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Crowding and disease: effects of host density on response to infection in a butterfly–parasite interaction

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Abstract. 1. Hosts experiencing frequent variation in density are thought to benefit from allocating more resources to parasite defence when density is high ('density-dependent prophylaxis'). However, high density conditions can increase intra-specific competition and induce physiological stress, hence increasing host susceptibility to infection ('crowding-stress hypothesis').

2. We studied monarch butterflies (*Danaus plexippus*) and quantified the effects of larval rearing density on susceptibility to the protozoan parasite *Ophryocystis elektroscirrha*. Larvae were inoculated with parasite spores and reared at three density treatments: low, moderate, and high. We examined the effects of larval density on parasite loads, host survival, development rates, body size, and wing melanism.

3. Results showed an increase in infection probability with greater larval density. Monarchs in the moderate and high density treatments also suffered the greatest negative effects of parasite infection on body size, development rate, and adult longevity.

4. We observed greater body sizes and shorter development times for monarchs reared at moderate densities, and this was true for both unparasitised and parasite-treated monarchs. We hypothesise that this effect could result from greater larval feeding rates at moderate densities, combined with greater physiological stress at the highest densities.

5. Although monarch larvae are assumed to occur at very low densities in the wild, an analysis of continent-wide monarch larval abundance data showed that larval densities can reach high levels in year-round resident populations and during the late phase of the breeding season. Treatment levels used in our experiment captured ecologically-relevant variation in larval density observed in the wild.

Key Words. *Danaus plexippus*, density-dependent prophylaxis, host–parasite interaction, melanism, monarch butterfly, neogregarine protozoan, *Ophryocystis elektroscirrha.*

Introduction

It is often assumed that animals living in larger groups or at higher population densities should experience a greater risk of acquiring infectious diseases (Alexander, 1974; Freeland, 1976; Møller *et al.*, 1993; Krause & Ruxton, 2002; Moore, 2002; Altizer *et al.*, 2003). This is mainly because host contact rates and the transmission of parasites spread by close proximity among individuals are predicted to increase with host population density (Anderson & May, 1979, 1981; McCallum *et al.*, 2001; Lloyd-Smith *et al.*, 2005). Several field and experimental studies support this assumption; mammals (Freeland, 1979; Hoogland, 1979, 1995; Wilkinson, 1985), birds (Brown &

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Brown, 1986; Shields & Crook, 1987), and insects (Dwyer & Elkinton, 1993; Knell *et al.*, 1996; Ryder *et al.*, 2005) exhibit positive relationships between measures of parasite prevalence or intensity, and host population density or group size. For example, in the African army worm (*Spodoptera exempta*), high host density results in increased host activity (Reeson *et al.*, 2000), which directly affects contact rates between susceptible hosts and pathogens.

As a result of increased parasite risk, animals living at higher population densities are predicted to invest more resources in resistance to infection, including behavioural and immune defences (Møller & Erritzoe, 1996; Møller *et al.*, 2001). Within a species, increased host resistance in response to crowding or higher host density has been termed 'density-dependent prophylaxis' (Reeson *et al.*, 1998; Wilson & Reeson, 1998). Under this scenario, greater investment in parasite resistance is presumed to counter the risk of increased transmission (Barnes & Siva-Jothy, 2000; Wilson *et al.*, 2001). There is substantial evidence that animals experiencing higher densities do indeed show greater resistance to infectious diseases (Kunimi & Yamada, 1990; Goulson & Cory, 1995; Reeson *et al.*, 1998; Wilson *et al.*, 2002). For example, one study showed that in a phase-polymorphic moth species, dark-coloured larvae (the high density phenotype) exhibited greater immune defences than pale-coloured larvae (based on higher haemolymph and cuticular phenoloxidase activity and a stronger encapsulation response; Cotter *et al.*, 2004). Other studies have shown that larvae reared under higher densities tend to develop darker cuticular melanism (Simmonds & Blaney, 1986; Hagen *et al.*, 2003; Lee & Wilson, 2006). This is important because for some species, an increase in external melanism correlates with an increase in immune effector traits (Reeson *et al.*, 1998; Barnes & Siva-Jothy, 2000; Wilson *et al.*, 2001; Cotter *et al.*, 2004), although this trend does not hold for all insect species (Robb *et al.*, 2003; Pie *et al.*, 2005; Hagen *et al.*, 2006). It is also important to note that some studies have found no effect of host density on measures of immunity, including field crickets (Adamo, 2006) and termites (Pie *et al.*, 2005).

Animals living in high density populations might also experience more intense competition for resources. Thus, a second key hypothesis is that high density leads to physiological or nutritional stress, and that animals in crowded conditions will be more susceptible to infectious diseases relative to less crowded hosts. In insects, this hypothesis was initially examined by Steinhaus (1958) in studies of caterpillars and their natural pathogens. More recent experimental studies on lepidopteran hosts have shown that animals reared at higher densities experience reduced disease resistance and/or decreased time to death (Goulson & Cory, 1995; Reilly & Hajek, 2008). However, Brown *et al.* (2003) found no effect of host-resource stress on infection or immunity in bumblebees.

