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The Effect of Parental Population Density on Offspring Immune Function

Dana Davis
University of Central Florida

THE EFFECT OF PARENTAL POPULATION DENSITY ON OFFSPRING IMMUNE FUNCTION

by

DANA B. DAVIS
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ABSTRACT

It is well known that an individual's environment, genetic code, and gene by environment interactions have an effect on its overall phenotype. However, there is a growing body of work that shows that parents can have an effect on their offspring's phenotype beyond the inherited genetic code. Studies have shown that parents may affect their offspring through physiological mechanisms such as egg provisioning and epigenetic effects and through behavioral mechanisms such as maternal care. In many of these cases, the parental effect is triggered by an environmental cue. Previous work has shown that density can impact immune function and cuticle color in insects - two phenotypic traits that are pleiotropically linked. Additional work has shown that parental density can have impacts on offspring immune function, as well. However, previous studies utilized insect species that show a strict density dimorphic phenotype where individuals reared at high densities exhibit increased immune function and much darker cuticles than their low density counterparts, which is not an accurate representation of most insect systems as most insect systems show a more continuous response to density effects. Also, previous work has not determined the parental origin of density effects on offspring immune function and cuticle color. It has been suggested that parental density effects may be due to maternal egg provisioning and that paternal effects may be minimal. However, knowledge of parental origin would give us a better insight into the possible mechanisms of these density driven parental effects and provide a direction for future research. In my study, we used *Drosophila melanogaster* in order to determine (1) if density affects immune function and cuticle color in a species that shows a continuous response to density, (2) if parental density affects offspring immune function and cuticle color, and (3) if the source of these parental effects are of a maternal origin only or if these effects are of a paternal origin, as well. We found that there is an effect of density on immune function and cuticle color in the parents in a more common insect system and parental density had an effect on offspring phenotype, as well. Most notably, we found that, in addition to the effects of maternal density, these parental effects on offspring phenotype were a response to paternal density, as well.

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INTRODUCTION

While it is known that an individual's genetic makeup, environment, and their interaction play a large role in that individual's phenotype, studies have increasingly shown that parents can also have an effect on their offspring's phenotype independent of its inherited genetic code. For example, a mother's choice of nesting site, which itself may be determined by uninherited or environmental effects, could affect the offspring's size, quality, or sex (Trivers and Willard 1973; Janzen 1993). These types of effects have been termed "parental effects" and they have been exhibited in a large majority of studied organisms (Mousseau and Fox 1998). Previously, parental effects were called "maternal effects" because many of the first studies focused on the effect by the mother and the effects by the father were thought to be minimal. However, we know now that parental effects of paternal origin also exist (Lacey 1998).

Many of the documented parental effects are adaptive. For instance, parents in a high stress environment may produce offspring that are better equipped to survive in that environment (Mousseau and Fox 1998). These adaptive phenotypes in the offspring can be facilitated through parental behavior (i.e. mate choice, egg provisioning, or maternal care) or through epigenetic effects (Kirkpatrick and Lande 1980; Mousseau and Fox 1998). There are two main mechanisms by which parental epigenetic effects operate. The first is DNA methylation in which methyl groups are added to the promoter region of a protein coding DNA strand blocking transcription factors and thereby preventing transcription. The opposite can also occur where methyl groups are removed from the promoter region (demethylation) promoting transcription (Wolf and Wade 2009). The second epigenetic mechanism is histone modification in which acetyl groups are added to a histone (acetylation) causing the DNA strand to unwind and transcription factors to bind to the promoter region. Alternatively, acetyl groups can be removed (deacetylation) so that the DNA strand tightens around the histone preventing transcription (Roth et al. 2001). No matter the mechanism, these parental effects have been show to influence

numerous offspring traits including size, growth, sex, and behavior (Allen et al. 2008; Zambrano et al. 2006; Weaver et al 2006; Grindstaff et al. 2003; Nager et al. 1999).

Recently, parental effects on offspring immunity have become of interest, considering that immunity is closely linked to survival and fitness for many organisms. Much of this work has been focused on insects (Grindstaff et al. 2003). There are two main reasons why immunity in insect systems have attracted interest. First, insect immune function is highly conserved across all animals and is relatively simple compared to vertebrate immune function, making the study of insect immunity of indirect importance to humans. Second, insect immunity plays a direct and vital role in human health. If insect pollinators are suffering from pathogenic infection, it could have major debilitating impacts on our agricultural system (e.g. honey bees). Moreover, failure of insects to defend against transmittable pathogens can facilitate transmission of human-centered diseases (e.g. malaria). While many previous studies have focused on the direct effect of environment on immune function, an increasing number of studies are beginning to go one step further and examine the indirect, transgenerational effects of parental environment on offspring immunity (Grindstaff et al. 2003). For instance, a study by Sadd and Schmid-Hempel showed that queen bees that were injected with bacteria produced eggs with a higher amount of antimicrobial activity compared to queen bees that were not injected (Sadd and Schmid-Hempel 2007). Another study by Yannick Moret showed that mealworm beetles injected with a bacteria produced offspring with a higher amount of antimicrobial peptides than those whose parents were not injected (Moret 2006). Furthermore, these parental effects have been showed to protect offspring from more than just bacterial infections, as Tidbury and colleagues (2011) showed that Lepidoptera that were injected with a virus produced offspring that were better suited to defend against that virus than offspring of uninfected parents (Tidbury et al. 2011).

