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Impacts on Fitness Associated with Acquisition of Gut Microbiota in Drosophila

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Gut Microbiota Acquisition in *Drosophila*

Abbreviations:

Carbeni – carbenicillin
MRB – Male, regular food, bleached eggs
FRB – Female, regular food, bleached eggs
MCB – Male, carbenicillin food, bleached eggs
FCB – Female, carbenicillin food, bleached eggs
MCW – Male, carbenicillin food, water eggs
FCW – Female, carbenicillin food, water eggs
NW – None, water eggs
NB – None, bleached eggs

Abstract:

Drosophila melanogaster is a model organism that has been studied to demonstrate the role of gut microbiota in fitness. It has already been established the gut microbiota is extremely important for the health of the organism, but the source of the microbiota has not been studied as thoroughly. In order to test if the source of microbiota affects the fitness of the individual, adult male and female flies were placed onto plates to defecate after being raised on standard food and food with carbenicillin. Stock eggs were washed in either water or a bleach solution, then placed on the defecation plates. There was a significant difference between the fecundity of FRB vs NB, as well as longevity of MRB vs FRB, FRB vs NB, and NW vs NB. This indicates that microbiota can be obtained from the fecal matter that is around the egg as the larvae hatches, and that it impacts fitness.

Introduction:

In order to research physiological aspects that are applicable to humans, model organisms such as *Drosophila* can be used because they are more easily manipulated. For example, the

antimicrobial immune system has already been genetically well characterized in *Drosophila*, or fruit flies. This is a valuable model to study insect and human innate immune defenses (Lazzaro, 2008). Experimental conditions are much easier to control and the flies produce much faster than more complex organisms. Previous studies have shown that bacteria in the gut of the flies affect their longevity and fitness. For example, a diet rich in probiotics and prebiotics may help prevent chronic age-related disease (Westfall, 2018). It has already been widely studied that diet affects microbiota. The microbiota adapts as the physiological needs, such as food, of the host change (Erkosar, 2013). Other studies have also shown that the larvae obtain bacteria via consumption of the chorion of their own eggs and other foods after hatching (Bakula, 1969). One of these other food sources may be the feces of the adult flies that laid the eggs. A 2009 study showed that the Nora virus can be transferred via the oral-fecal route, implying that other microbes can be transferred as well (Habayeb, 2009). Not only has it been demonstrated that microbes can be transferred from feces to mouth, but also that fecal matter contains pheromones that affect behavior. The presence of fecal matter increases adult feeding and aggregation (Keesey, 2016). Given this information, it is likely that newly hatched larvae consume the feces left by the adult flies. There is little research about how the different sources of microbiota may affect the overall success and fitness of *Drosophila.* There could be variation if the larvae are hatched with feces from males vs. females that were grown on antibiotics vs. normal growth conditions. This experiment will elaborate on these ideas, extending it to the next generation of *Drosophila.* Does the source of the microbiota affect the fitness of the larvae that consume it?

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Materials and Methods:

Preparing Egg Plates

In order to make the plates for egg collection, 18 g of agar and 600 mL of water were mixed in a large, glass bottle. In a separate bottle, 20 g of sucrose and 200 mL of apple juice were mixed. These were both autoclaved for about 30 mins, then mixed together. The majority of the solution was poured into individual plates. With about 100 mL of the solution remaining, 100 µL of carbenicillin was added, then mixed and used to pour the remaining plates.

Carbenicillin Food Preparation

Most flies were raised on standard food. However, some of the parents were raised on standard food with carbenicillin. To do this, 1 tsp of powdered food was added to a fly vial. Next, 20 mL of water and 28 µL of carbenicillin were mixed together. This solution was divided among four vials, so 5 mL of water/carbenicillin was added for every 1 tsp of powdered food.

Parent Generation

Stock flies were raised and aged to be between 3-7 days old. These were divided evenly among four eggs plates, two of which were standard, and two included carbenicillin. These were incubated overnight at 25°C. The following day, eggs were collected from the egg plates and placed onto Whatman paper. The Whatman paper was placed into the appropriate food vials. Eggs from standard egg plates were placed onto standard food, with approximately 100 eggs each in two vials. This was repeated for the carbenicillin eggs onto the carbenicillin food. These eggs were left at 25°C in order to be raised into the “parent” generation. Once they began hatching, virgin females were separated from males. This was done to prevent females from

laying fertile eggs, adding an additional variable. These flies were then placed onto standard eggs plates to create "defecation plates". Five flies were placed onto each plate overnight following Table 1.