Here we ask how host rearing density affects the outcome of infection by a common protozoan parasite, *Ophryocystis elektroscirrha*, in monarch butterflies, *Danaus plexippus*. Parasitism by *O. elektroscirrha* occurs in all monarch populations examined to date, but prevalence varies widely both within and among populations (Leong *et al.*, 1997; Altizer *et al.*, 2000). Monarch densities in the wild vary over space and time (Ackery & Vane-Wright, 1984; Prysby & Oberhauser, 2004). Hence, it is possible that effects of host density on susceptibility to infection will affect patterns of infection in the wild. We reared monarch larval stages under low (single larva), moderate, and high densities and compared infection probability, development, and survival among treatments. We expected that monarchs reared under the highest density treatment would develop the fastest (based on time to pupation and adulthood), and show smaller body sizes than monarchs reared at lower densities. Monarchs reared under the highest densities could show greater susceptibility to infection, in support of the stress-disease hypothesis, and would thus experience greater lethal and sub-lethal effects of parasitism on host fitness. Alternatively, monarchs reared under high densities could invest more in disease resistance, in support of the density-dependent prophylaxis hypothesis. This could be manifested by darker wing coloration, which may correlate with resistance to infection (Barnes & Siva-Jothy, 2000).

Materials and methods

Host–parasite system

Monarch butterflies inhabit islands and continents worldwide (Ackery & Vane-Wright, 1984), migrate annually in temperate North America and Australia (Urquhart & Urquhart, 1978; James, 1993; Brower, 1995), and form resident populations that breed year-round in tropical locations such as South Florida and Hawaii (Stimson & Berman, 1990; Knight, 1998). Although monarchs generally lay eggs singly on host plants (Zalucki & Kitching, 1982; Farrey & Davis, 2004; Prysby & Oberhauser, 2004), multiple larvae can occupy the same plant, especially in areas where host plants are patchily distributed or rare. In support of this, observations of monarchs breeding year-round in South Florida indicate that it is not unusual to find plants with several larvae feeding on them (e.g. Brower, 1964; Farrey & Davis, 2004). By comparison, across the large breeding range of monarchs in North America, host plants (including common milkweed, *Asclepias syriaca*) are common and widespread, and larval densities per plant can be exceedingly low, with a single larva occurring on roughly one out of every 30–50 host plants examined (Prysby & Oberhauser, 2004). Other studies have shown that per-plant larval densities can vary over time and space in response to weather-related abiotic factors (Zalucki & Rochester, 1999; Fig. 1), and as monarch numbers increase over the course of a breeding season (Prysby & Oberhauser, 2004).

The neogregarine protozoan parasite *Ophryocystis elektroscirrha* occurs naturally in wild monarch populations (Leong *et al.*, 1997; Altizer *et al.*, 2000) and is transmitted when adult butterflies scatter parasite spores on eggs and milkweed leaves. After spores are ingested by larvae, emerging sporozoites penetrate the gut wall, migrate to the larval hypoderm, and undergo vegetative schizogony (McLaughlin & Myers, 1970). During the host pupal stage, the parasite undergoes sexual reproduction and haploid spores are formed 2–3 days

Fig. 1. Fifth instar monarch larvae feeding on *Asclepias syriaca* (common milkweed) in a field near Forestport, New York, USA, during summer 2007. Wild monarch larvae at this location are typically scattered at low density (a single larva per plant; Maureen Clark, MLMP pers. Obs.). However, in 2007, up to 8 eggs were laid on single plants, and multiple late instar larvae were seen feeding on some milkweeds, probably owing to low rainfall and scarcity of milkweeds during this year. (Photograph: Maureen Clark).

before adult butterflies eclose from their pupal cases. Infected butterflies emerge covered with dormant parasite spores on the outside of their bodies, concentrated primarily on the abdomen (McLaughlin & Myers, 1970; Leong *et al.*, 1992). Negative effects of *O. elektroscirrha* depend on the initial dose and the stage at which hosts are infected (De Roode *et al.*, 2007). They include pre-adult mortality, shorter adult longevity, smaller adult body sizes, smaller forewings, and lower flight ability (Altizer & Oberhauser, 1999; Bradley & Altizer, 2005; De Roode *et al.*, 2007; Lindsey & Altizer, 2008).

All monarch populations examined to date have been parasitised by *O. elektroscirrha,* and prevalence is highly variable across different regions (Leong *et al.*, 1997; Altizer *et al.*, 2000). Monarchs in resident populations that breed year round (i.e. in southern Florida and Hawaii) bear the highest parasite loads (over 70% heavily infected). Approximately 30% of monarchs from a migratory population in western North America are heavily infected (Leong *et al.*, 1992; Altizer *et al.*, 2000). Less than 8% of monarchs from the eastern migratory population (longest-distance migrants) are heavily infected (Altizer *et al.*, 2000). These differences among populations have persisted for many years and could be caused by differences in monarch migratory behaviour (Altizer *et al.*, 2000; Bradley & Altizer, 2005), local population densities, or environmental variation among sites. In support of a role for monarch density in affecting parasite prevalence, prevalence of *O. elektroscirrha* in eastern N. America increases from early spring to late summer, as might occur with increases in adult and larval abundance during the summer months (S. Altizer, unpubl. data, http://www.monarchparasites.org).