Interestingly, infections are not the only environmental cues that affect insect immune function. Research on *Lepidoptera* species by Wilson et al. (2001) has shown that individuals reared at high

densities have darker cuticles and heightened immune function. Both of these responses appear to serve as a preventative measure in order to ameliorate the increased risk of disease transmission expected under high densities, a phenomenon termed "density-dependent prophylaxis" (DDP). Specifically, Wilson and colleagues found that individuals reared at higher densities had increased cuticle melanin, increased phenoloxidase titers in the cuticle and hemolymph, and an improved ability to defend against ectoparasitoid eggs and fungal pathogens (Wilson et al. 2001). Importantly, phenoloxidase is an enzyme circulating in the hemocoel that protects individuals against bacteria, viruses, protists, and fungi (Kutch et al. 2015). Darkened cuticles also provide a direct immunological benefit by (i) reducing the probability of pathogens breeching the cuticle (darker cuticles are thicker and stronger), and (ii) containing non-circulating phenoloxidase, which limits the passage of pathogens into the hemocoel (Arakane et al. 2009). As it stands, density-dependent prophylaxis may be a highly conserved trait in insects (Barnes and Siva-Jothy 2000; Wilson et al. 2002; Wilson and Reeson 1998; Mills 2012; Bailey et al. 2008; Ruiz-González et al. 2009).

However, the potential for the immunological effects of density-dependent prophylaxis to be transmitted between generations is dramatically understudied. The first study on transgenerational density-dependent prophylaxis was performed by Miller et al. (2009) using locusts. Their results showed that individuals reared at higher densities produced offspring with weaker immune function compared to offspring with parents reared at lower densities (Miller et al. 2009). This seems counter intuitive based on work by Wilson and colleagues, and two mechanisms have been proposed to explain those results (Wilson et al. 2001). First, the reduction in offspring immunity could be due to the mother allocating more immune resources towards herself leaving few resources to be transmitted to her offspring. Alternatively, the mother may have allocated too many immune related substances, so that the offspring's own immune function was under-stimulated during development (Grindstaff et al. 2003). Unfortunately, the Miller et al. study did not test the immune function of the parents in order to determine if the parents were effected by density and if it was in the same direction as the study by Wilson et al. (2001). A recent

study by Wilson and Graham found that the parents reared at high densities are better able to defend against infection. However, similar to the study by Miller et al. (2009), they found that the offspring of high density parents had lower survival post infection compared to offspring of low density parents. This study showed that moths reared at lower densities produced offspring with better survival when challenged with a virus (Wilson and Graham 2015). Thus, the effect of density on offspring immune functions seems to be maladaptive for the offspring and contradictory to parental immune function. More research should be done in order to determine if this is a general pattern among insects and to understand the mechanisms behind this phenomenon.

To help elucidate the general pattern of density-dependent transgenerational immune effects, current research should focus on if these effects are maternal or paternally influenced. For instance, if these effects are only seen in the offspring of mothers reared at high densities, then that would suggest the effect is likely due to egg provisioning. But if the effects are seen only in the offspring of fathers reared at high densities, then that would suggest that the effects are likely epigenetic (although this pattern is also consistent with cryptic female choice). Moreover, both the Miller et al. (2009) and Wilson and Graham (2015) studies were conducted on species that exhibit dimorphic density-dependent prophylaxis (i.e. a strict high density and low density morphotypes). If density-dependent prophylaxis is ubiquitous among insects, future work should use a tractable insect system that exhibits the more common continuous distribution of physiological response to density.

In this study, we address density-related transgenerational effects in the fruit fly *Drosophila melanogaster*. To date, no studies have investigated *D. melanogaster* for density-dependent prophylaxis. However, recent work has shown that its cuticle color and immune function are sensitive to changes in the environment; including temperature, humidity, and ultraviolet radiation (Kutch et al. 2014; Seema and Chandana 2013; Bastide et al. 2014). Moreover, the environmentally induced changes in cuticle color are continuous, not discrete like that in organisms traditionally used to investigate DDP, suggesting that flies

are more representative of a typical insect's response to density. In short, we address three hypotheses regarding transgenerational effects of DDP in *D. melanogaster*: (1) high population densities influence cuticle color and immune function, (2) parental densities influence offspring cuticle and immune phenotypes, and (3) both maternal (resource provisioning and/or epigenetically based) and paternal effects (epigenetically based) underlie the transgenerational phenotypic effects.

In order to address these hypotheses, we reared *D. melanogaster* larvae at a high or low density and assessed their cuticle color and immune function. We then mated these treatments in a fully factorial design (**Figure 1**). The resulting offspring were reared at a common density and their adult phenotypes assayed. We predicted that parents reared at high densities would exhibit darker cuticles and improved immune function. However, we predicted that the direction of the transgenerational effect in offspring would be dependent on the sex of the parent. Fathers and mothers would epigenetically try to improve offspring immune function and darken their cuticles in order to prepare the offspring for a high density environment but a mother in high density would allocate immune resources towards herself leaving fewer resources for egg provisioning and the lack of resources would have a larger effect than the epigenetic efforts by both parents (**Figure 2**). Therefore, we predicted that individuals from high density fathers and low density mothers would have the highest immune function because fathers would be epigenetically improving immune function and darken cuticles and mothers from the low density would have immune resources for egg provisioning. We predicted the next group to follow the first with higher immune function and darker cuticles would be the group with both parents from low densities because neither parent would epigenetically prepare offspring for a high density environment but the mother would have resources for egg provisioning which has a much larger effect. The next group to follow the first two would be the group with both parents from higher densities because, while mothers are lacking in resources, there is some effort by both parents to epigenetically improve offspring immune function and cuticle darkness. Finally the group with the lowest immune function and lightest cuticles would be the group with low density fathers and high density mothers because father would not try to epigenetically

improve offspring immune function and cuticle color, and at the same time, mothers would lack resources for egg provisioning (**Figure 1**: C > A > D > B).

