No Evidence for Density-Dependent Prophylaxis in Response to Adult Crowding in *Drosophila melanogaster*

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Department of Biological Sciences
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"….Time only remains for us to ripe

like a harvest in green soil…."

-- Jibanananda Das

Dedicated to my parents

Certificate of Examination

This is to certify that the dissertation titled -"No evidence for density-dependent prophylaxis in response to adult crowding in *Drosophila melanogaster*" submitted by Mr. Paresh Nath Das (Reg. No. MS15175) for the partial fulfillment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The dissertation work represents original research carried out at IISER, Mohali under the supervision of Dr.N.G.Prasad, Associate Professor, Department of Biological Sciences during the academic year 2019-2020. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

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Dr. N.G. Prasad	Dr. Rhitoban Ray Choudhury	Dr. Manjari Jain

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr. N.G. Prasad at the Indian Institute of Science Education and Research Mohali.

This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

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Paresh Nath Das

(Candidate)

Dated: 4th May,2020

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

…...……

Dr.N.G.Prasad

(Supervisor)

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List of figures

List of Tables

Contents

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Abstract

Increasing densities also increases chances of pathogen transmission, which has been hypothesized to prompt organisms to mount a prophylactic immune response when living in dense conditions (density-dependent prophylaxis). Alternatively with increase in density the per capita resource availability falls and this is expected to manifest in form of deteriorating physiological state of individuals, including a compromised immune system. I tested for these hypotheses by crowding adult fruit flies (*Drosophila melanogaster*) at different densities, and then measuring their immune function against infection with bacterial pathogens and starvation resistance. My results indicate that with increase in density, immune function remains unchanged or is compromised, depending on the pathogen. This negates the density dependent prophylaxis hypothesis. I did not observe reduction in starvation resistance because of crowding, so these results are unlikely to be caused by differential availability of resources.

Chapter 1

Introduction

Density-dependent prophylaxis (DDP) is a phenomenon, where animals heighten their disease resistance with increase in density to counter greater infection risk. This was first proposed as a hypothesis, and experimentally verified, by Wilson and Reeson (1998). DDP hypothesis states that, when in high density, increased social contact might signal greater infection risk and prompt a prophylactic upregulation of immunity.

Fighting pathogens is a costly affair, in terms of resources, and therefore continuous activation of immune response in not ideal. So, disease resistance should show some kind of phenotypic plasticity depending upon the threat present in the immediate environment. Studies have shown that animals living at high density experience a greater risk of contracting infectious diseases (Alexandar, 1974; Freeland et al.1976; Møller et al., 1993; Krause and Ruxton, 2002; Moore, 2002; Altizer et al., 2003). The reason for this is that at high density host contact rates increase and it is easier for pathogens to spread (Anderson and May,1979,1981; McCallum et al., 2001; Lloyd-Smith et al., 2005). Studies across many species support this argument: this has been shown in insects (Dwyer and Elkinton, 1993; Knell et al., 1996; Ryder et al., 2005; Lindsey et al., 2009), birds (Brown and Brown, 1986 ; Sheilds and Crook, 1987), echinoderms (Lessios, 1988; Lafferty, 2004), mollusks (Lafferty and Kuris, 1993), reptiles (Godfrey et al., 2009), and mammals (Freeland, 1979; Hoogland,1979, 1995; Wilkinson, 1985). Density also decreases per capita resource availability and changes population growth parameters. According to the hypothesis, this high risk will act as a cue of increased threat from pathogens and a phenotypically plastic immune response will be generated to counter that. So, in higher density more resources will be invested in resistance to infection and prophylactic increase of immunity will be observed (Wilson and Reeson, 1998: Cotter et al., 2004).

There is some empirical evidence in support of the DDP hypothesis. First evidence comes from *Spodoptera exempta* where at high density, higher disease resistance against viral pathogen (Reeson et al., 1998) and fungal pathogen (Wilson et al., 2001) were observed. In *Tenebrio molitor*, a general higher immunity against entomopathogenic fugus was observed

in individuals reared in high density (Barnes and Siva-Jothy, 2000). It is also known that resistance to fungus increases with density in several other insect species like *Spodoptera littoralis* (Wilson et al., 2001), *Schistocerca gregaria* (Wilson et al., 2002). DDP was also found in armyworm *Mythimna seperata* (Mitsui and Kunimi, 1988; Kumini and Yamada, 1990).

As major evidences come from phase polyphenic insects, initially it was considered that there might be a correlation between phase polyphenism and larval density driven DDP (Wilson and Cotter, 2009). But later evidences contested this view. DDP was also found in non polyphenic insects like *Zootermopsis angusticollis* (Rosengaus et al., 1998) and *Acromyrmex echinatior* (Hughes et al., 2002), where increase in resistance to fungus was found with increase in population density.

Gonzalez and colleagues (2009) claimed that DDP is not just dependent upon larval conditions but can also manifest due to changes in adult environment. Their results showed that in social insects, like bumble bee (*Bombus terrestris*), rapid plasticity in immune function can be observed in response to high adult density, especially among worker bees. This was the first study which showed DDP in social animals (Ruiz-Gonzalez et al., 2009). DDP due to changes in adult density has also been reported in Mormon crickets (Bailey et al.,2008).

Other than insects DDP is also present in other invertebrates like sea star species *Acanthaster planci*, where adults reared at high density were more resistant to bacterial pathogens (S.C.Mills, 2012).

In cabbage moth *Memestra brassicae* the relationship between population density and resistance to viral pathogens was found to be complex: positively correlated over a certain range but reduced immunity when extreme densities were reached, suggesting that under very crowded condition this adaptive responses to density might break down (Goulson and Cory, 1995).

Yet other studies have failed to find DDP like response in certain insects, like termites (Pie et al., 2005) and field cricket (*Gryllus texensis*) (Adamo, 2006).