Monarch sources and mating design

Monarchs used in this experiment were the great-grand progeny of monarchs collected as larvae and adult butterflies from three sites in eastern N. America during August to October 2004: Virginia (Giles County), Georgia (Dekalb County), and New York (Tompkins County). All monarchs were examined for the presence of *O. elektroscirrha* according to Altizer *et al.* (2000) and only uninfected individuals were used to obtain progeny ($N_{\text{initial}} = 33$ adults). Captive monarchs were reared from egg to adult using a breeding design that eliminated the possibility of full-sib mating and maximised the contribution of initial founders to each generation (with N per generation >200). Eggs for this experiment were obtained from 15 females that oviposited onto potted greenhouse-reared *Asclepias incarnata*. Plants were transferred to a laboratory and maintained at 24 °C, and larvae remained on their natal plants until they reached the second instar.

Inoculation and host rearing

We used a fully factorial design where infection treatment (parasitised and control) and larval rearing density (low, moderate, and high) were experimental factors (Table 1). Parasite inoculum was derived from the abdomen of a monarch captured in Atlanta, GA, U.S.A. Following Altizer and Oberhauser (1999), we vortexed the abdomen for 5 min in 10 ml of distilled water and calibrated inoculum to a dose of 300 spores per larva using a haemocytometer. Control inoculum was prepared by vortexing the abdomen of an uninfected eastern adult monarch. We inoculated second instar larvae individually by pipetting 10-μl drops of inoculum onto 1-cm^2 milkweed pieces placed on dampened filter paper inside sterile 8.5-cm Petri dishes. Larvae were maintained singly in the Petri dish until they consumed all of the plant material, which occurred within 48 h.

After inoculation, larvae were transferred to plastic 3.8-l containers with mesh-screen lids and reared to adulthood in a laboratory exposed to ambient light (∼LD 14:10 h) and maintained at 26 °C. A total of 420 larvae were randomly assigned to density treatment groups as follows: one larva/container (low density), five larvae/container (moderate density), and 10 larvae/container (high density; Table 1). We checked containers twice daily, and at least once per day added fresh cuttings of greenhouse-raised milkweed (*A. incarnata*) to each container, removed frass, and maintained a clean, moist paper lining. We adjusted the total food supply so that the number of leaves per larva remained relatively constant by provisioning approximately five leaves per larva to each container per feeding. Milkweed cuttings were

Table 1. Number of monarchs used to initiate the experiment, and per cent surviving to adult eclosion, shown separately for each parasite treatment and larval rearing density.

	Density treatment			
Parasite treatment	Low (1 larva per container)	Moderate (5 larvae per container)	High (10 larvae per container)	Total
Control				
Initial number	40	60	110	210
Third Instar	40	55	88	183
Adult	22	28	47	97
Egg to adult survival*	55%	47%	43%	46%
Third instar to adult survival*	55%	51%	53%	53%
Parasitised	0	0	0	0
Parasitised				
Initial number	40	60	110	210
Third instar	39	56	105	200
Adult	18	40	33	91
Egg to adult survival*	45%	67%	30%	43%
Third instar to adult survival*	46%	71%	31%	46%
Parasitised	14 (78%)	34 (85%)	29 (88%)	77 (85%)

*Survival estimates used in the statistical analysis were based on the number of adult monarchs divided by the number of third instar larvae, because deaths of larvae from earlier instars were difficult to observe (larvae were frequently missing but no carcass was found). Overall survival (egg to adult) is shown for comparison.

held in florist tubes and sterilised by soaking in a 20% bleach solution for 20 min, and rinsing thoroughly in tap water prior to use.

After all monarchs in a container had pupated, containers were transferred to an adjacent laboratory maintained at 26 °C to avoid contaminating the larval rearing area with parasite spores. Pupal mass was measured on an analytic balance to the nearest 0.0001 g. Pupae were transferred to single 0.5-l plastic containers to avoid transfer of parasite spores among individual butterflies. We recorded the development time of monarchs based on the number of days from oviposition to pupation and eclosion. After adults emerged, we recorded the sex of each butterfly and placed adults individually into glassine envelopes 6–12 h post-eclosion. Monarchs were held at 24 °C without feeding, and mortality counts were taken daily to record adult longevity (in days). We used latex gloves to handle milkweed, monarchs, and inoculum; gloves were frequently changed and laboratory surfaces and utensils were sterilised with 20% bleach solution to prevent unintentional transmission of parasite spores.

Quantifying infection and monarch wing parameters

We assessed the infection status of each adult by estimating the total number of spores on the insects' abdomens. Upon death, the abdomen of each monarch was removed and placed into a vial containing 5 ml of deionised water. After vortexing at high speed for 15 min, a haemocytometer counting chamber was used to estimate the number of spores per butterfly based on replicate counts for each sample.

We used digital image analysis to quantify adult monarch wing size and the degree of melanism (dark coloration). We removed left and right forewings from preserved adults and scanned them using a flatbed HP scanner set to 300 dpi using the same exposure settings for each scan. Measurements were made using Adobe Photoshop software with the Image Processing Tool Kit plugin (Reindeer Graphics, Inc., Asheville, NC, U.S.A.). Total forewing area (mm^2) and two measures of wing melanism were obtained for both forewings of each adult butterfly, according to Davis *et al.* (2005). First, we quantified the proportion of forewing area encompassed by black pigmentation. Second, we estimated the density of black pigmentation, an indicator of the intensity or level of opacity of black. The scoring measurement of density is on a 0–255 scale, with 0 being completely black (greatest colour density). Average measures per individual were based on results for L and R forewings.