Treatment Eggs

Stock flies aged between 1-5 days old were placed on standard egg plates overnight at 25°C. The following day, eggs were collected and treated with either bleach or water, following Table 1. In order to bleach the eggs, a paintbrush was used to move them into a drop of water. Once enough eggs were collected, 8 drops of 50% bleach was added and swirled for about 15 seconds. It was quickly diluted with water, then taken off and rinsed three times with more water. For each treatment, 50 eggs were collected onto Whatman paper and placed onto the respective defecation plate. In order to treat eggs with water, the same procedure was used, but lacking bleach. The first defecations plates were made from February 17-18, then eggs were placed on February 19. On February 22, the larvae from each defecation plate were moved onto standard food, remaining separated by their treatments listed in Table 1. This was repeated to produce three trials in total.

Measuring Fecundity

On March 9, the first trial of eggs was aged at approximately 10-12 days old. From each treatment, 5 females and 2 males were placed into egg cups onto standard egg plates. They were left overnight at 25°C, and this was repeated for each trial. The following day, the number of eggs was counted and recorded, so the number of eggs laid per female could be calculated.

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Measuring Longevity

All remaining flies not used to measure fecundity were pooled into one vial per treatment. The starting populations differed between treatments. Females remained in the vials, but only males were counted. The flies were placed onto new food every 2-3 days and the number of deceased males was recorded each time. They were always stored at 25°C.

Results:

The number of eggs laid per female was calculated for each treatment. The average of all three trials is shown in Figure 1, including standard error bars. The overlap in most standard error bars indicates few statistical differences. Excel was used to run a two-tailed t-test on the data (Table 2). There was a significant different between treatments with no defecation and bleached eggs vs females on standard food and bleached eggs. In this comparison, the only variable is the presence of defecation. There was also a significant different between females on standard food and bleached eggs vs males on carbenicillin and bleached eggs. However, there are two variables in this comparison: sex and food of parent providing defecation.

The percent of living males was calculated every 2-3 days. After 26 days, all flies in all treatments were deceased. Flies coming from bleached eggs and defecation from a male on standard food had the lowest survival curve. The highest survival curves included NW, FRB, and FCW (Figure 2). There were significant differences between MRB vs FRB, FRB vs NB, and NW vs NB (Table 3). There were more significant differences, but because they contained more than one variable, a clear comparison cannot be made.

Discussion:

The results show that the source of the gut microbiota of *Drosophila* may play a role in fitness. Longevity was measured because a study showed that lifespan is reduced under axenic conditions, which indicates that fitness is reduced without microbiota (Brummel, 2004). Another study showed that the removal of gut bacteria represses oogenesis and fecundity, therefore, fecundity should also be a key indicator of fitness (Elgart, 2015). Using fecundity data, there was a significant difference between FRB vs NB and FRB vs MCB (Table 2). A clear comparison cannot be drawn between FRB and MCB because there are two variables. However, NB and FRB can be more easily compared. This difference illustrates that the flies hatched with defecation from a standard female laid significantly more eggs than flies hatched with no defecation at all. Both of these sets of eggs were rinsed with bleach, so that should not have an effect. It is possible that the eggs hatched, then the larvae ate the defecation, obtaining gut microbiota. This seems to be beneficial, because the eggs without defecation laid fewer eggs. Additionally, the FRB flies lived longer than the NB flies (Table 3). Both measures show similar results, so there is most likely a benefit to eggs being hatched with defecation from adult flies.

Another significant difference was seen between the longevity of MRB and FRB. The FRB lived significantly longer than MRB. This indicates that it may be more beneficial to obtain microbiota from defecation of a female rather than a male. There were also differences between the eggs hatched without defecation, but some rinsed with water vs others in bleach. Eggs rinsed with water lived significantly longer that those rinsed with bleach. This indicates that flies may obtain microbiota from the surface of their own eggs, which is consistent with previous studies (Bakula, 1969). Therefore, flies may ingest microbiota from various sources, including

defecation from adult flies, and the surface of their eggs. The source of the microbiota appears to have an effect on the fitness of the larvae that consumes it, supporting the original hypothesis.