Other studies have reported lower disease resistance at high host densities. Steinhauss (1958) first hypothesized and verified this in caterpillars of various species and their natural pathogens. This hypothesis states that high density conditions increase intraspecific competition, which creates physiological stress or nutrient limitation, and therefore host becomes more susceptible to the infection; the 'Crowding stress hypothesis'. There are other empirical evidences for this. In cabbage moth (*Mamestra brassicae*), organisms reared in extreme high density have lower disease resistance (Goulson and Cory, 1995). Studies done on larvae of gypsy moth (*Lymantria dispar*) (Reilly and Hajek, 2008) and monarch butterfly (*Danaus plexippus*) (Lindsey et al.,2009) also support this hypothesis.

In *Drosophila melanogaster*, one study checked immunity against bacteria in solitary and paired (single sex) treatments and found that paired individuals perform same or better than single flies in terms of post-infection lifespan, and concluded in favour of DDP present (Leech et al., 2019).

Beyond immunity, density also has major impact on other life history traits. It is also known that adult density has an effect on longevity; crowding adults in early life has been shown to significantly reduce longevity in *Drosophila melanogaster* (Graves and Mueller,1993: Joshi et al., 1998). Crowding adults primarily alters their age independent mortality rate (Joshi and Mueller, 1997). In *Drosophila melanogaster* adults housed in same sex pairs have reduced longevity than adults housed in isolation (Leech et al., 2017).

Studies have shown that increasing adult density increases progeny number per culture upto a maximum and then decreases in *Drosophila melanogaster* and *Drosophila simulans* (Sameoto and Miller, 1966; Baker, 1973). Trans-generational effects of adult density also has been reported in *Drosophila melanogaster* (Nandy et al., 2019).

It has been hypothesized that starvation resistance might also show some degree of density dependent adaptive plasticity. Crowding, or resultant decline food quality or quantity, can act as a cue to prompt a greater starvation resistance to deal with upcoming period of starvation (Rion and Kawecki, 2007).

In this study I have used a lab adapted population of *Drosophila melanogaster* to investigate the effects of manipulating adult density on immune function and resistance to starvation.

Since the population used here has been maintained in a constant and controlled lab environment for the past 200 generations (see Materials and Methods), it gives us the liberty to explore the effects of changing ecological parameters on organismal response with ease, without any confounding effects of population history. In the experiments reported in this thesis I have quantified immunity in terms of survival post infection with bacterial pathogens because this is considered to be a more holistic measure of immune function (Neyen et al., 2014) compared to quantifying individual components of the immune system.

Chapter 2

Materials and Methods

Fly populations and general handling

All experiments described in this thesis were conducted on a large outbred population of *Drosophila melanogaster*, the BRB2. The BRB1-5 (Blue Ridge Baselines) were established by hybridizing 19 wild-caught iso-female lineages, and thereafter splitting the obtained population into five independent replicates (Gupta et al., 2013). Since then these populations have been maintained under standard laboratory conditions for more than 200 generations prior to these experiments. BRB2 is maintained in a 14-day discrete generation cycle on standard banana-jaggery-barley-yeast medium, with a census population size of about 2800 adults per generation. The BRB2 population has been well characterized in terms of immune function and life-history parameters (Basu et al, unpublished) and hence was used for this study.

Deriving flies for experiments

For all experiments described below, eggs were collected from population cages at a density of 60-80 eggs per vial (vial dimensions: 25 mm diameter × 90 mm height) with 8 mL of standard food medium, similar to the general maintenance of BRB2 population. Vials were incubated at 25 ^{O}C and 12:12 hours LD cycle; under this conditions the egg-to-adult development time for these flies is about 9-10 days. On day 12 post-egg laying (day of egg collection is demarcated as day 1), adults were sorted under light CO_2 anesthesia into their respective treatments (see below) and shifted to fresh food vials. Hereafter adults were maintained by flipping into fresh food vials every 2-3 days till the day of experiment. The amount food provided in the vials was *ad libitum* with respect to their density treatments to prevent starvation.

Immunity assay

To assay for immune function of the adult flies, two bacterial pathogens were used for infection: *Erwinia c. carotovora* and *Enterococcus faecalis*. 5 mL lysogeny broth (Luria-Miller-Hinton, HiMedia) was inoculated with a stab of bacterial glycerol stock and incubated overnight at 37 ^{O}C with aeration. Secondary culture was established by inoculating 10 mL lysogeny broth using 100 uL of this culture and allowed to grow till the culture was confluent. The bacterial cells were then pelleted down via centrifugation and re-suspended in sterile $MgSO_4$ buffer at 1.0 OD_{600}. Flies were infected by pricking them in the thorax under light CO_2 anesthesia with 0.01 mm Minutein pins (Fine Scientific Tools, USA) dipped in the bacterial slurry. Sham infections were done similarly except that the pins were dipped into sterile buffer. Flies were then placed in fresh food vials, and were again shifted to fresh food vials once at about 72 hours post-infection. Vials were monitored every 4-6 hours and mortalities were recorded for 120 hours post-infection. For all infection experiments, sample size per density treatment was 160 males and females (1:1 sex ratio) for infections and 80 males and females for sham-infections.

Starvation resistance assay

To assay for starvation resistance, flies were placed into glass vials with 2 mL of non-nutritive agar (2%) gel. Flies were transferred to new vials every 2-3 days, and checked for mortality every 8-10 hours till every fly was dead. For all starvation resistance experiments, sample size per density treatment was 160 males and females (1:1 sex ratio).

Statistical analysis

Survival analysis was done in R (R Core Team 2019, v3.6.2) using the package *survival* (Therneau 2015, v2.38). All analysis was done after pooling data across both replicates (see

below). Combined effects of density and sex was tested using Pairwise Log Rank tests on survival data, using the Benjamini and Hochberg (1995) method to adjust p-values for multiple comparisons. Survival curves were plotted using the package *survminer* (Kassambara et al., 2019, 0.4.6).