Regional and temporal variation in monarch density

We used data from the Monarch Larva Monitoring Project (MLMP; Prysby & Oberhauser, 2004; Oberhauser & Prysby, 2008), a citizen science programme, to further indicate the degree to which monarch butterfly larval densities vary over time and space in eastern N. America. Volunteer observers for the MLMP began collecting weekly abundance data during the monarch's breeding season in 1997, with per plant densities of monarch egg and larval (reported to individual stadia) stages on milkweed plants available for 32 states and provinces across North America. Data included the total number of larvae and milkweed plants observed at a specific time and location. As a high proportion of monarchs die as eggs and early instar larvae, we calculated average larval density per site based on count data for the final three instars (3, 4, and 5) only. All sites used in the analysis had been monitored for more than 1 year with a minimum of 4-weekly observations per year.

We divided observations from 1997 through to 2006 in the eastern U.S. and Canada into three geographic regions: Midwest

(MN, WI, MI, IA, IL, IN, MO, OH, and NE), Northeast (VT, MA, NY, NJ, PA, MD, ON, and DC), and South (TX, GA, NC, VA, and TN), and three temporal breeding phases: early (before June first), middle (June first–July 31st), and late (after July 31st) to examine changes in larval abundance. Geographical regions were selected based on previously described patterns of monarch spring re-colonisation, whereby adults returning from Mexico lay eggs in the southern-most states during April–May (here represented by the region denoted 'South'), and a second generation continues the journey north followed by a brief time lag (Malcolm *et al.*, 1993; Howard & Davis, 2004; Davis & Howard, 2005). In addition, northeastern and mid-western states were examined separately, because these areas are associated with two major fall migratory flyways at the end of the breeding season (Howard & Davis, 2009). We analysed the average larval density per site based on the total number of larvae divided by the number of milkweed plants examined each week, and averaged the weekly density values for each site within a given phase. We then excluded zero density reports (where sites were monitored but no larvae were reported for a given phase), and log-transformed the remaining density estimates prior to analysis.

Statistical analysis

Analyses to examine the effects of parasite treatment and rearing density were conducted using average values for all monarchs reared in a container as the unit of observation. Dependent variables included pupal mass, adult forewing area, development time from inoculation to adulthood, adult spore load, adult longevity, and two measures of wing melanism (proportion of black and density of black on forewings). Count data were log-transformed prior to calculating container means. We tested for equal variances between density treatment groups using Fisher's *F*-test, for normally distributed data, and Levene's Test, for non-normally distributed data, at significance levels of $\alpha = 0.05$. Variances between density treatments were equal for spore load data and all continuous response variables.

Analysis of variance was used to examine effects of design variables on one count variable, final spore load, and all continuous variables: pupal mass, adult forewing area, and two measures of wing melanism (proportion of black and density of black on forewings). The non-parametric Kaplan–Meier analysis was used to examine treatment effects on development time and adult longevity, and multinomial logistic regression was used to examine treatment effects on the proportion of monarchs that survived to adulthood, the proportion of adults infected with *O. elektroscirrha*, and the proportion of adults with deformed wings. For analyses of adult measures, monarch sex (M/F) was included as a fixed factor, and the final density of larvae (based on the actual number of monarchs that survived to pupation per container) was included as a continuous covariate (full model: dependent variable = parasite treatment + density treatment + final density + sex + parasite*density + infection*sex + density*sex).

Analyses were performed in SPSS (ver. 15.0; SPSS, Inc., Chicago, IL, U.S.A.) and we used comparisons of Akaike's Information Criterion (AIC) and Hurvich and Tsai's Criterion (AICC) for model simplification according to Crawley (2002). In Table 2, we report significance values only for variables included in the final minimum adequate model. Bonferroni's pairwise comparisons of means were used to further examine differences between the three density treatments in cases where rearing density was significant, and results are reported in the figure legends. We examined the distribution of residuals for each minimum adequate model, and in most cases, found that these approximated a normal distribution.

Results

Regional and temporal variation in monarch density

Analysis of MLMP data indicated that the average number of larvae per plant differed among breeding phases (early, middle, and late) and between regions in eastern N. America (Midwest, Northeast, and South). The final data set included a total of 641 density estimates by sampling location and breeding phase, as recorded by 78 observers over all 10 years. The number of density values (calculated for individual sites within a given breeding phase) used for each region by phase combination ranged from 14 (Northeast, early phase) to 307 (Midwest, middle phase), after reports of zero density were removed from the data set. Average densities per location were highest in the South during the early phase of the breeding season (Fig. 2). During the middle of the breeding season, monarch density was very low in the South, and increased again late in the breeding season. In the

Table 2. Analysis of pupal mass and adult forewing characteristics as a function of experimental design variables (density and infection treatment) and sex (full model: Response variable = rearing density + parasite treatment + sex + rearing density*treatment + final larval density + rearing density*sex + treatment*sex + error term). Model simplification was performed as described in Methods text. *F*- and *P*-values are shown only for those explanatory variables that remained in the final model; adjusted R^2 is shown for the final reduced models. All analyses use container means as the unit of observation.