In order to possibly obtain more statistically significant data, more eggs should be used in each treatment. Even though 50 eggs were placed on each treatment plate, not all 50 hatched. Only about 20-30 of the larvae could be placed onto food. After that, still not all larvae successfully developed into adults, leaving only about 10-15 adults per treatment within some trials. Because there are so many steps in which individuals are lost, a larger sample size in the beginning would be beneficial. Additionally, fecundity may not be the best measure of fitness compared to longevity. In Elgart's study, they found that the main impact on oogenesis was related to the lack of *Acetobacter* species specifically. Because this study was not completely free of microbes, this species may have been present still. The gut microbiota in general has a larger impact on longevity than fecundity. In future studies, it could be beneficial to look at the offspring of the flies studied in this experiment. There may also be variations within the fitness of the next generation. One treatment that was not run was standard female defecation, then eggs rinsed with water. Standard females with bleached eggs had the highest fecundity, followed by no defecation with eggs rinsed in water. Both of these treatments showed increased longevity relative to the other treatments as well. Eggs that can obtain microbiota from both the surface of their egg, as well as defecation from females, may show an even greater increase in fitness. If this experiment is repeated, that treatment should be included, as well as beginning with more eggs per treatment. Additionally, there should be an equal number of flies per vial. There may have been differences in longevity due to crowding or resource availability.

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This experiment shows that not only is gut microbiota important for the fitness of *Drosophila*, but the source of the microbiota may also play a role. More experiments must be conducted in order to gain more evidence for this hypothesis. For example, a more sterile experiment could be performed in which the eggs and egg-laying surfaces are completely axenic. After ensuring that everything is completely free of microbes, defecation could be placed around the eggs. The defecation could come from males and females raised on different media, similar to the previous experiment. This would allow for more control of the source of the microbes. In the previous experiment, the eggs rinsed with water may still have microbes from their biological parents, in addition to the defecation from other flies. A future experiment could include defecation from biological vs non-biological parents to show if paternity plays a role. It is possible that obtaining microbiota from a biological parent may be more beneficial than a non-parent. Therefore, the proposed experiment would expand on the idea that the source of gut microbiota affects fitness and determine if paternity has an effect. If this is true for *Drosophila* and supported by future experiments, it is possible that the source of microbiota in the gut of many organisms can affect fitness.

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Tables and Figures:

Table 1: Treatment Hatch Plates: The following adult flies were placed onto each hatch plate to defecate, then were removed. 50 eggs per treatment were treated with bleach or water, then added to the plates.							
1 (MRB) Bleach	2 (FRB) Bleach	3 (MCB) Bleach	4 (FCB) Bleach	5 (MCW) Water	6 (FCW) Water	7 (NW) Water	8 (NB) Bleach
Males	Virgin females	Males carbeni	Virgin females carbeni	Males carbeni	Virgin females carbeni	None	None

Table 2: Fecundity t-test. The average number of eggs laid per female was calculated for each treatment. A two-tailed t-test was run in Excel to determine p-value for significance. Values in green indicate significance.

t-test 2,1	MRB	FRB	MCB	FCB	MCW	FCW	NW	NB
MRB		0.402291717	0.837827893	0.895319526	0.837827893	0.895319526	0.814479374	0.373562058
FRB			0.027687705	0.389742847	0.348870253	0.325659782	0.919257341	0.037190813
MCB				0.575712074	0.66823496	0.980703704	0.460804145	0.657482211
FCB					0.781597414	0.64445822	0.623742482	0.690742621
MCW						0.772336753	0.660127768	0.752647126
FCW							0.136826968	0.913040434
NW								0.584644131
NB								

Table 3: Post- hoc analysis of longevity. SPSS was used to compare each treatment in terms of longevity. Cells in green indicate significance.

Multiple Comparisons

Dependent Variable: time
LSD

(I) intervention		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-5.02273*	2.32641	.033	-9.6225	-.4230
	3	-4.60294	2.41592	.059	-9.3797	.1738
	4	-3.67308	2.27819	.109	-8.1775	.8313
	5	-2.83333	2.57195	.273	-7.9185	2.2519
	6	-4.95000*	2.35723	.038	-9.6107	-.2893
	7	-5.20238*	2.34114	.028	-9.8312	-.5735
	8	-1.34524	2.34114	.566	-5.9741	3.2836
2	1	5.02273*	2.32641	.033	.4230	9.6225
	3	.41979	1.81961	.818	-3.1779	4.0175
	4	1.34965	1.63232	.410	-1.8777	4.5770
	5	2.18939	2.02218	.281	-1.8088	6.1876
	6	.07273	1.74093	.967	-3.3694	3.5149
	7	-.17965	1.71908	.917	-3.5786	3.2193
	8	3.67749*	1.71908	.034	.2786	7.0764
3	1	4.60294	2.41592	.059	-.1738	9.3797
	2	-.41979	1.81961	.818	-4.0175	3.1779
	4	.92986	1.75754	.598	-2.5451	4.4048
	5	1.76961	2.12455	.406	-2.4310	5.9702
	6	-.34706	1.85885	.852	-4.0223	3.3282
	7	-.59944	1.83840	.745	-4.2343	3.0354
	8	3.25770	1.83840	.079	-.3771	6.8925
4	1	3.67308	2.27819	.109	-.8313	8.1775
	2	-1.34965	1.63232	.410	-4.5770	1.8777
	3	-.92986	1.75754	.598	-4.4048	2.5451
	5	.83974	1.96651	.670	-3.0484	4.7279
	6	-1.27692	1.67595	.447	-4.5906	2.0367
	7	-1.52930	1.65324	.357	-4.7980	1.7394
	8	2.32784	1.65324	.161	-.9409	5.5966
5	1	2.83333	2.57195	.273	-2.2519	7.9185
	2	-2.18939	2.02218	.281	-6.1876	1.8088
	3	-1.76961	2.12455	.406	-5.9702	2.4310
	4	-.83974	1.96651	.670	-4.7279	3.0484
	6	-2.11667	2.05756	.305	-6.1848	1.9515