Experimental design

Experiment 1(a): Effect of density (32 adults vs. 8 adults) on immune function, two-day conditioning

2-3 day old adult flies were sorted into fresh food vials at densities of 8 individuals or 32 individuals in each vial, with 1.5-2 mL of food, in 1:1 sex ratio. The flies were held in these vials for two days, the conditioning period. After the conditioning the flies were subjected to infections as described above, and housed at density of 4 males and 4 females per vial. 20 infection vials were set up per density treatment and 10 sham-infection vials were set up per treatment. The experiment was replicated twice.

Experiment 1(b): Effect of density (32 adults vs. 8 adults) on immune function, ten-day conditioning

2-3 day old adult flies were sorted into fresh food vials at densities of 8 individuals or 32 individuals in each vial, with 1.5-2 mL of food, in 1:1 sex ratio. The flies were held in these vials for ten days, the conditioning period. After the conditioning the flies were subjected to infections as described above, and housed at density of 4 males and 4 females per vial. 20 infection vials were set up per density treatment and 10 sham-infection vials were set up per treatment. The experiment was replicated twice.

Experiment 2: Effect of density (200 adults vs. 50 adults) on immune function

2-3 day old adult flies were sorted into fresh food vials at densities of 50 individuals or 200 individuals in each vial, with 1.5-2 mL of food, in 1:1 sex ratio. The flies were conditioned in these densities for two days. After the conditioning the flies were subjected to infections as described above, and housed at density of 4 males and 4 females per vial. 20 infection vials were set up per density treatment and 10 sham-infection vials were set up per treatment. The experiment was replicated twice.

Experiment 3: Effect of density (32 adults vs. 8 adults) on starvation resistance

2-3 day old adult flies were sorted into fresh food vials ad densities of 8 individuals or 32 individuals in each vial, with 1.5-2 mL of food, in 1:1 sex ratio. The flies were conditioned in these densities for two days. After the conditioning the flies were placed in agar vials as described above, and housed at density of 4 males and 4 females per vial. 20 vials were set up per density treatment. The experiment was replicated twice.

Experiment 4: Effect of density (200 adults vs. 50 adults) on starvation resistance

2-3 day old adult flies were sorted into fresh food vials ad densities of 50 individuals or 200 individuals in each vial, with 1.5-2 mL of food, in 1:1 sex ratio. The flies were conditioned in these densities for two days. After the conditioning the flies were placed in agar vials as described above, and housed at density of 4 males and 4 females per vial. 20 vials were set up per density treatment. The experiment was replicated twice.

Chapter 3

Results

Experiment 1(a): Effect of density (32 adults vs. 8 adults) on immune function, conditioning for two days

For flies infected with *Erwinia c. carotovora* (hereafter, Ecc), sex was a major determinant of post-infection survival, with females always surviving more than males irrespective of the density treatment. Within each sex, adults conditioned at a lower density had significantly greater survival compared to flies crowded at higher density (males: $p < 0.001$; females: $p = 0.024$; Pairwise Log Rank tests reported in table 1(a)).

For flies infected with *Enterococcus faecalis* (hereafter, Ef) neither sex nor density treatment had any effect on post-infection survival of the adults (table 1(a)).

Experiment 1(b): Effect of density (32 adults vs. 8 adults) on immune function, conditioning for 10 days

For flies infected with Ecc, sex was the major determinant of survival post infection, with females surviving more than the males. Density had no effect on survival for either sex (table 1(b)).

For flies infected with Ef, neither sex nor density treatment had any effect on post-infection survival of the adults (table 1(b)).

Experiment 2. Effect of density (200 adults vs. 50 adults) on immune function, conditioning for two days

For flies infected with Ecc, females in general survived better than males, irrespective of density treatment. Within each sex, adults subjected to a lower density had significantly

greater survival compared to flies conditioned at higher density (males: $p = 0.010$; females: $p = 0.005$; Pairwise Log Rank tests reported in table 2).

For flies infected with Ef, neither sex nor density treatment had any significant effect on post-infection survival of the adults (table 2).

Experiment 3. Effect of density (32 adults vs. 8 adults) on starvation resistance, conditioning for two days

I measured starvation resistance as time to death when adults were not allowed any access to food continuously, with ad libitum supply of water in form of 2% agar gel.

Females in general survived better than males when starved, but survival was not influenced by conditioning at different densities (table 3).

Experiment 4. Effect of density (200 adults vs. 50 adults) on starvation resistance, conditioning for two days

Females in general survived better when starved compared to males, with a strong Sex X Density interaction. Among the females, adults conditioned at higher density were more resistant to starvation than adults housed at lower density ($p = 0.013$), but there was no observable effect of density on starvation resistance among the males ($p = 0.563$; table 4).

Tables and figures

Table 1(a). Effect of density (32 adults vs. 8 adults) on immune function after two days of conditioning: Pair-wise Log Rank test on survival data (p-values adjusted for multiple comparisons in Benjamini & Hochberg (1995) method)

Pathogen: *Enterococcus faecalis*				
	HD (Female)	HD (Male)	LD (Female)	LD (Male)
HD (Female)	-			
HD (Male)	0.99	-		
LD (Female)	0.24	0.24	-	
LD (Male)	0.24	0.24	0.99	-
Pathogen: *Erwinia c. carotovora*				
	HD (Female)	HD (Male)	LD (Female)	LD (Male)
HD (Female)	-			
HD (Male)	<0.001	-		
LD (Female)	**0.02479**	<0.001	-	
LD (Male)	0.00594	**<0.001**	<0.001	-

Table 1(b). Effect of density (32 adults vs. 8 adults) on immune function after ten days of conditioning: Pair-wise Log Rank test on survival data (p-values adjusted for multiple comparisons in Benjamini & Hochberg (1995) method)

Pathogen: *Enterococcus faecalis*				
	HD (Female)	HD (Male)	LD (Female)	LD (Male)
HD (Female)	-			
HD (Male)	0.78	-		
LD (Female)	**0.78**	0.78	-	
LD (Male)	0.78	**0.86**	0.78	-
Pathogen: *Erwinia c. carotovora*				
	HD (Female)	HD (Male)	LD (Female)	LD (Male)
HD (Female)	-			
HD (Male)	<0.001	-		
LD (Female)	**0.21**	<0.001	-	
LD (Male)	<0.001	**0.22**	<0.001	-