Independent variable	Pupal mass	Wing area	Wing proportion black	Wing black density
Rearing density	$F_{2,104} = 10.38$ $P = 0.000$	$F_{2,102} = 12.71$ $P = 0.000$		
Parasite treatment	$F_{1,104} = 4.80$ $P = 0.032$	$F_{1,102} = 2.13$ $P = 0.148$	$F_{1,107} = 3.81$ $P = 0.053$	
Sex	$F_{1,104} = 39.07$ $P = 0.000$	$F_{1,102} = 4.97$ $P = 0.028$	$F_{1,107} = 388.99$ $P = 0.000$	$F_{1,108} = 209.94$ $P = 0.000$
Final density		$F_{1,102} = 3.88$ $P = 0.052$		
Rearing density*parasite treatment	$F_{2,104} = 4.14$ $P = 0.019$	$F_{2,102} = 3.94$ $P = 0.022$		
Adjusted R-square	0.389	0.254	0.788	0.657

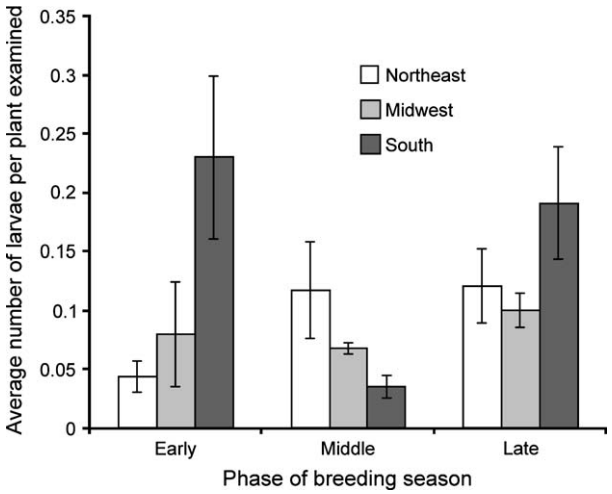

Fig. 2. Average densities of monarch butterfly larvae reported by MLMP (measured as the total number of larvae divided by the number of milkweed stalks examined per site) for three regions in eastern North America: Northeast (open bars), Midwest (gray bars), and South (black bars). Phases are early (before Jun 1st), middle (Jun 1st - July 31st), and late (after July 31st). Error bars represent standard errors.

Northeast and Midwest, average larval density increased from early to late in the breeding season (Fig. 2). Effects of phase of breeding season ($F_{2,627} = 3.71$, $P = 0.025$) and the two-way interaction between breeding phase and region ($F_{4,627} = 2.82$, $P = 0.024$) were highly significant, but the main effect of region was not significant ($F_{2,627} = 0.56$, $P = 0.574$).

It is important to note that average larval densities reported in Fig. 2 underestimate the actual numbers of larvae per host plant. This is because plants with larvae and those without were included equally in the count of plants examined. Since the number of larvae on a single plant for sites with monarchs present must be at least 1.0, averages shown in Fig. 2 could be higher if only those plants with larvae had been counted. Moreover, within each region, the maximum larval density for any given site was greater than 1.0 in several cases, leaving no doubt that some plants carried >1 larva (e.g. Fig. 1). Finally, an observer in Delray Beach, FL (excluded from the analysis here because of its close proximity to the S. Florida resident monarch population) reported average numbers of 7.0 and 6.5 larvae per plant examined during the middle phase of the breeding season in both 2005 and 2006, respectively. This observation indicates that the numbers of larvae per plant could reach higher levels in year-round resident populations as compared with the relatively low larval densities experienced by the eastern migratory population.

Survival, infection status, and parasite load

Monarchs in the density-infection experiment experienced high larval and pupal mortality across all treatments; only 49% of all monarchs survived from third instar to the adult stage (Table 1). On average, the probability of survival to adulthood did not differ between control and parasite-treated monarchs ($\chi^2 = 7.01$, d.f. = 6, $P = 0.320$) or across density treatments ($\chi^2 = 17.43$, d.f. = 12, $P = 0.134$), and the interaction between rearing density and parasite treatment was also not significant ($\chi^2 = 35.24$, d.f. = 12 $P = 0.998$). Based on variation in survival within the parasite-treated monarchs (Table 1), we ran a separate analysis focused on survival within this treatment group, which showed that the effect of rearing density was only nearly significant ($\chi^2 = 19.83$, d.f. = 12, $P = 0.070$).

All surviving control monarchs were parasite-free (N = 97), whereas 85% of the parasite-treated monarchs (N = 91) were infected with *O. elektroscirrha.* The proportion of infected monarchs within the parasite-treated class increased with larval rearing density (Table 1). This effect of density on infection status was significant ($\chi^2 = 23.32$, d.f. = 10, $P = 0.010$). The average parasite load per infected monarch was 3.08×10^5 spores (range: 5.56×10^3–1.16×10^6), and we noted a trend of increasing spore load with increased rearing density (average spore load by rearing density: Low 2.46×10^5; Moderate 2.91×10^5; High 3.39×10^5). However, analysis within the subset of parasitised monarchs showed that average spore load was not significantly affected by either rearing density ($F_{2,47} = 0.73$, $P = 0.490$) or sex ($F_{1,47} = 0.32$, $P = 0.573$).

