | 0.94 |
| **Doubles Inoculated - Tens Control == 0** | **2.34 (0.76)** | **3.10** | **0.02** |
| Tens Inoculated - Tens Control == 0 | 1.78 (0.75) | 2.38 | 0.16 |
| Doubles Inoculated - Singles Inoculated == 0 | 1.62 (0.78) | 2.09 | 0.29 |
| Tens Inoculated - Singles Inoculated == 0 | 1.06 (0.77) | 1.37 | 0.74 |
| Tens Inoculated - Doubles Inoculated == 0 | -0.56 (0.75) | -0.75 | 0.98 |

**Table S11.** Post hoc pair-wise Tukey contrast results on forewing area model (food unlimited experiment; Fig. 1E). Significant effect contrasts (P-value ≤ 0.05) appear in bold.

| Contrast | Estimate (SE) | z value | P value |
|---|---|---|---|
| Doubles Control - Singles Control == 0 | -0.10 (0.17) | -0.60 | 0.99 |
| Tens Control - Singles Control == 0 | -0.18 (0.17) | -1.05 | 0.90 |
| Singles Inoculated - Singles Control == 0 | -0.16 (0.17) | -0.97 | 0.93 |
| Doubles Inoculated - Singles Control == 0 | -0.25 (0.17) | -1.48 | 0.68 |
| Tens Inoculated - Singles Control == 0 | -0.06 (0.17) | -0.38 | 1.00 |
| Tens Control - Doubles Control == 0 | -0.08 (0.17) | -0.46 | 1.00 |
| Singles Inoculated - Doubles Control == 0 | -0.06 (0.17) | -0.38 | 1.00 |
| Doubles Inoculated - Doubles Control == 0 | -0.15 (0.17) | -0.88 | 0.95 |
| Tens Inoculated - Doubles Control == 0 | 0.04 (0.17) | 0.21 | 1.00 |
| Singles Inoculated - Tens Control == 0 | 0.01 (0.18) | 0.08 | 1.00 |
| Doubles Inoculated - Tens Control == 0 | -0.07 (0.17) | -0.41 | 1.00 |
| Tens Inoculated - Tens Control == 0 | 0.11 (0.18) | 0.65 | 0.99 |
| Doubles Inoculated - Singles Inoculated == 0 | -0.08 (0.17) | -0.49 | 1.00 |
| Tens Inoculated - Singles Inoculated == 0 | 0.10 (0.17) | 0.58 | 0.99 |
| Tens Inoculated - Doubles Inoculated == 0 | 0.19 (0.17) | 1.07 | 0.89 |

**Table S12.** Post hoc pair-wise Tukey contrast results on hindwing area model (food unlimited experiment; Fig. 1F). Significant effect contrasts (P-value ≤ 0.05) appear in bold.

| Contrast | Estimate (SE) | z value | P value |
|---|---|---|---|
| Doubles Control - Singles Control == 0 | -0.08 (0.18) | -0.43 | 1.00 |
| Tens Control - Singles Control == 0 | -0.13 (0.18) | -0.71 | 0.98 |
| Singles Inoculated - Singles Control == 0 | -0.19 (0.18) | -1.06 | 0.90 |
| Doubles Inoculated - Singles Control == 0 | -0.41 (0.18) | -2.30 | 0.19 |
| Tens Inoculated - Singles Control == 0 | -0.21 (0.18) | -1.16 | 0.86 |
| Tens Control - Doubles Control == 0 | -0.05 (0.18) | -0.29 | 1.00 |
| Singles Inoculated - Doubles Control == 0 | -0.12 (0.18) | -0.63 | 0.99 |
| Doubles Inoculated - Doubles Control == 0 | -0.34 (0.18) | -1.86 | 0.43 |
| Tens Inoculated - Doubles Control == 0 | -0.13 (0.18) | -0.73 | 0.98 |
| Singles Inoculated - Tens Control == 0 | -0.06 (0.19) | -0.34 | 1.00 |
| Doubles Inoculated - Tens Control == 0 | -0.28 (0.19) | -1.53 | 0.65 |
| Tens Inoculated - Tens Control == 0 | -0.08 (0.19) | -0.43 | 1.00 |
| Doubles Inoculated - Singles Inoculated == 0 | -0.22 (0.19) | -1.19 | 0.84 |
| Tens Inoculated - Singles Inoculated == 0 | -0.02 (0.19) | -0.09 | 1.00 |
| Tens Inoculated - Doubles Inoculated == 0 | 0.20 (0.19) | 1.11 | 0.88 |