Table 2. Effect of density (200 adults vs. 50 adults) on immune function after two days of conditioning: Pair-wise Log Rank test on survival data (p-values adjusted for multiple comparisons in Benjamini & Hochberg (1995) method)

Pathogen: *Enterococcus faecalis*				
	HD (Female)	HD (Male)	LD (Female)	LD (Male)
HD (Female)	-			
HD (Male)	0.37	-		
LD (Female)	**0.36**	0.93	-	
LD (Male)	0.08	**0.36**	0.36	-
Pathogen: *Erwinia c. carotovora*				
	HD (Female)	HD (Male)	LD (Female)	LD (Male)
HD (Female)	-			
HD (Male)	<0.001	-		
LD (Female)	**0.0106**	<0.001	-	
LD (Male)	0.1057	**0.0053**	<0.001	-

Table 3. Effect of density (32 adults vs. 8 adults) on starvation resistance after two days of conditioning: Pair-wise Log Rank test on survival data (p-values adjusted for multiple comparisons in Benjamini & Hochberg (1995) method)

	HD (Female)	HD (Male)	LD (Female)	LD (Male)
HD (Female)	-			
HD (Male)	0.00276	-		
LD (Female)	**0.21230**	<0.001	-	
LD (Male)	<0.001	**0.66437**	<0.001	-

Table 4. Effect of density (200 adults vs. 50 adults) on starvation resistance after two days of conditioning: Pair-wise Log Rank test on survival data (p-values adjusted for multiple comparisons in Benjamini & Hochberg (1995) method)

	HD (Female)	HD (Male)	LD (Female)	LD (Male)
HD (Female)	-			
HD (Male)	<0.001	-		
LD (Female)	**0.013**	<0.001	-	
LD (Male)	<0.001	**0.563**	<0.001	-

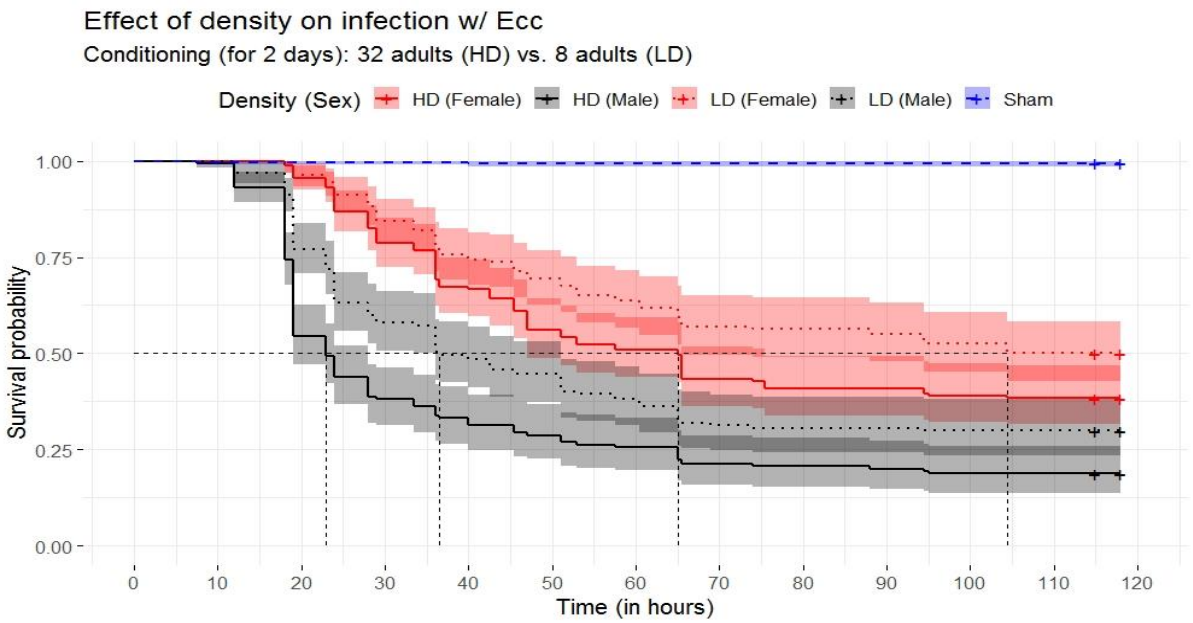

Figure 1(a). Survival curve: Effect of density (32 adults vs. 8 adults) on immune function with Ecc after two days of conditioning.

