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The effect of honey supplementation on drosophila melanogaster hemocyte count

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Abstract
Immune diseases, particularly the COVID-19 pandemic, are a growing problem; according to the CDC, an estimated 3,049 people died daily from 2020 to 2021 of COVID-19 immune complications, surpassing death rates of even heart disease and cancer. As a result, many people have turned to honey to help boost immunity. Natural honey has remained a popular treatment for infected wounds and ingredient in numerous naturally-made immune medications, despite there being no justified studies on honey's immune health benefits. Thus, there arises a need to confirm whether honey is safe to boost immunity. The effects of honey on Drosophila melanogaster's immune system were tested. Hemocyte count was a proxy measurement for immune health; hemocytes are cells that destroy pathogens and trigger a fast-acting immune response. Using a needle to puncture the larvae, the hemocytes for each larva were drained into wells and evaluated by a cell counter. 80 trials were conducted for each of the two groups, and the 2061 hemocytes per larva for the non-honey-fed larvae was statistically significantly larger than the 1319 hemocytes per larva for the honey-fed larvae. Despite the long-held belief that honey is beneficial for the immune system, the research conducted supports the contrary.

Keywords: immunity, honey, drosophila, hemocytes, larvae

Introduction
The number of people contracting COVID-19 has risen substantially since the discovery of severe acute respiratory syndrome coronavirus 2 in 2019. The virus peaked between 2020 and 2021, where around 3,049 people died daily of the virus, surpassing death rates of even heart disease and cancer that year (CDC, 2021). COVID-19 is an example of an immune disease, or a virus that affects the immune system significantly in its virulence. They are particularly tricky to treat, namely because of the close ties the immune system has to overall body health. This attack specificity in pathogens narrows treatment options significantly and, depending on the type of pathogen involved, can even spell grave consequences for those who do not receive another method of treatment quickly enough. Although scientists are locked in a progressive "arms race" against these diseases, with each racing to find ways to respectively attack and withstand each other, there may be a source of new potency in natural treatments. One particular naturally-occurring substance, although it is a common household item added to sweet beverages and baked with bread, may hold further knowledge that could present a new way of looking at the new threats lying in immune diseases: honey.

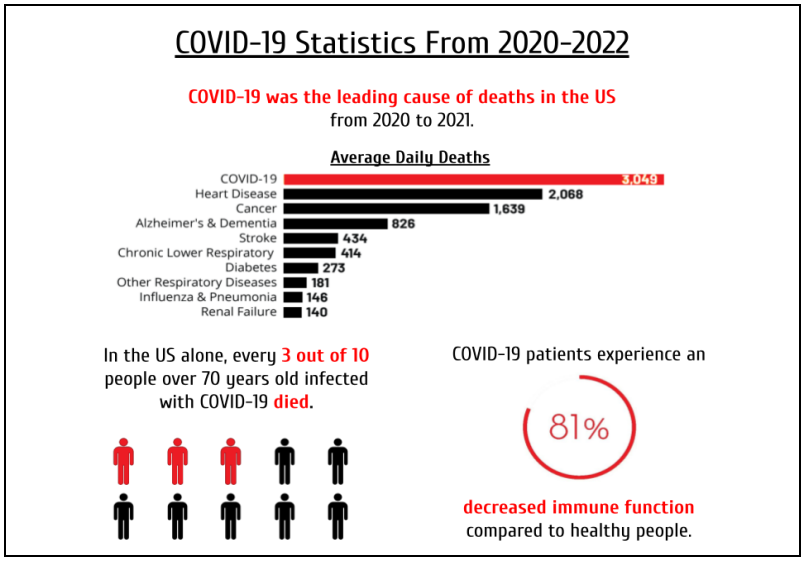

Fig 1: Statistics of COVID-19 between 2020 and 2021. Adapted from (CDC, 2021).

Honey

Most strikingly, honey has notable anti-inflammatory abilities, meaning it can reduce inflammation, swelling, and infection progression around wounds. Natural honey consists of 75% monosaccharide sugars, 10–15% disaccharide sugars, and a mixture of enzymes, minerals, vitamins, amino acids, flavonoids, and phenolic compounds (Masad, 2021) [4]. Monosaccharides are simple sugars like glucose, a naturally made plant sugar produced from photosynthesis; research using glucose to test immune health of flies has been previously explored as well (Ponton, 2019) [7]. Flavonoids are compounds that have been shown to reduce inflammation at wound sites and protect cells from oxidative damage. Oxidative damage is a phenomenon that occurs when pathogenic infections stimulate overproduction of reactive oxygen species in a cell, resulting in overoxidation of substances like protein, lipids, and even DNA in the cell. This upsets the balance of intracellular vital redox reactions, resulting in the shutdown of several cellular processes, degradation of nutrients, and sometimes even the death of the whole cell. Phenolic compounds, apart from being antioxidants (substances that can bond with reactive oxygen species in order to prevent overoxidation and thus oxidative damage), help signal defense response mechanisms in the fly, including parts of the immune response.