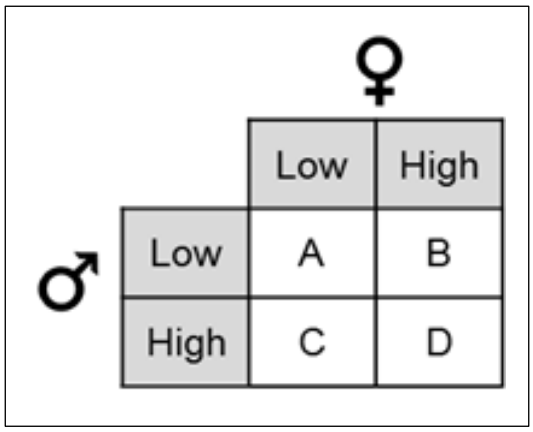

Figure 1. Fully factorial scheme between males and females reared at low and high densities (letters reflect different treatments). We predicted that high density fathers induced improved offspring immunity via epigenetic effects, but that high density mothers produce offspring of reduced immunity due to reduced maternal provisioning (C >A > D > B).

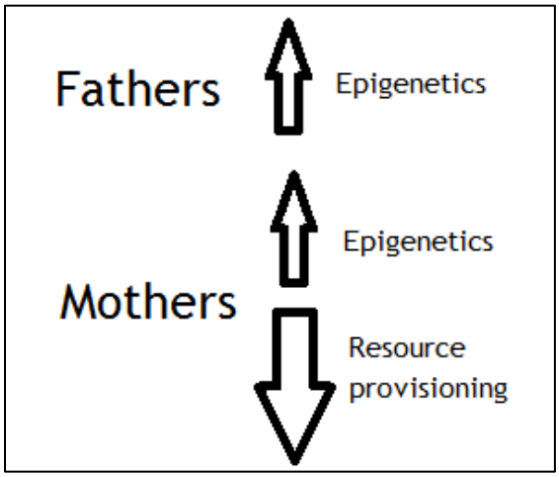

Figure 2. At high densities, fathers and mothers epigenetically increase offspring immune function and cuticular melanization in order to prepare offspring for a denser environment. However, lack of resources for egg provisioning would have a larger effect decreasing immune function for offspring and therefore, the overall effect of high parental densities would cause offspring to have lower immune function and lighter cuticles.

METHODS

Experimental Design

Flies used in the experiment were from an outbred Orlando population. This population was created from 40 gravid females collect in Orlando in 2010 and maintained with several hundred adults per generation. To establish the parental densities, ten mating pairs were placed into 177ml bottles and allowed to mate over four days, at which point the adults were culled and larvae allowed to develop. The resulting adult offspring (the P1 generation) were allowed to eclose and mate within their native bottles (**Figure 3**). Four days after the first eclosion, P1 flies were transferred to grape agar for oviposition in groups of 30 (15 of each sex) for 24 hours. 12 hours after P1 adults were cleared, the top halves of the bottles were removed. F1 larvae were collected from the plates and randomly assigned to either 50 or 150 larval density treatment, which were housed in 46ml drosophila vials. After a nine day development period, the F1 adults were collected as virgins and held at a density of 15 same sex adults per vial for four days. These adults were then randomly split into two groups: group 1 were used to generate the next generation, while group 2 was used to assess the cuticle melanization and immune function. The group 2 adults provided the direct effects of density on organismal physiology while the F2 offspring of group 1 provided the transgenerational effects of density. To create the F2 generation four mating treatments were created that captured all sex by density combinations including (A) $females_{50}$ X $males_{50}$, (B) $females_{150}$ X $males_{50}$, (C) $females_{50}$ X $males_{150}$, and (D) $females_{150}$ X $males_{150}$. These combinations allowed the decomposition of parental effects into maternal and paternal components. All mating pairs were placed on grape agar to oviposit. F2 larvae were collected from each mating pair and placed in vials at a medium density of 90 individuals. Upon adult eclosion, the F2 generation was held as virgins in groups of 15 for four days, after which they had their cuticle melanization and immune function assayed (**Figure 3**).