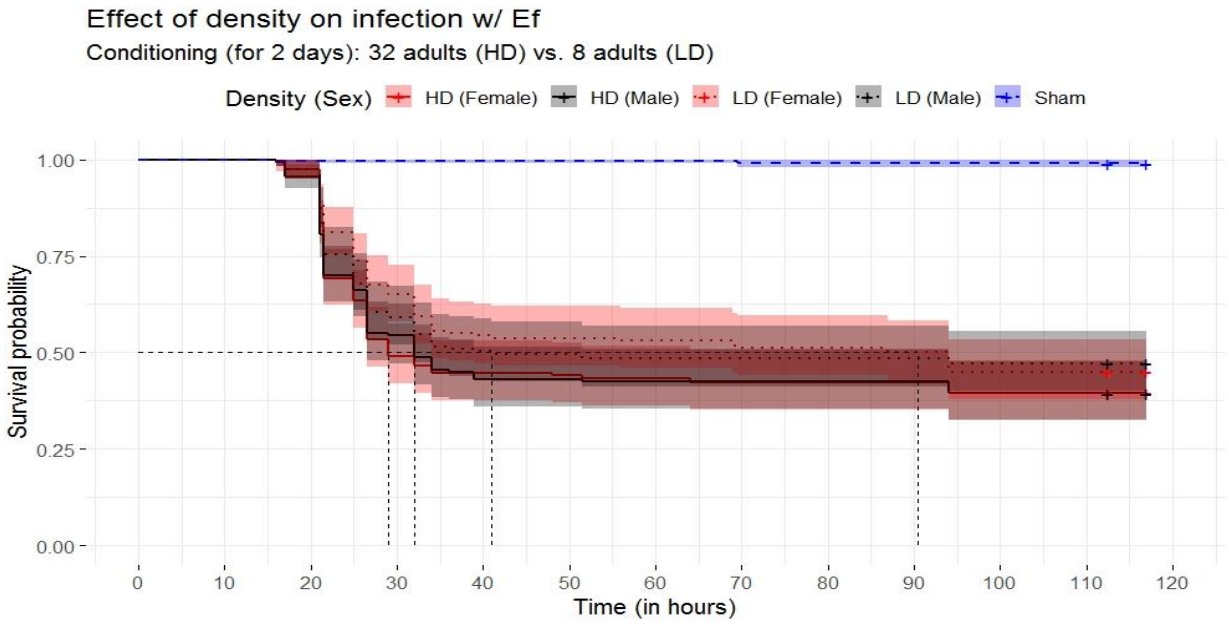

Figure 1(b). Survival curve: Effect of density (32 adults vs. 8 adults) on immune function with Ef after two days of conditioning .

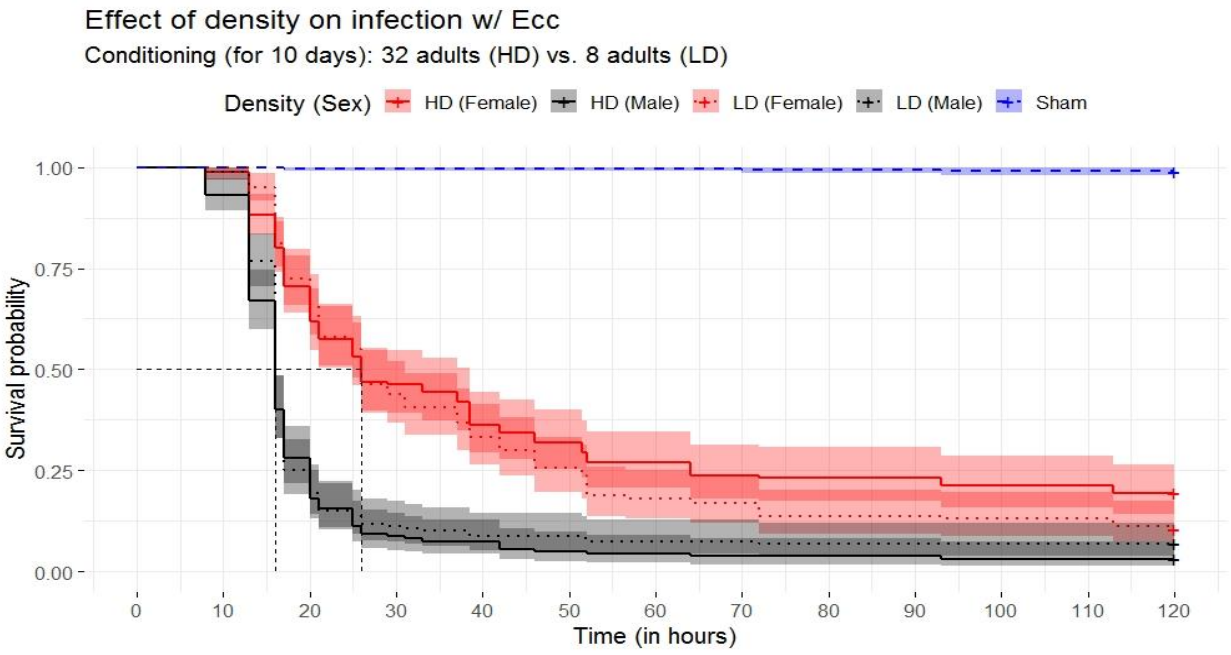

Figure 2(a). Survival curve: Effect of density (32 adults vs. 8 adults) on immune function with Ecc after ten days of conditioning.

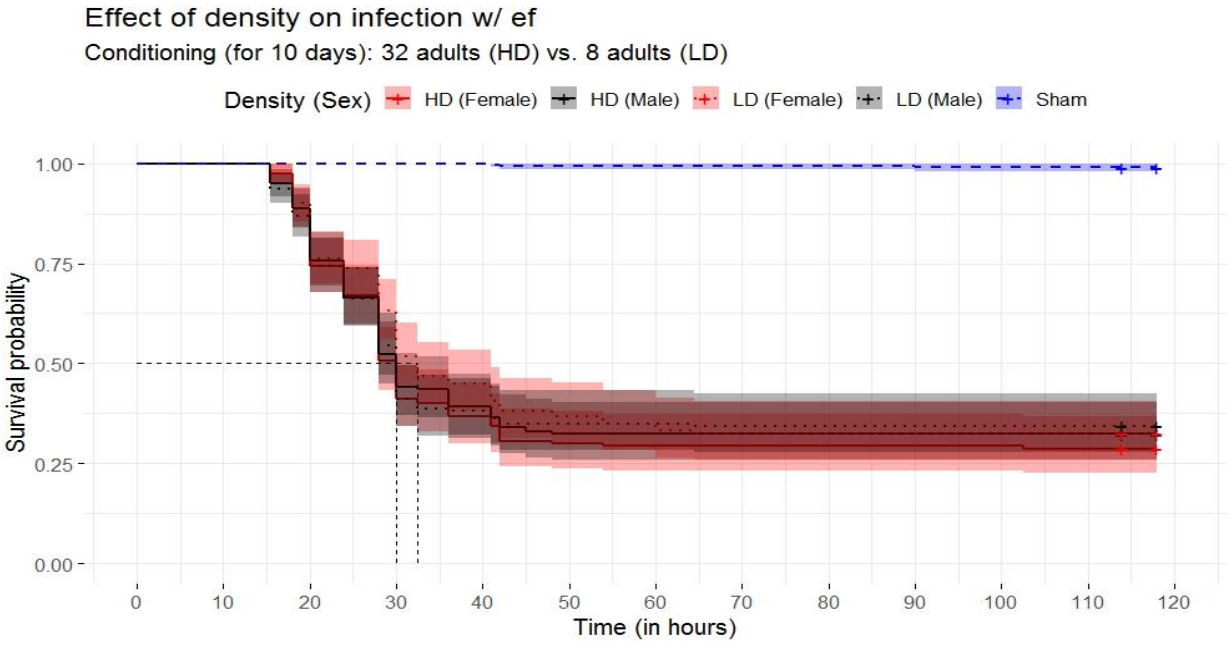

Figure 2(b). Survival curve: Effect of density (32 adults vs. 8 adults) on immune function with Ef after ten days of conditioning.