Pupal mass and development time

Pupal mass was greatest in the moderate density treatment (Fig. 3a) and this effect was highly significant (Table 2). Average pupal mass was lower among parasite-treated monarchs in both

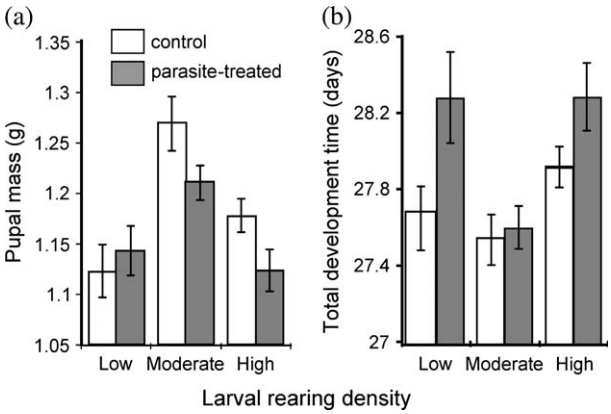

Fig. 3. Effects of larval rearing density and parasite treatment on (a) pupal mass and (b) development time. Data are shown for unparasitized (open bars) and parasitized (gray bars) treatment groups within each larval rearing density (low: 1 larva per container; moderate: 5 larvae per container; high: 10 larvae per container). Error bars represent standard errors. Bonferroni pairwise comparisons showed that mean pupal mass for monarchs reared under moderate densities were significantly greater than means for high and high densities ($p < 0.05$). whereas means for high and low density treatments were statistically similar ($p > 0.50$). Bonferonni comparisons also showed that mean development time for monarchs reared at moderate densities were significantly shorter than means for monarchs reared at high densities ($p < 0.03$), whereas means for all other combinations were statistically similar ($p > 0.30$).

the moderate and high density treatments, but parasite-treated and control monarchs had similar pupal mass in the low density treatment (Fig. 3a). Statistical analysis showed a significant main effect of parasite treatment and a significant two-way interaction between parasite treatment and density (Table 2). Pupal mass was also greater in males than females across all treatment categories, and this effect was highly significant (Table 2).

Parasite-treated monarchs developed more slowly (based on time from hatching to adult eclosion) than monarchs in the control treatment (Log Rank: $\chi^2 = 6.89$, d.f. $= 1$, $P = 0.009$), and this effect was strongest in the low and high density treatments (Fig. 3b). Moreover, larvae in the moderate density treatment developed faster than those in the low and high density treatments, and this effect of rearing density on development time was highly significant (Log Rank: $\chi^2 = 8.77$, d.f. $= 2$, $P = 0.013$). There was also a significant interaction between parasite treatment and density (Log Rank: $\chi^2 = 15.11$, d.f. $= 5$, $P = 0.013$). Specifically, development time differed across rearing densities only within parasite-treated monarchs, as demonstrated by further analysis within control (Log Rank: $\chi^2 = 1.01$, d.f. $= 2$, $P = 0.605$) and parasite-treated monarchs (Log Rank: $\chi^2 = 9.70$, d.f. $= 2$, $P = 0.008$). In addition, female monarchs developed more quickly than males across all parasite and density treatments (Log Rank: $\chi^2 = 8.447$, d.f. $= 1$, $P = 0.004$).

Adult longevity and wing traits

Parasite-treated monarchs experienced a 23% reduction in adult longevity as compared with control monarchs, and this effect of parasite treatment was highly significant (Fig. 4; Log Rank: $\chi^2 = 48.06$, d.f. $= 1$, $P < 0.001$). Adult longevity was not affected by larval density alone (Log Rank: $\chi^2 = 3.95$, d.f. $= 2$, $P = 0.139$). However, there was a significant interaction between parasite treatment and density (Log Rank: $\chi^2 = 58.31$, d.f. $= 5$, $P = 0.000$; Fig. 4), such that adult longevity decreased with increasing larval density within parasite-treated, but not control monarchs.

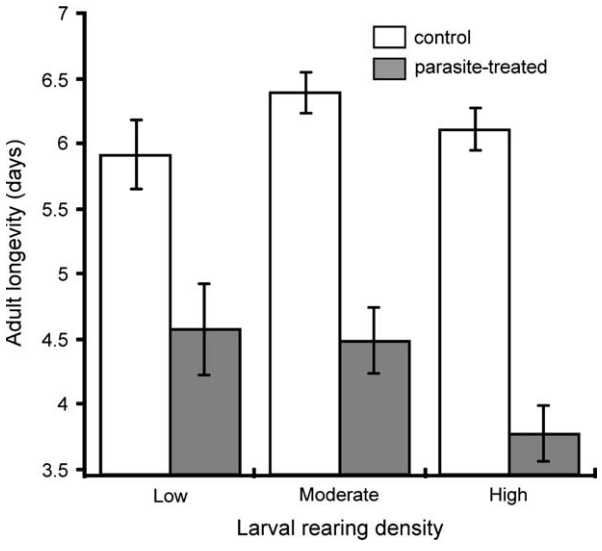

Fig. 4. Effects of larval rearing density and parasite treatment on adult longevity (in days). Data are shown for unparasitized (open bars) and parasitized (gray bars) treatment groups within each larval rearing density (low: 1 larva per container; moderate: 5 larvae per container; high: 10 larvae per container). Error bars represent standard errors.