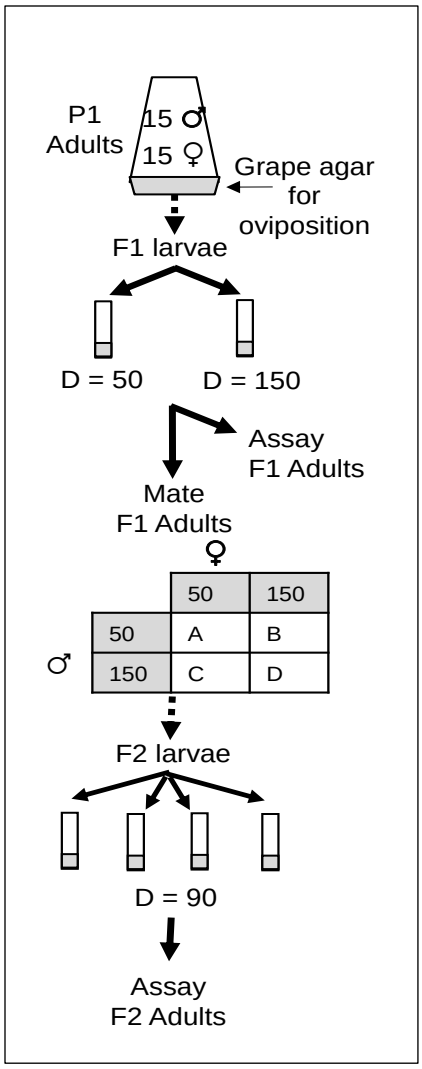

Figure 3. Experimental Design. Vertical rectangles are fly vials. Irregular hexagon is a fly bottle. Dotted lines represent generational transitions. D = larval density.

Immune Assays

Phenoloxidase Assay

To estimate the amount of phenoloxidase contained in the hemolymph, 20 individuals of the same sex and treatment were pricked in the thorax and all placed in a 0.5 microliter centrifuge tube with a hole in the bottom. Twelve microliters of PBS were added to the centrifuge tube. Then, it was placed into a 1.5 microliter centrifuge tube and centrifuge at 8,000 RPM for five minutes. PBS and hemolymph drained into the larger tube. Both tubes were placed in a freezer at -80 degrees Celsius. The individuals remaining in the smaller tube were used for cuticular melanization later. Each tube of hemolymph was considered one sample. There were at least thirty samples of hemolymph for each sex and treatment. Later, the tubes of hemolymph were removed from the freezer for the phenoloxidase assay. 10 microliters of each sample were placed randomly on a 96-well-plate. Fourteen microliters of alpha-chymotrypsin were added to each well, and 90 microliters of L-DOPA were added to each well containing a sample. The phenoloxidase enzymes in each sample will catalyzed the reaction of L-DOPA into melanochrome. At 0 minutes, 30 minutes, and 60 minutes the absorbance of each well was determined. Wells with greater absorbance had more melanin, which is the result of samples with high phenoloxidase concentrations. Melanin is essential for innate immune function in insects. It encapsulates a pathogen which prevents the pathogen from persisting in the insect. Therefore, phenoloxidase concentration is a good indicator of immune function with samples with higher phenoloxidase concentration having better melanin-based immune function.

Cuticular Melanization Assay

As noted in the introduction, a stereotypical density-dependent prophylactic response to high density is a darkened, highly melanized cuticle. Dark cuticles are also genetically correlated with

circulating phenoloxidase concentrations (Armitage and Siva-Jothy 2005; Rolff et al 2005), which protects against immunological insults that reach the hemocoel. To this end, the F1 and F2 individuals frozen above were retrieved and placed on 20 slides with each slide containing four individuals of both sexes from every treatment (**Figure 4**). Pictures were taken of the dorsal sides of the flies using the BK Imaging System. Then, Image J was used to assess the darkness of the cuticle of each individual by assigning an average gray scale value (AGV) to it. For each fly, the grayscale value of a blank space in close proximity of the fly was also assessed to act as a control image in order to account for variation within slides due to spatial variation in lighting. We also assigned a unique number to each slide in order to account for variation in light intensity between slides due to temporal variation in lighting.

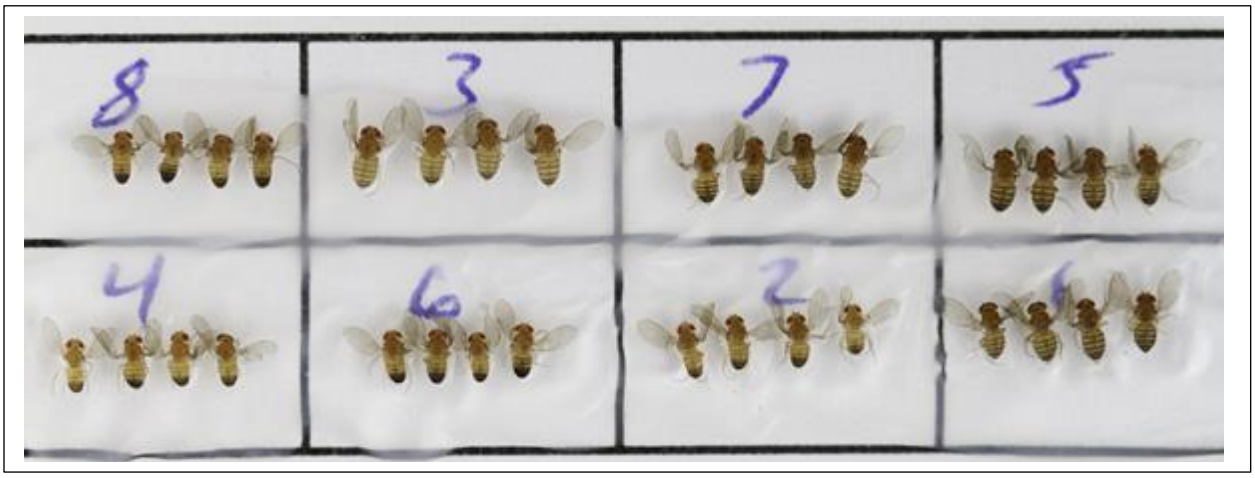

Figure 4. Cuticle melanization assay slide. A number was assigned to each sex and treatment and a random number set generator was used to randomize each group's position on each slide. This photographic slide was one of the slides used to address hypothesis 2 showing male and female offspring from four different mating pairs

Survival Assay

Several flies from each sex and treatment were pricked with a sterile needle that had been dipped in an LD_{50} pathogen solution of *Pseudomonas Aeruginosa*. After 48 hours, the number of flies that survived the pathogen load and the number of flies that died were counted. Then, the percent survival for each sex and treatment was calculated.