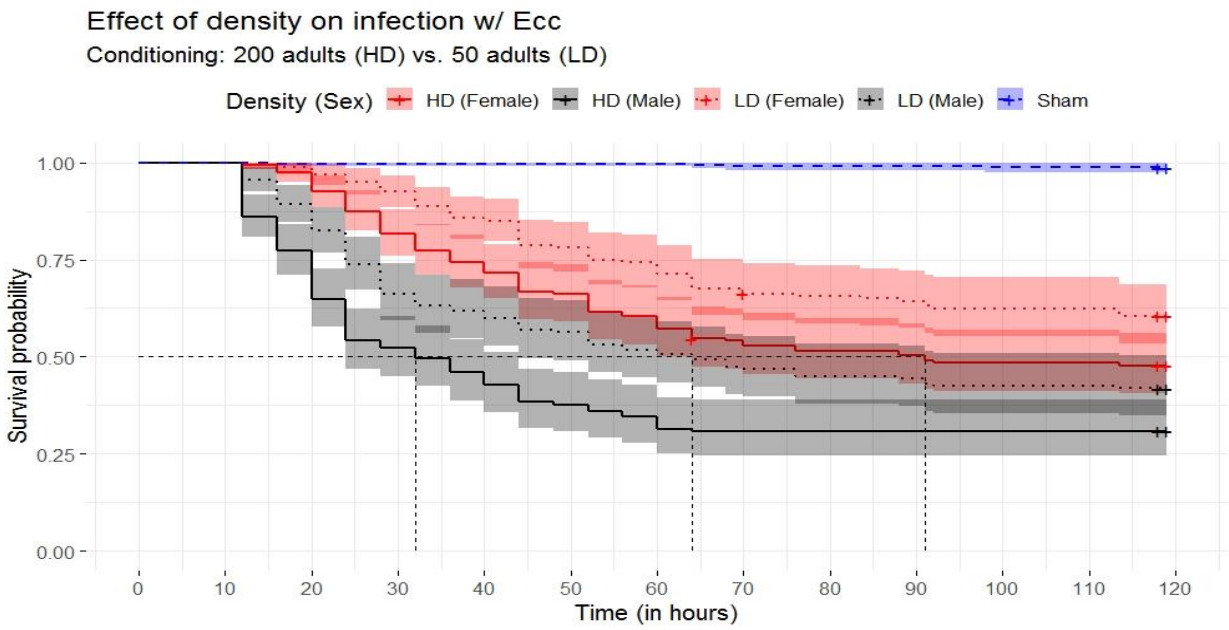

Figure 3(a). Survival curve: Effect of density (200 adults vs. 50 adults) on immune function with Ecc after two days of conditioning.

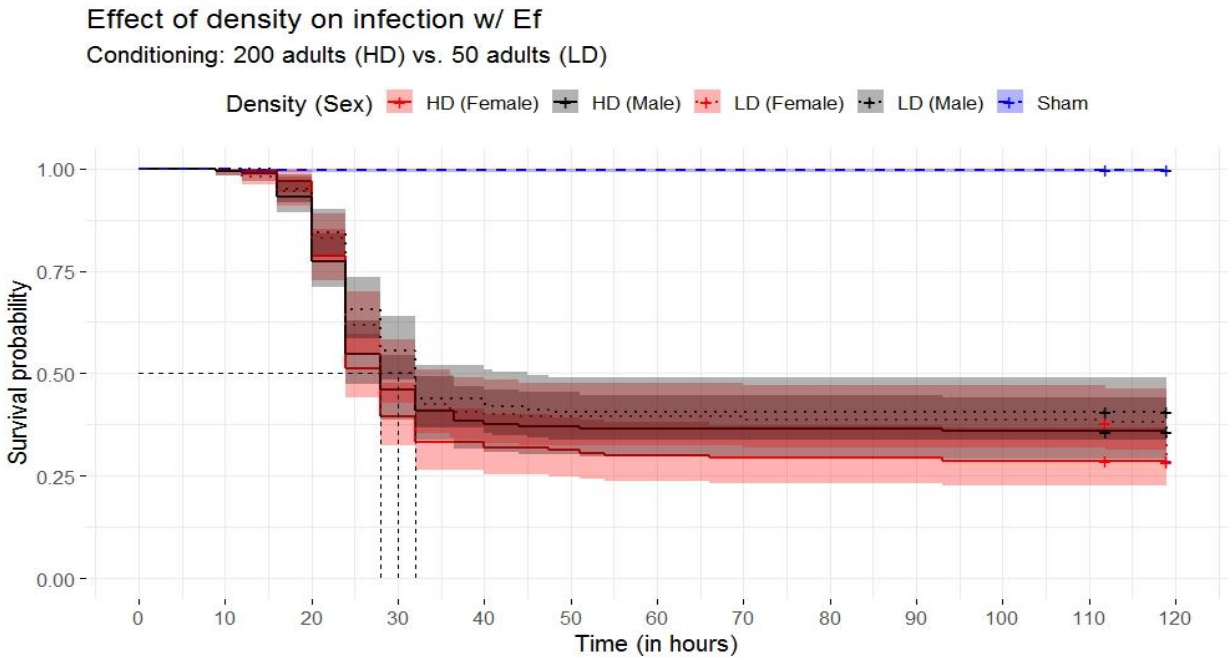

Figure 3(b). Survival curve: Effect of density (200 adults vs. 50 adults) on immune function with Ef after two days of conditioning.