**Table S13.** Post hoc pair-wise Tukey contrast results on proportion infected model (food unlimited experiment; Fig. 2A). Significant effect contrasts (P-value ≤ 0.05) appear in bold.

| Contrast | Estimate (SE) | z value | P value |
|---|---|---|---|
| Doubles Inoculated - Singles Inoculated == 0 | 0.02 (0.07) | 0.22 | 0.97 |
| Tens Inoculated - Singles Inoculated == 0 | 0.07 (0.08) | 0.86 | 0.66 |
| Tens Inoculated - Doubles Inoculated == 0 | 0.05 (0.08) | 0.66 | 0.79 |

**Table S14.** Post hoc pair-wise Tukey contrast results on $\log_{10}$ parasite spore load model (food unlimited experiment; Fig. 2B). Significant effect contrasts (P-value ≤ 0.05) appear in bold.

| Contrast | Estimate (SE) | z value | P value |
|---|---|---|---|
| Doubles Inoculated - Singles Inoculated == 0 | 0.19 (0.41) | 0.47 | 0.89 |
| Tens Inoculated - Singles Inoculated == 0 | 0.36 (0.43) | 0.84 | 0.68 |
| Tens Inoculated - Doubles Inoculated == 0 | 0.17 (0.43) | 0.39 | 0.92 |

**Table S15**. Post hoc pair-wise Tukey contrast results on survival model (food limited experiment Fig. 3A). Significant effect contrasts (P-value ≤ 0.05) appear in bold.

| Contrast | Estimate (SE) | z value | P value |
|---|---|---|---|
| Tens Control - Singles Control == 0 | -3.53e-02 (9.85e-02) | -0.36 | 0.98 |
| Singles Inoculated - Singles Control == 0 | 1.49e-16 (1.08e-01) | 0.00 | 1.00 |
| Tens Inoculated - Singles Control == 0 | -1.17e-01 (9.83e-02) | -1.19 | 0.63 |
| Singles Inoculated - Tens Control == 0 | 3.53e-02 (9.85e-02) | 0.36 | 0.98 |
| Tens Inoculated – Tens Control == 0 | -8.19e-02 (8.81e-02) | -0.93 | 0.79 |
| Tens Inoculated - Singles Inoculated == 0 | -1.17e-01 (9.83e-02) | -1.19 | 0.63 |

**Table S16.** Post hoc pair-wise Tukey contrast results on total development model (food limited experiment Fig. 3B). Significant effect contrasts (P-value ≤ 0.05) appear in bold.

| Contrast | Estimate (SE) | z value | P value |
|---|---|---|---|
| **Tens Control - Singles Control == 0** | **1.75 (0.65)** | **2.70** | **0.04** |
| Singles Inoculated - Singles Control == 0 | 0.36 (0.70) | 0.52 | 0.96 |
| Tens Inoculated - Singles Control == 0 | 0.83 (0.66) | 1.26 | 0.59 |
| Singles Inoculated - Tens Control == 0 | -1.39 (0.65) | -2.15 | 0.14 |
| Tens Inoculated - Tens Control == 0 | -0.92 (0.61) | -1.52 | 0.43 |
| Tens Inoculated - Singles Inoculated == 0 | 0.47 (0.66) | 0.71 | 0.89 |

**Table S17.** Post hoc pair-wise Tukey contrast results on adult lifespan model (food limited experiment, Fig. 3C). Significant effect contrasts (P-value ≤ 0.05) appear in bold.