Statistical Analysis

Direct Density Effects

In order to determine the effect of density on immune phenotypes and cuticle melanization in the F1 generation, we examined the effect of density on phenoloxidase activity, darkness of abdomen and thorax, pronotum area (a proxy for body size), and survival 48 hours post prick infection. To determine the effect of larval density on pronotum area we employed an ANOVA (n=480) while controlling for variation in sex and photographic slide. For cuticle melanization, we examined the effect of density on thorax and abdomen darkness in males and females using an ANOVA (n=480) while controlling for sex, slide, and size. Photographic slide was included as a factor due to variation across slides driven by temporal variation in the BK imaging light settings. The control image nested within slide was also included due to spatial variation in lighting within a slide. In order to determine if there was an effect of larval density on phenoloxidase activity, we examined change in ocular density over 60 minutes (removing zero and negative data) as a function of density while for controlling sex, initial ocular density, plate and cohort (first or second data collections) using an ANOVA (n=112). In order to determine the effect of density on pathogen survival, we first removed individuals from the analysis that died immediately after infection because this indicated that death was the result of unintentional damage and not the infection. With the remaining individuals we examined survival (dead or alive after 48 hours) as a function of density while controlling for sex, and set (all experiments were performed twice to confirm repeatability; therefore, set refers to first or second data collections) using a general linear model on the binomial distribution (n=1580). We also examined pathogen survival of each replicate separately using a general linear model on the binomial distribution (n=790). For all models, all second order interactions were assessed and those that remained significant were kept in the model.

Transgenerational Density Effects

To determine the effect of parental density on cuticle and immune phenotypes, we compared offspring from different larval density treatments (for each density treatment, parental density was constant; i.e. ♀50 x ♂50 versus ♀150 x ♂150). In order to determine the effect of parental density on offspring size we used an ANOVA (n=320) with pronotum area as a function of parental density while controlling for sex and photographic slide. Next, we examined cuticle melanin by using an ANOVA (n=320) with thorax and abdomen darkness as a function of parental density while controlling for variation due to sex, slide, and cohort. For phenoloxidase activity, we once again, used an ANOVA (n=86) with change in ocular density as a function of parental density while controlling for sex, initial ocular density, plate, and cohort after removing zero and negative data. Lastly, we determined the effect of parental density on survival by removing individuals that died immediately and then using a general linear model on the binomial distribution (n=1040) with survival as a function of density while controlling for sex, and replicate.

Source of Transgenerational Effects

We assessed whether the transgenerational effects were of maternal or paternal origin by comparing offspring from the four mating pairs (i.e. ♀50 x ♂50, ♀150 x ♂150, ♀50 x ♂150, ♀150 x ♂50). First we determined whether maternal or paternal density had an effect on the offspring cuticle melanization by using an ANOVA (n=640) with the darkness of the offspring thorax and abdomen as a function of maternal or paternal density while controlling for sex slide and cohort. Next, we examined whether phenoloxidase activity was affected by maternal or paternal density by using an ANOVA (n=192) with change in ocular density as an effect of maternal or paternal density while controlling for variation due to sex, initial ocular density, plate, and cohort and removing negative and zero data. Lastly, we used a general linear model on the binomial distribution (n=2080) with offspring survival 48 hours after a bacterial prick infection as a function of maternal or paternal density while controlling for sex and set.

RESULTS

Direct Density Effects

We found that the density of F1 individuals during development had a significant influence on body size after controlling for a variation in sex and photographic slide ($F_{1,477} = 103.4$, $P < 0.0001$), with higher density individuals being smaller. Considering that size can influence cuticle darkness independent of density (Fedorka et al., 2013), size was included in our cuticle analysis. We found that higher larval density resulted in a darker thorax in both males and females (**Figure 5** and **Table 1**), which supports the existence of density-dependent prophylaxis in fruit flies. Density did not significantly influence abdomen darkness (P=0.1050). In general, females exhibited darker thoraxes than did males (mean + standard error: 170.5 + 1 versus 165.1 + 0.9, respectively; **Table 1**), but lighter abdomens (153.8 + 1.3 versus 158.3 + 1.2, respectively). With regard to immune function, we found no effect of density on phenoloxidase activity (P=0.1135). When survival against bacterial infection was analyzed, we uncovered an interaction between density and replicate. When the replicates were analyzed separately, we found greater survival against the pathogen in the higher density treatment in the first replicate (0.82 + 0.02 versus 0.87 + 0.02; **Table 2**), but the second replicate show no effect of density. These data are consistent with the expectations of density-dependent prophylaxis.