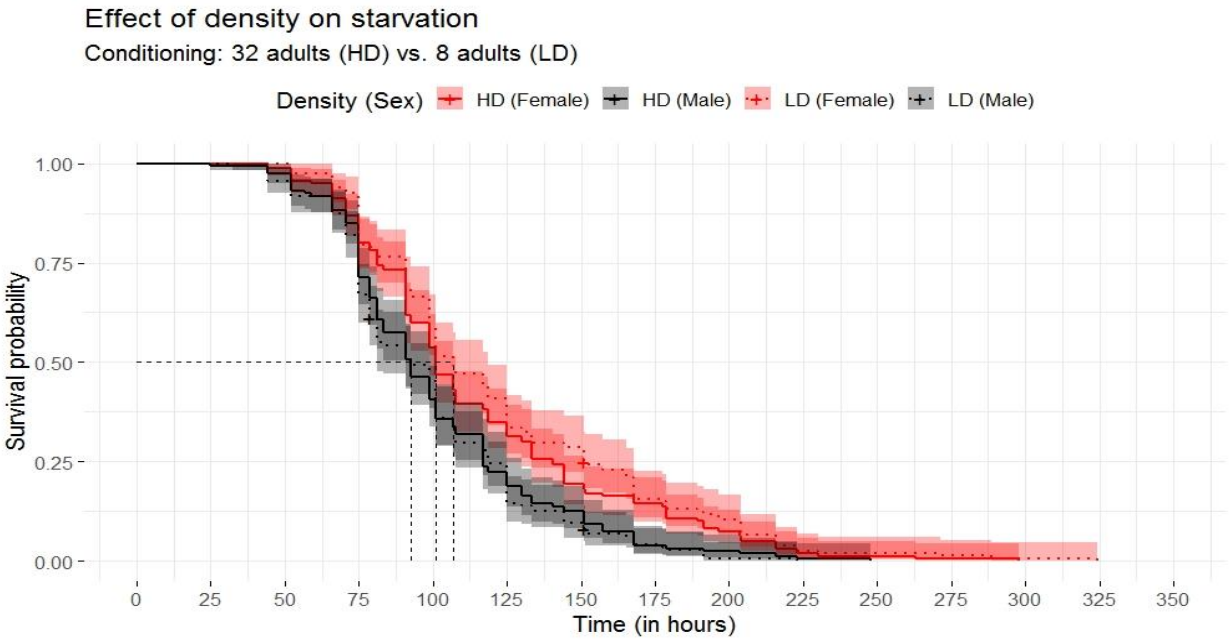

Figure 4. Survival curve: Effect of density (32 adults vs. 8 adults) on starvation resistance after two days of conditioning.

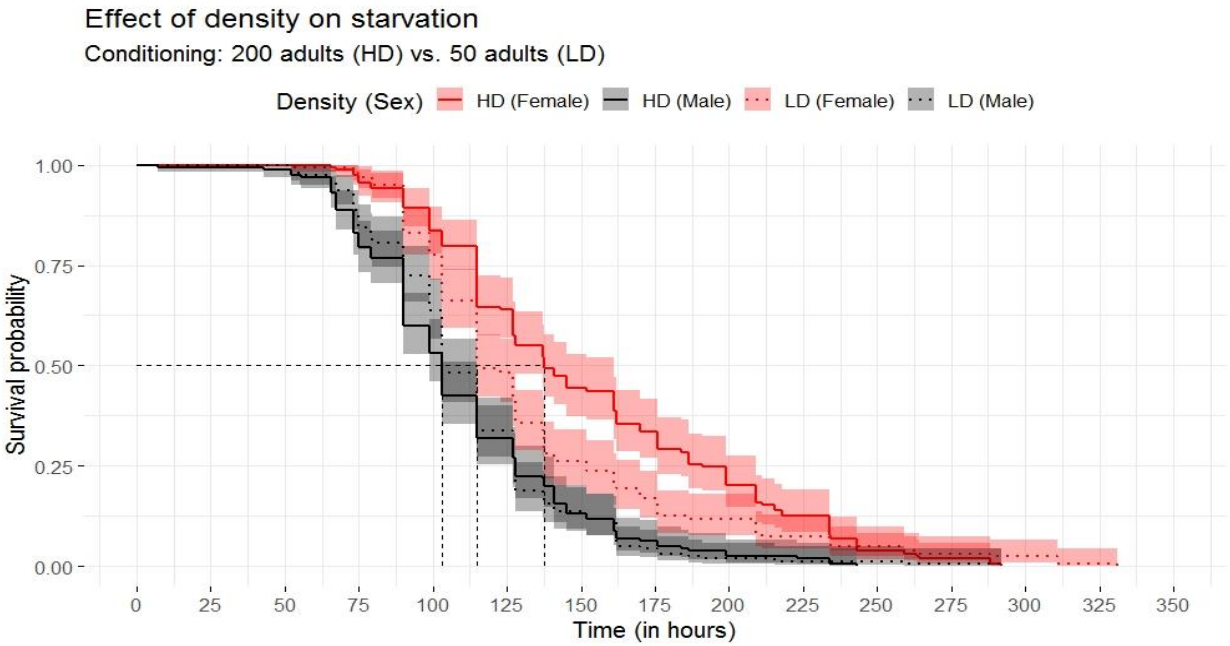

Figure 5. Survival curve: Effect of density (200 adults vs. 50 adults) on starvation resistance after two days of conditioning.

Chapter 4

Discussion

Various studies in the past have tested for correlations between host population density and host immune response. The density dependent prophylaxis (DDP) hypothesis suggests that due to anticipated increase in risk of infection when population densities increase individual organisms upregulate their immune system as a counter measure (Wilson and Reeson, 1998). Alternatively, the crowding stress hypothesis proposes that at high population density organism's physiological capabilities are compromised due to resource limitation and stress, which leads to individuals having sub-optimal immune function (Steinhauss, 1958).