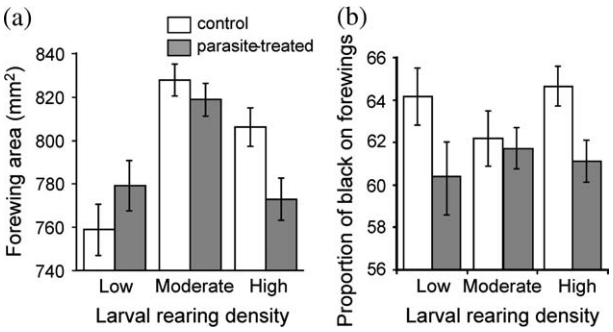

Fig. 5. Effects of larval rearing density and parasite treatment on (a) adult forewing area and (b) proportion of back pigmentation on adult forewings. Data are shown for unparasitized (open bars) and parasitized (gray bars) treatment groups within each larval rearing density. Error bars represent standard errors. Bonferroni pairwise comparisons showed that mean forewing area for monarchs reared under moderate density was significantly greater than means for monarchs reared at low and hogh densities ($p < 0.002$), whereas means for high and low density treatments were statistically similar ($p = 0.098$). Rearing density was not significantly associated with the proportion of back pigmentation on monarch forewings.

Only two unparasitised monarchs ($n = 105$) emerged with wing deformities, whereas five parasitised adults ($n = 77$) had deformed wings. Four out of the five parasitised monarchs with wing deformities were in the high density treatment. However, neither parasite treatment nor rearing density was significantly associated with wing deformities (parasite treatment: $\chi^2 = 5.03$, d.f. $= 3$, $P = 0.170$; rearing density: $\chi^2 = 9.93$, d.f. $= 6$, $P = 0.127$).

Adult forewing area was significantly influenced by rearing density, parasite treatment, and sex (Table 2). Males were larger than females, and monarchs in the moderate density treatment had the largest forewings, whereas those in the low density treatment had the smallest forewings (Fig. 5a). Forewing area was also affected by the density of parasite treatment interaction (Table 2). Specifically, control monarchs had larger forewings than parasite-treated monarchs in both the moderate and high density treatments, whereas parasite-treated monarchs had larger forewings in the low density treatment (Fig. 5a).

The proportion of black coloration on monarch forewings was greater for control monarchs than for parasite-treated butterflies (63% vs 61%), but this trend was marginally non-significant ($P = 0.053$). Females were darker than males across all treatment groups (Fig. 5b, Table 2). The density of wing pigmentation, a measure of the opacity or intensity of black, was greater for female than for male monarchs, but this variable was not significantly affected by parasite treatment (Table 2). Finally, neither measure of wing coloration was significantly affected by rearing density based on main or interaction effects (Table 2).

Discussion

Both larval rearing density and infection by the protozoan *O. elektroscirrha* affected measures of size and development in monarch butterflies. First, we observed significant negative effects of parasite treatment on monarch fitness, with infection resulting in decreased monarch pupal mass, slower development rate, reduced wing area, and shorter adult longevity. This is consistent with previous studies demonstrating that high replication of *O. elektroscirrha* within monarch hosts results in substantial negative consequences for adult lifespan, body size, wing size, mating success, and flight ability (Altizer & Oberhauser, 1999; Bradley & Altizer, 2005; De Roode *et al.*, 2007; Lindsey & Altizer, 2008).

While monarch infection probability increased significantly with increasing larval densities, average spore loads after exposure to *O. elektroscirrha* increased only slightly with larval densities. The effect of density treatment on host infection status, weakly suggests increased susceptibility to infection with increasing larval density. Thus, in the case of rearing density, results here do not support the 'density-dependent prophylaxis' hypothesis, which predicts that increased resistance to parasitism can result from increased rearing density (Reeson *et al.*, 1998; Barnes & Siva-Jothy, 2000; Wilson *et al.*, 2003). On the one hand, as monarchs appear to experience ecologically relevant variation in density in the wild, they should, in theory, benefit from tailoring levels of immunity or resistance to variation in host density. This is because infection patterns by *O. elektroscirrha* in wild populations show that prevalence increases from early to late summer within migratory populations (S. Altizer, unpubl. data) and is higher in year-round breeding populations as compared with migratory populations (Leong *et al.*, 1997; Altizer *et al.*, 2000, 2004). Since both of these situations also correlate with higher numbers of larvae on plants (e.g. Fig. 2), it seems likely that monarchs in high-density populations could also experience higher risks of infection. On the other hand, the two species for which the 'density-dependent prophylaxis' hypothesis has found the most support (armyworm and desert locust; Reeson *et al.*, 1998; Wilson *et al.*, 2002; Wilson *et al.*, 2003) are both polyphenic outbreak species, with specific high and low density morphs. These insects experience much greater fluctuations in population density on a regular basis than monarchs, thus the selective benefit of greater immune defences under high density conditions is likely to be higher in these species than in monarchs. Additionally, monarch immune defences can be costly (Lindsey & Altizer, 2008). Hence, although greater measures of immunity correlate with higher host survival after infection with *O. elektroscirrha*, these defences might not be readily mobilised under high-risk conditions.

An alternative hypothesis to density-dependent prophylaxis is the 'crowding and stress' hypothesis, which predicts that individuals reared at lower population densities will be in better physical condition, and hence will be better able to resist the negative effects of parasitism (Goulson & Cory, 1995; Adamo, 2006; Reilly & Hajek, 2008). This idea is based on the assumption that increased stress and intra-specific competition will make hosts more susceptible to infection, or less able to tolerate the negative consequences of parasitism. In support of this hypothesis, monarchs in the moderate and high density treatments generally experienced slightly higher infection rates and suffered the greatest negative effects of infection (based on differences between the parasite-treated and untreated groups). Moreover, monarchs in the moderate and high density rearing treatments also suffered the greatest negative effects of infection on development and body size (based on relative differences between the parasite-treated and untreated class). By comparison, parasitised monarchs in the low density treatment had mean values of pupal mass, development rate, and adult wing size that were similar to or slightly greater than uninfected monarchs. Collectively, these effects of rearing density on host infection and fitness measures suggest that monarchs under high-density conditions are more susceptible to parasite infection and its costly effects.