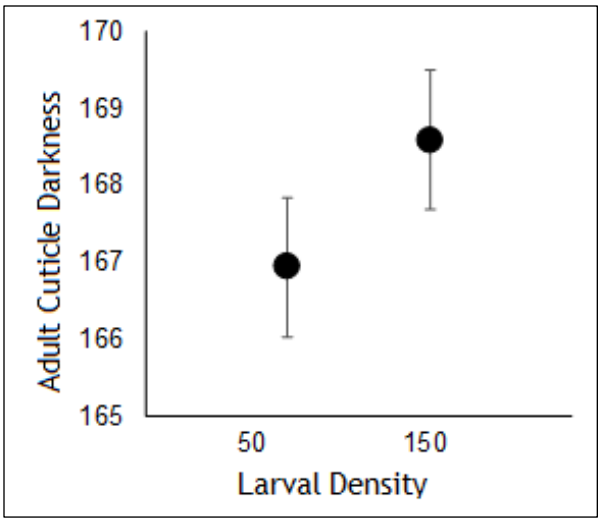

Figure 5. Individuals from the high density treatment had darker thoraxes.

Table 1. Larval density had a significant effect on cuticle melanization of the thorax with a p-value of 0.0303 but there was not a significant effect on abdomen darkness.

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Source	DF	SS	F	P
Thorax				
Density	1	224.4	4.7	0.0303
Sex	1	1218.7	25.6	0.0001
Size	1	21180.9	15.4	0.0001
Slide	29	429.1	9.1	0.0028
Control (Slide)	30	3826.5	2.7	0.0001
Abdomen				
Density	1	216.9	2.6	0.1050
Sex	1	852.6	10.4	0.0014
Size	1	160.6	1.9	0.1629
Slide	29	29916.4	12.5	0.0001
Control (Slide)	30	5949.6	2.4	0.0001

Table 2. Effect of F1 Larval Density on Mortality

Source	DF	X2	P
Mortality (reps 1 + 2)			
Density	1	0.18	0.6747
Sex	1	2.03	0.1541
Replicate	1	1.66	0.197
Density x Rep	1	5.11	0.0239
Mortality (rep 1)			
Density	1	3.85	0.0498
Sex	1	2.34	0.1262
Mortality (rep 2)			
Density	1	1.58	0.2090
Sex	1	0.21	0.6510

Transgenerational Density Effects

To test for the presence of transgenerational effects on cuticle and immunity, we compared the offspring from the treatments whose parents shared the same density treatment (i.e. ♀50 x ♂50 versus ♀150 x ♂150). Unlike the F1 generation, we found no size difference among these treatments in the F2 generation ($P = 0.4591$) and therefore did not included size in our cuticle color analysis. We found that parents reared at high densities produced offspring that exhibited a lighter thorax and abdomen compared with offspring of their low density counterparts (**Figure 6**; **Table 3**); therefore, the pattern for F2 offspring was opposite of their parents. There was a significant interaction between parental density and the photographic slide, which was driven by variation among slides in the treatment differences. With regard to immune function, we found no transgenerational effect of density on phenoloxidase activity ($P = 0.3330$) or on survival against a bacterial pathogen ($P = 0.2998$). These data suggest that density has transgenerational effects on cuticle, but not on immunity.

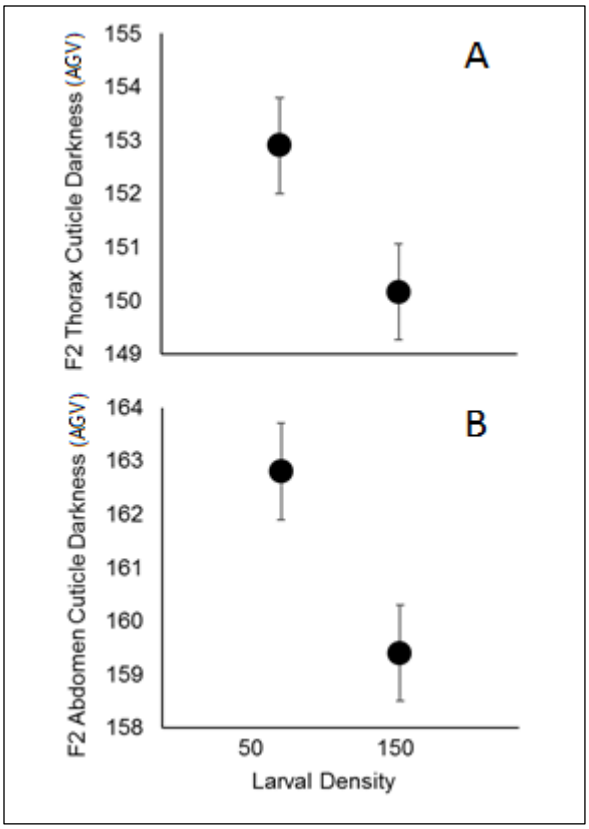

Figure 6. Offspring of high density parents had lighter thoraxes (A) and abdomens (B) than offspring from low density parents.