In most studies exploring the effect of population density on organism's immune function subjected the juveniles to crowding and tested for prophylaxis in adults or in later life stage (Barnes and Siva-Jothy, 2000; Wilson et al., 2002; Reeson et al., 1998; Kumini and Yamada, 1990; Wilson and Cotter, 2009). Positive results obtained via such study designs prompted the idea that DDP manifests primarily through developmental plasticity. But it has been shown that prophylaxis in adults can be induced by crowding just the adult organisms (Bailey et al., 2008; Ruiz-Gonzalez et al., 2009). Yet results are still equivocal as some studies have reported negative or no effect of population density on immune function (Steinhauss 1958 ; Goulson and Cory, 1995; Reilly and Hajek, 2008; Lindsey et al.,2009; Pie et al., 2005; Adamo, 2006).

Another drawback that plagues investigations into DDP is that very few studies have measured fitness directly in terms of survival post-infection with pathogens (SC Mills 2012; Barnes and Siva-Jothy, 2000; Wilson et al., 2002). This is a problem since it is often observed that changes in physiological immune components do not always translate to differences in survival (Fedorka et al., 2007; Woestmann and Saastamoinen, 2016).

Leech and colleagues (2019) did not found any predictable effect of social environment in *Drosophila melanogaster* but they showed that with one pathogen *P aeruginosa*, post infection lifespan is significantly higher for paired (same sex) individuals compared to

isolated individuals in older age (52 days). (Leech et al., 2019). This might not be because of DDP rather because of the effect of *isolation* as it is known that isolation and social contact effect differently across traits.(Bailey and Moore, 2018). Also those flies were held as virgin through-out their life, and it is possible that mating status in itself changes how a fly responds to social cues.

For this thesis, I subjected adult flies to two different density ranges, and then quantified their resistance against bacterial pathogens and starvation. The idea behind using two different density ranges was to test for the effect of increasing density with and without changing the amount of physiological stress suffered by the focal organisms. In the experiments comparing between 32 adults vs. 8 adults per vial, there was no differential mortality during the conditioning period, or even after that (shams died equally and negligibly in both treatments). But in experiments comparing between 200 adults vs. 50 adults per vial, considerable mortality was recorded during the two-day conditioning period in case of adults at higher density but not in the low density treatment (preliminary data, not shown here) ; post-conditioning mortality was not different between treatments in this case too (shams died equally and negligibly in both treatments). For the lower range of density (32 adults vs. 8 adults per vial) treatments, I also tested if conditioning period had any differential effect on density dependent immune function; longer duration of conditioning was not possible for the higher density range because of logistic issues and high mortality in the density treatment of 200 adults per vial.

To make my immune function experiments generalizable, I tested immunity of the focal organisms against two bacteria pathogens, one Gram negative (*Erwinia c. carotovora*, hereafter Ecc) and one Gram positive (*Enterococcus faecalis*, hereafter Ef). Previous research into *Drosophila* immunity has established that the mechanisms employed to counter these two types of pathogens are significantly different, with some level of cross-talk (Buchon et al., 2014).

The results from my immunity experiments seem to indicate an absence of DDP in response to adult crowding. When infected with Ecc, across both density range comparisons, flies housed at lower density had better immune function than flies housed at higher density (figures 1(a) and 3(a)). The effect is surely due to the differences during the conditioning

window as during the experiments (post infection) all adults were housed at equal density. Interestingly, increasing the conditioning period to ten days from two days seems to eliminate the effect of conditioning at different density (figure 2(a)), although this may be due to loss of plasticity with increasing age. Experiments with two-day conditioning and ten-day conditioning were done on flies aged 4-5 days and 12-13 days as adults, respectively.

For flies infected with Ef, no effect of conditioning at different density was apparent in any of the experiments (figures 1(a), 2(a), and 3(a)). This difference between pathogens may be due to differences in resistance mechanism used by the insect to fight these two pathogens.

The canonical alternative to DDP hypothesis is the crowding stress hypothesis, which argues that higher density leads to lack of resources, and therefore should compromise immune function. The results from experiments with Ecc seem to agree very well with this idea, and in that case the differences between results with Ecc and Ef can be attributed to differential energetic costs of immunity against these two pathogens. Fighting off against Ef may be cheaper and therefore is free of any negative effect of increasing density; although it is difficult to confirm this idea.

If the results of immune function experiments are indeed caused by differences in availability of resources during the conditioning period, this should also reflect in starvation resistance assays. Differential availability of resources is expected to translate into different levels of stored resources, which should lead to differential survival when subjected to starvation. This is assuming equal rates of resource utilization. By this argument adults housed at low densities should survive for a longer time when starved. Yet I see no such pattern in the results from starvation assays.

When comparing between 32 individuals vs. 8 individuals housed together, there was no observable effect of density (figure 4). And, in comparison between 200 individuals vs. 50 individuals housed in a single vial, females from high density treatment survived better than their low-density counterparts, while there was no effect of density on survival of males (figure 5). Indeed, it has been proposed before that organisms subjected to higher density may invest into a prophylactic stress response (Rion and Kawecki, 2007).

My results thus indicate that increasing density has opposite effects on immune function and resistance to starvation. It is difficult to explain these differences simply in terms of differential availability/acquiring of resources during the conditioning period. *Drosophila melanogaster* adults subjected to high density have been previously reported to only be different from low-density controls with respect to early life mortality only, which can be attributed to stress due to crowding (Joshi and Mueller, 1997). This might indicate that being housed at different densities do not necessarily imply differential levels of teneral resources. Also, differences in resistance to starvation can be due to differences in rate of resource use, independent of amount of resource stored in the body. The mechanisms that underly the plasticity in either immune function or stress resistance is little understood. Hence it is not possible as of yet to explain the full set of results under a single theoretical framework.

In conclusion, I found no indication of induction of density dependent prophylaxis by crowding of adults in *Drosophila melanogaster*. Results suggest that flies at lower densities either have better or equal immune proficiency as the flies at higher densities. Whether this result is driven by differences in teneral resources remains contested.

Chapter 5

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