Contrary to our initial expectations, we observed a non-linear relationship between larval density and measures of development and body size for both parasite-treated and untreated larvae. Specifically, each of these variables was greatest for monarchs reared under moderate density, and averages were 10–20% lower for monarchs reared singly and at the highest density. Previous studies of Lepidoptera and other insect species have shown that immature stages reared under high density conditions experience decreased survivorship, slower development rates, and/or achieve smaller adult body sizes (Mercer, 1999; Tammaru *et al.*, 2000; Gibbs *et al.*, 2004). On the other hand, cabbage moth *Mamestra brassicae* larvae reared both singly and at the highest densities weighed less than larvae reared at intermediate densities (Goulson & Cory, 1995). Our findings are consistent with those of Goulson and Cory (1995), and suggest that for both healthy and infected monarchs, the ideal rearing conditions are to be neither solitary nor in a large group, but to occur at moderately low densities, provided that food resources are not limiting.

Observations of greater body size and faster development rate for intermediate density conditions, irrespective of host infection status, might be best explained by two different mechanisms. Given that high densities could cause greater intra-specific competition under natural conditions, larvae might feed more rapidly to attain a large body size before food supplies are depleted. As food supplies, ultimately, were not limited in this experiment, this could have resulted in both more rapid development *and* greater size at pupation when compared with solitary larvae. In our experiment, monarch larvae reared under the highest density conditions (10/container) could have suffered from physical or developmental stress associated with overcrowding or interference competition. For example, Gibbs *et al.* (2004) observed aggressive encounters, including head and tail biting and head butting, among speckled wood butterfly larvae (*Pararge aegeria*) reared at high densities. Although we did not quantify feeding behaviours in this study, monarchs engage in similar aggressive interactions in captivity, and larvae can also bear integument scars and missing tubercules from previous injuries (S. Altizer, pers. obs.). Finally, we note that in experiments described here, a greater number of host plant leaves provisioned to containers with more larvae could have altered the micro-environmental conditions in ways that enhanced

monarch survival and development in the moderate density treatment.

External melanism (i.e. dark body coloration) is often thought to correlate positively with resistance to infection (Barnes & Siva-Jothy, 2000), but not in all insect species (Robb *et al.*, 2003). Moreover, previous work has shown that body or wing melanism can increase under crowded host conditions (Wilson *et al.*, 2001). For example, polyphenism in Lepidoptera can occur such that the high density phenotype is darker than the low density phenotype (e.g. as demonstrated for *Spodoptera littoralis*; Cotter *et al.*, 2004). Within monarchs, our results did not support an effect of rearing density (or a density by parasite treatment interaction) on measures of dark coloration in adult butterflies. In addition, although we found that unparasitised adults had darker forewings (based on the proportion of black pigmentation) than parasite-treated butterflies, this trend was not statistically significant.

Field data from the MLMP do not allow us to determine per plant larval densities for occupied plants alone, and thus it is impossible to determine how often the experimental larval densities examined here occur in the wild. However, MLMP data do clearly demonstrate that per-plant monarch densities vary up to five-fold across space and time in North America, and we have anecdotal reports of densities of late-instar larvae similar to those used in our study (Fig. 1). It is therefore possible that the faster development observed here for monarchs in the moderate density treatment is an evolved response to crowding that occurs in nature.

Ultimately, hosts that live in variable environments can experience changes in population structure and extrinsic forces that influence variation in disease risk. These risks are particularly relevant to monarch butterflies that have been threatened at their overwintering sites and breeding habitats in recent years (Brower & Malcom, 1991; Zalucki & Rochester, 1999; Brower *et al.*, 2002; Oberhauser & Peterson, 2003). Specifically, destruction of overwintering sites, climate warming, and planting of tropical milkweed host plant species in non-native regions can alter the ecological dynamics of migratory populations, potentially resulting in the replacement of the large migratory populations with smaller remnant populations that breed year-round. These non-migratory populations could experience higher local population densities, and are also likely to become heavily parasitised (e.g. Altizer *et al.*, 2000, 2004).

As indicated by this study, both parasite infection and larval density can affect monarch butterfly fitness, although results reported here were not always consistent with predictions from previous work. Specifically, we found that contact with other larvae at moderate (but not high) local densities could increase pre-adult survival, stimulate larval growth, and increase pupal and adult body size, irrespective of host infection status. However, it is important to note that this finding probably relies on non-limiting food resources given modest increases in density. Moreover, our study indicates that highly crowded conditions can increase the potential for monarchs to suffer from the negative consequences of disease, probably owing to increased stress and intra-specific competition. In summary, being in a group may have beneficial impacts on a number of fitness traits, even for species such as monarchs that do not have gregarious larval stages. However, high density conditions appear to have negative effects on monarch resistance and tolerance to infection, and results such as those reported here can help reveal the mechanisms that underlie the positive and negative consequences of host density for individual performance.

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