Table 3. Transgenerational Effect of Density on F2 Cuticle Darkness

Source	DF	SS	F	P
Thorax				
Density	1	1067.2	11.7	0.0007
Sex	1	2655.3	29.2	0.0001
Slide	14	14767.7	11.6	0.0001
Control (Slide)	15	6720.7	4.9	0.0001
Cohort	1	3184.1	35.0	0.0001
Density X Slide	14	3370.2	2.6	0.0012
Abdomen				
Density	1	1141.9	10.5	0.0013
Sex	1	902.3	8.3	0.0043
Slide	14	12803.5	8.4	0.0001
Control (Slide)	15	7400.9	4.5	0.0001
Cohort	1	3031.5	27.9	0.0001
Density X Slide	14	4265.3	2.8	0.0006

Source of Transgenerational Effects

To assess whether the transgenerational effect was of maternal and/or paternal origin, we compared offspring from all four mating treatments. As expected, we found that when maternal density increased, offspring thorax darkness decreased (**Table 4**; **Figure 7A**; abdomen darkness was not significant). Contrary to expectation, when male density increased, offspring thorax (**Figure 7B**) and abdomen darkness (**Figure 7C**) also decreased (**Table 4**). There was no effect of F1 maternal or paternal density on F2 phenoloxidase activity or bacterial survival. These data suggest that both epigenetic and provisioning mechanisms may be influencing the transgenerational influence of density on offspring phenotypes in the same direction.

Table 4. Source of Transgenerational Density Effect

Source	DF	SS	F	P
Thorax				
Maternal Denstiy	1	890.4	8.6	0.0035
Paternal Density	1	380.2	3.7	0.0558
Sex	1	5197.3	50.2	0.0001
Slide	14	24221.7	16.7	0.0001
Control (slide)	15	11322.1	7.3	0.0001
Cohort	1	9898.6	95.4	0.0001
Slide X Maternal	14	3925.8	2.7	0.0007
Abdomen				
Maternal Denstiy	1	259.5	2.3	0.1293
Paternal Density	1	1003.4	8.9	0.0029
Sex	1	3663.9	32.6	0.0001
Slide	14	20654.8	13.1	0.0001
Control (slide)	15	12403.2	7.4	0.0001
Cohort	1	11342.4	100.9	0.0001
Slide X Maternal	14	4012	2.5	0.0015

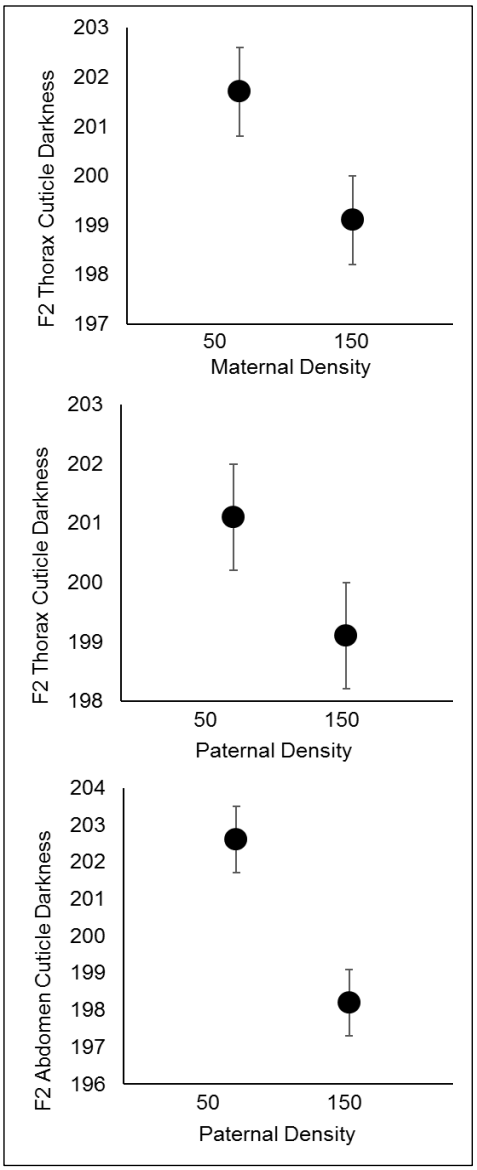

Figure 7. (A) Mothers reared at the high density produced offspring that had lighter thoraxes than low density mothers. (B) Fathers reared at the high density also produced offspring that had lighter thoraxes than low density fathers. (C) Fathers reared at the high density produced offspring with lighter abdomens than low density fathers.

DISCUSSION

A basic tenet of epidemiology and disease ecology is that as population density increases, the risk of disease transmission also increases. To minimize this risk, individuals in high density environments are expected to invest heavily in immune preventative measures that reduce pathogen susceptibility. Density-dependent prophylaxis (DDP) represents such a measure. The preponderance of evidence for DDP has come from phase polyphenic insects (insects that exhibit distinct high and low density morphotypes) that tend to exhibit darker cuticles and improved immune defense in high density environments (Kong et al., 2013). Moreover, two recent phase polyphonic insect studies have shown that DDP can reduce next generation offspring immunity (Miller et al. 2009; Wilson and Graham 2015), which should have a profound influence on multigenerational disease dynamics. However, the source (maternal or paternal) and the mechanism (provisioning or epigenetic) underlying the transgenerational effect is currently unclear and there is little evidence that DDP exists outside of the small group of phase polyphenic insects. In this study, we addressed three questions regarding transgenerational density-dependent prophylaxis: (1) does DDP exist in a non-phase polyphenic system, (2) do transgenerational effects of DDP exist in non-phase polyphenic systems, and (3) are transgenerational effects due to resource provisioning or epigenetic effects (or both)?