Legend

1 MRB
2 FRB
3 MCB
4 FCB
5 MCW
6 FCW
7 NW
8 NB

	7	-2.36905	2.03910	.247	-6.4007	1.6626
	8	1.48810	2.03910	.467	-2.5436	5.5198
6	1	4.95000*	2.35723	.038	.2893	9.6107
	2	-.07273	1.74093	.967	-3.5149	3.3694
	3	.34706	1.85885	.852	-3.3282	4.0223
	4	1.27692	1.67595	.447	-2.0367	4.5906
	5	2.11667	2.05756	.305	-1.9515	6.1848
	7	-.25238	1.76056	.886	-3.7333	3.2286
	8	3.60476*	1.76056	.042	.1238	7.0857
7	1	5.20238*	2.34114	.028	.5735	9.8312
	2	.17965	1.71908	.917	-3.2193	3.5786
	3	.59944	1.83840	.745	-3.0354	4.2343
	4	1.52930	1.65324	.357	-1.7394	4.7980
	5	2.36905	2.03910	.247	-1.6626	6.4007
	6	.25238	1.76056	.886	-3.2286	3.7333
	8	3.85714*	1.73895	.028	.4189	7.2954
8	1	1.34524	2.34114	.566	-3.2836	5.9741
	2	-3.67749*	1.71908	.034	-7.0764	-.2786
	3	-3.25770	1.83840	.079	-6.8925	.3771
	4	-2.32784	1.65324	.161	-5.5966	.9409
	5	-1.48810	2.03910	.467	-5.5198	2.5436
	6	-3.60476*	1.76056	.042	-7.0857	-.1238
	7	-3.85714*	1.73895	.028	-7.2954	-.4189

*. The mean difference is significant at the 0.05 level.