| Contrast | Estimate (SE) | z value | P value |
|---|---|---|---|
| **Tens Control - Singles Control == 0** | **-4.67 (1.50)** | **-3.13** | **0.01** |
| **Singles Inoculated - Singles Control == 0** | **-7.11 (1.76)** | **-4.05** | **< 0.001** |
| **Tens Inoculated - Singles Control == 0** | **-7.34 (1.51)** | **-4.86** | **< 0.001** |
| Singles Inoculated - Tens Control == 0 | -2.44 (1.49) | -1.64 | 0.35 |
| Tens Inoculated - Tens Control == 0 | -2.67 (1.20) | -2.22 | 0.12 |
| Tens Inoculated - Singles Inoculated == 0 | -0.23 (1.52) | -0.15 | 0.99 |

**Table S18**. Post hoc pair-wise Tukey contrast results on wing melanin score model (food limited experiment, Fig. 3D). Significant effect contrasts (P-value ≤ 0.05) appear in bold.

| Contrast | Estimate (SE) | z value | P value |
|---|---|---|---|
| **Tens Control - Singles Control == 0** | **5.99 (1.13)** | **5.30** | **< 0.001** |
| **Singles Inoculated - Singles Control == 0** | **7.32 (1.26)** | **5.79** | **< 0.001** |
| **Tens Inoculated - Singles Control == 0** | **14.03 (1.16)** | **12.10** | **< 0.001** |
| Singles Inoculated - Tens Control == 0 | 1.32 (1.15) | 1.16 | 0.65 |
| **Tens Inoculated - Tens Control == 0** | **8.03 (1.04)** | **7.76** | **< 0.001** |
| **Tens Inoculated - Singles Inoculated == 0** | **6.71 (1.18)** | **5.69** | **< 0.001** |

**Table S19.** Post hoc pair-wise Tukey contrast results on forewing area model (food limited experiment, Fig. 3E). Significant effect contrasts (P-value ≤ 0.05) appear in bold.

| Contrast | Estimate (SE) | z value | P value |
|---|---|---|---|
| **Tens Control – Singles Control == 0** | **-2.12 (0.24)** | **-9.01** | **< 0.001** |
| Singles Inoculated – Singles Control == 0 | -0.14 (0.27) | -0.50 | 0.96 |
| **Tens Inoculated – Singles Control == 0** | **-2.05 (0.24)** | **-8.59** | **< 0.001** |
| **Singles Inoculated – Tens Control == 0** | **1.99 (0.23)** | **8.57** | **< 0.001** |
| Tens Inoculated – Tens Control == 0 | 0.07 (0.19) | 0.38 | 0.98 |
| **Tens Inoculated – Singles Inoculated == 0** | **-1.92 (0.24)** | **-8.15** | **< 0.001** |

**Table S20.** Post hoc pair-wise Tukey contrast results on hindwing area model (food limited experiment, Fig. 3F). Significant effect contrasts (P-value ≤ 0.05) appear in bold.

| Contrast | Estimate (SE) | z value | P value |
|---|---|---|---|
| **Tens Control – Singles Control == 0** | **-2.18 (0.24)** | **-9.09** | **< 0.001** |
| Singles Inoculated – Singles Control == 0 | -0.17 (0.28) | -0.62 | 0.93 |
| **Tens Inoculated – Singles Control == 0** | **-2.17 (0.24)** | **-8.94** | **< 0.001** |
| **Singles Inoculated – Tens Control == 0** | **2.01 (0.24)** | **8.37** | **< 0.001** |
| Tens Inoculated – Tens Control == 0 | 0.01 (0.20) | 0.03 | 1.00 |
| **Tens Inoculated – Singles Inoculated == 0** | **-2.00 (0.24)** | **-8.23** | **< 0.001** |

**Table S21.** Post hoc pair-wise Tukey contrast results on proportion infected model (food limited experiment, Fig. 4A). Significant effect contrasts (P-value ≤ 0.05) appear in bold.

| Contrast | Estimate (SE) | z value | P value |
|---|---|---|---|
| Tens Inoculated - Singles Inoculated == 0 | 0.08 (0.66) | 0.12 | 0.91 |

**Table S22.** Post hoc pair-wise Tukey contrast results on $\log_{10}$ parasite spore load model (food limited experiment, Fig. 4B). Significant effect contrasts (P-value ≤ 0.05) appear in bold.

| Contrast | Estimate (SE) | z value | P value |
|---|---|---|---|
| **Tens Inoculated - Singles Inoculated == 0** | **-0.21 (0.10)** | **-2.10** | **0.04** |