With regard to the first question, we found that the non-phase polyphenic fruit fly, *Drosophila melanogaster*, exhibited a darker thorax (but not abdomen) when reared at a high population density. We also found that high density flies were better able to defend against a live bacterial infection in one of two experimental replicates. One reason why only one of the replicates showed a significant difference may be due to the high survival rates (~85%). When more individuals survive an infection, variation between the density treatments is diminished making the detection of a significant difference difficult. We also did not observe a significant difference in phenoloxidase concentration between low and high densities, which may be due to the difficulty inherent in acquiring a standardized amount of hemolymph from *D. melanogaster*. The method for hemolymph extract employed here used pin pricks followed by mass

centrifugation on 20 individuals into a buffered solution, which may have significantly increased phenoloxidase sample variation. Alternatively, phenoloxidase may not be sensitive to the density treatments employed here. Another possibility for not observing strong immune responses to density is that previous studies that have determined the effect of density on immune function assessed larval and juvenile immune function while my study assessed adult immune function and perhaps the effect of density on immune function diminishes when an individual matures into an adult. Regardless, the cuticle and survival data support the hypothesis that DDP exits in non-phase polyphenic taxa and is likely a basal trait in insects.

The second question addressed whether DDP transgenerational effects also exist in non-phase polyphenic systems. When the offspring of the density treatments were reared under a standard density, we found that darker, high density parents produced offspring with lighter cuticles. This pattern is consistent with previous work in phase polyphenic insects (Miller et al. 2009; Wilson and Graham 2015). However, we did not detect any transgenerational immunity effects (i.e. parental density had no effect on offspring infection survival and phenoloxidse activity). This is curious, as the DDP hypothesis centers on improved immunity. Given that cuticles exhibited a more robust and consistent response to density than did immune defense *per se* suggests that cuticle darkness is the target of density selection and not immune function. If true, then our data suggest an alternative hypothesis to density-dependent prophylaxis. In short, cuticle darkening in high density environments may occur to improve thermoregulation, not immune defense. Darker cuticles allow for the absorption of more solar radiation, which increases metabolic rates. When densities are high and food resources scarce, darker individuals may possess an advantage by acquiring resources more quickly and/or by reaching adulthood more quickly, effectively outcompeting their lighter cuticle counterparts. The improved immunity seen in most DDP studies may be an indirect response considering that cuticle melanin and phenoloxidse are genetically correlated (Armitage and Siva-Jothy 2005; Rolff et al 2005). Although darker cuticles may not translate into much of a thermoregulatory advantage in *D. melanogaster* due to its small size, this response likely evolved

much earlier in evolutionary time when *D. melanogaster* ancestors may be profited from such a response. The cuticle response to density in *D. melanogaster* persists today most likely because it has a weak association with fitness and is therefore not selected against.

The third question addressed the source of the transgenerational effect by mating male and female parents from both densities in a fully factorial design. We found that mothers reared in high density were themselves darker, but produced offspring with lighter cuticles compared with the offspring of low density parents. Surprisingly, fathers reared at high densities showed the same pattern. To date, the prevailing hypothesis for why high density mothers produce offspring with weakened immunity was a lack of maternal provisioning of immune-related products, which are instead used for the mother's immune needs. However, the observation that high density fathers produce the same phenotype suggests that the effect is epigenetically based and adaptive. One potential adaptive reason for this pattern may be that parents expect the next generation to experience a low population density. Although this hypothesis is speculative, it may be driven by boom and bust cycles like that seen in swarming pest species such as locust. Alternatively, the pattern may be an artifact of experimental design. High density parents were reared at a density of 150 larvae per vial, low density parents were reared at 50 larvae per vial, and the offspring of each were reared at 90 larvae per vial. Thus, the offspring of high density parents experienced a reduction in density while the offspring of low density parents experienced an increase in density. In other words, the offspring experienced a different environment than they were epigenetically programed for, resulting in the pattern reversals. If true, then it suggests that offspring are somehow equipped to deal with inaccurate transgenerational signals, which is an interesting prospect. Another possibility is that density is not directly causing the patterns that we see but density affects another aspect in insect systems which then has an effect on offspring cuticle darkness. For instance, high densities could play a role in sexual conflict and fathers may invest differently in offspring when they are reared at higher densities. The mechanism underlying the pattern that we see from both parents on offspring cuticle color is unclear, but we know that there is a significant response from offspring of both mothers

and fathers reared at high densities and that the response is in the same direction for each. Therefore, we now have a direction for future research and more questions that can be answered concerning the mechanisms behind that pattern that we found in this study.

In short, we show that DDP exists in common insects that do not exhibit strict light/dark density morphotypes, suggesting the trait is basal to the insect phylogeny. Most notably, we show that paternal environment has a significant impact on offspring phenotype when it was previously thought that parental effects of density on offspring immune function was solely a maternal effect. Now we know that high density fathers are producing offspring with lighter cuticles which is the same pattern seen for offspring of high density mothers. Immune-related traits, however, showed no transgenerational effect. These data suggest that the transgenerational effect of cuticle color is epigenetic. Furthermore, the lack of transgenerational immune effects but strong cuticle effects suggest that cuticle darkening is the target of density selection and not immunity, supporting an alternative hypothesis to DDP where cuticle darkening in high densities aims to improve competitiveness for resources or perhaps future research will support an alternative mechanism behind the results found in this study.

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