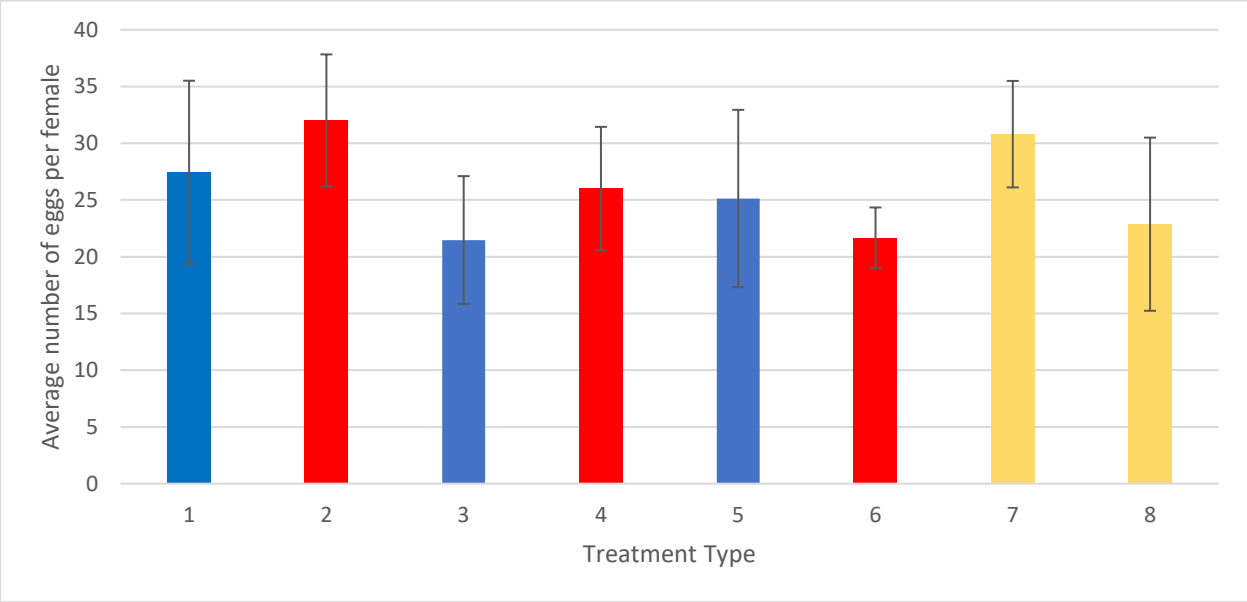

Figure 1: Average fecundity per female. Blue=male. Red=female. Yellow=none. Treatments follow Table 1. Standard error bars are shown.

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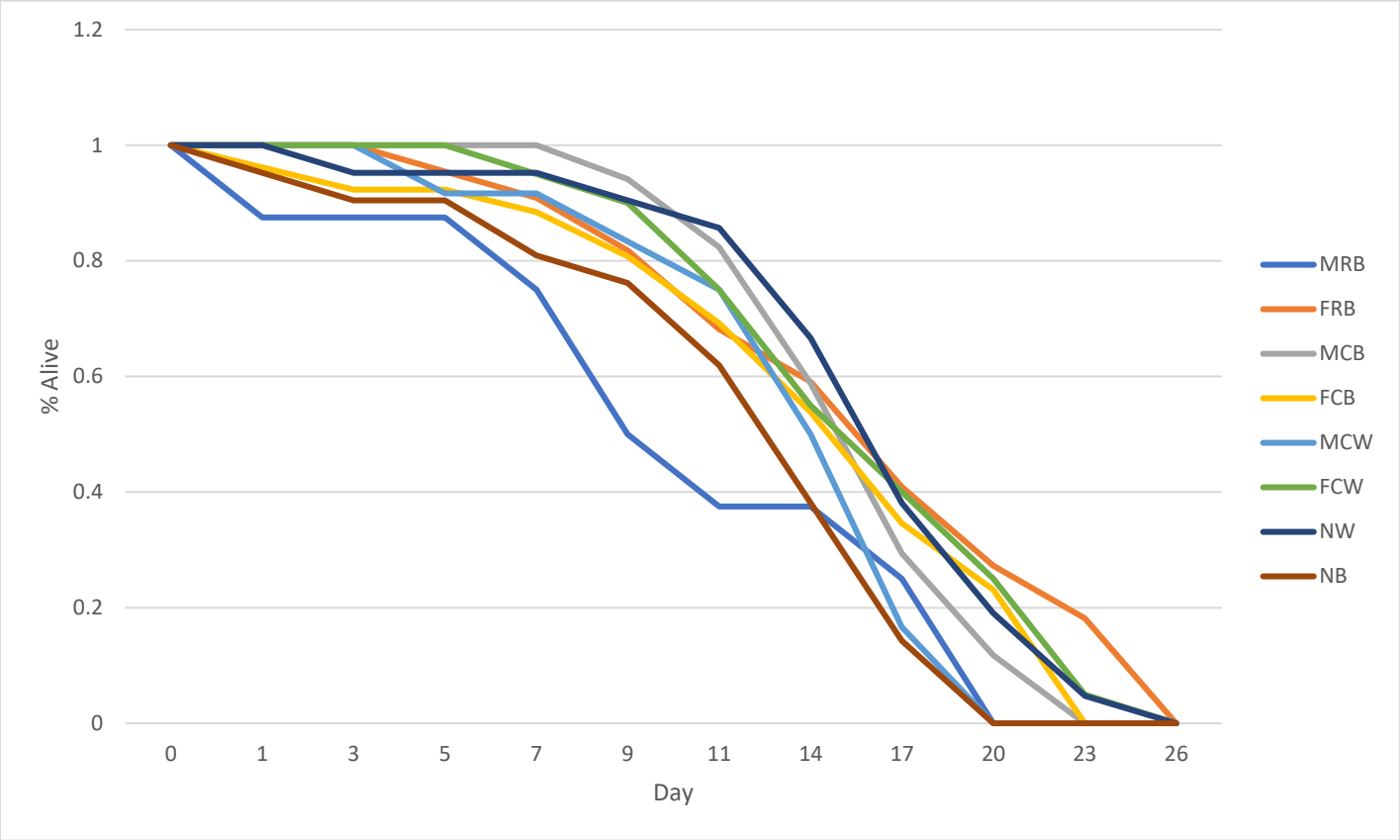

Figure 2: Longevity of males. Males and females were kept together at 25°C, but only males were counted for longevity. All flies were deceased within 26 days.