Several more studies have shown that honey has antimicrobial and anticancer effects. (Samarghandian, 2017) [7]. Investigated honey's anticancer properties and found that honey interferes with multiple cell-signaling pathways that induce apoptotic, antimutagenic, and antiproliferative properties in nearby cells. Apoptotic properties are properties that promote or accelerate apoptosis, which is a form of cell death. Consequently, honey has been indicated to prevent cell proliferation, cause apoptosis, modify the cell cycle, and cause mitochondrial membrane depolarization in several types of cancer cells in the kidneys, liver, prostate, bladder, lungs, mouth, and bones, among other areas in the human body. Mitochondrial membrane depolarization is a process built into many cells that decreases the difference between the electric potential of the exterior and interior of a cell, causing the stagnation of oxygen production and sending the cell into apoptosis. Honey also displays stunning antimicrobial activity through its acidic pH level, low protein content, high hydrogen peroxide content, and low redox potential; all of these qualities create an environment in which microbes like bacteria cannot grow (Samarghandian, 2017) [7]. Redox potential is the tendency of electrons to be gained or lost to an electrode in a solution; since honey has a low redox potential, it can act as a stabilizer of overflowing reactive oxygen species in the event of a pathogenic infection.

With all of this research, it is no surprise that honey is regularly used in natural medications that prevent itching or swelling, as well as a natural way of disinfecting wounds. It is already quite popular as a disinfectant for most scrapes, bruises, and cuts for humans. In fact, honey has been shown to weaken over 60 species of bacteria, including pathogens that can cause potent immune reactions in humans: Bacillus anthracis, Corynebacterium diptheriae, Haemophilus influenzae, Klebsiella pneumoniae, Listeria monocytogenes, Mycobacterium tuberculosis, Salmonella diarrhoea, Salmonella typhi, Shigella dysentery, Staphylococcus aureus, Streptococcus faecalis, Strep. mutans, Strep. Pneumoniae, Strep. pyogenes and Vibrio cholerae, all of which can cause anthrax, diphtheria, influenza, pneumonia, listeriosis, tuberculosis, diarrhea, typhoid fever, dysentery, strep throat, the common cold, and cholera among other diseases. Honey is also commonly used to treat abrasions, incisions, and lacerations of the skin lest they become infected and/or inflamed (Najafi, 2018). Honey has been discussed to have two general properties of antimicrobial activity: it dehydrates bacteria by removing moisture from the environment and its low pH deters growth of bacteria. Thus, honey is a sought-after all-natural immune treatment for its role in curing and or inhibiting a number of bacterial diseases, as well as its role in reducing inflammation and rate of infection in open cuts or bruises.

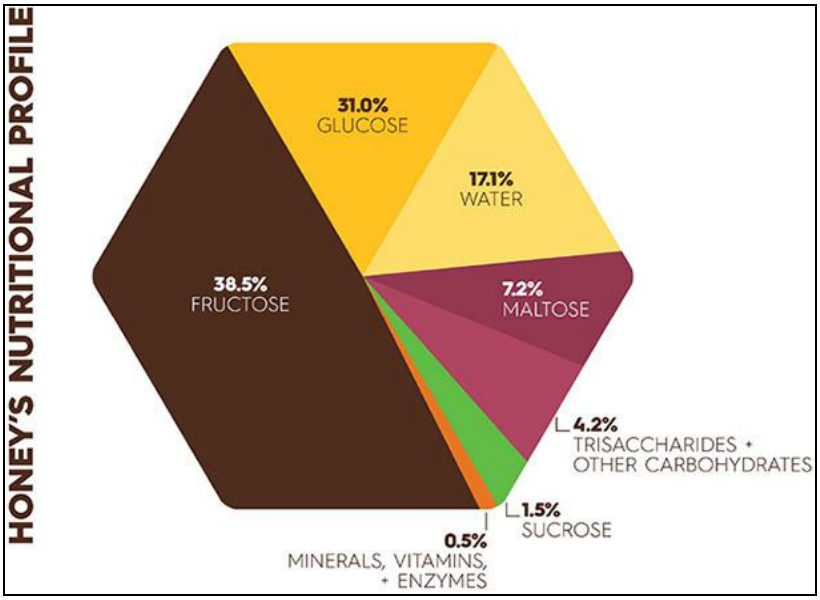

Fig 2: Molecular content of honey. Adapted from (Masad, 2021) [4].

Hemocytes

Drosophila was used as a model in this experiment due to its innate immunity being similar to that of humans'. Drosophila's connection between metabolism and immune health is mainly connected by hemocytes. Hemocytes are circulatory cells found in invertebrates like Drosophila that supply sufficient iron to Drosophila's other body cells. Hemocytes are directly related to the immune system, as there are types of hemocytes that act like macrophages found in human bodies; they mark pathogens with antibodies and obliterate them. Lamellocytes, a particular type of hemocyte, is produced in situations where homeostasis is upset. When mutated lamellocytes that were unable to function were studied as part of the immune response, the fly with the mutated cells had less resistance to being infected by bacteria and fungi, particularly by S. typhimurium and L. monocytogenes. Coupled with similar responses from dysfunctional Toll and Imd pathways, it is clear that the systemic pathway response from the fat body synergizes with the hemocyte-driven immune response. The Toll and IMD pathways are important signal systems that are able to find and mark pathogens in Drosophila, as well as alert other immune system cells of foreign substances and/or microorganisms. Additionally, general depletion of hemocytes in Drosophila reduces the likelihood of the fly surviving future infections. This heavily supports the relationship between hemocytes and the immune system; not only are hemocytes able to trigger a response of their own, but the response also actually works together with the systemic fat body response in order to maximize the immune response for defending against pathogens. Compromising the hemocyte response has a similar effect when you compromise the Toll/Imd pathways, showing that both play a major role in the response. The hemocyte response can actually be compared to the quick antibody response in humans, followed by the Toll/Imd pathways, which can be compared to the slow but powerful macrophage response (Banerjee, 2019) [2]. In fact, certain cells like plasmatocytes, crystal cells, and lamellocytes find close human equivalents in the form of macrophages, platelets, and giant cells.

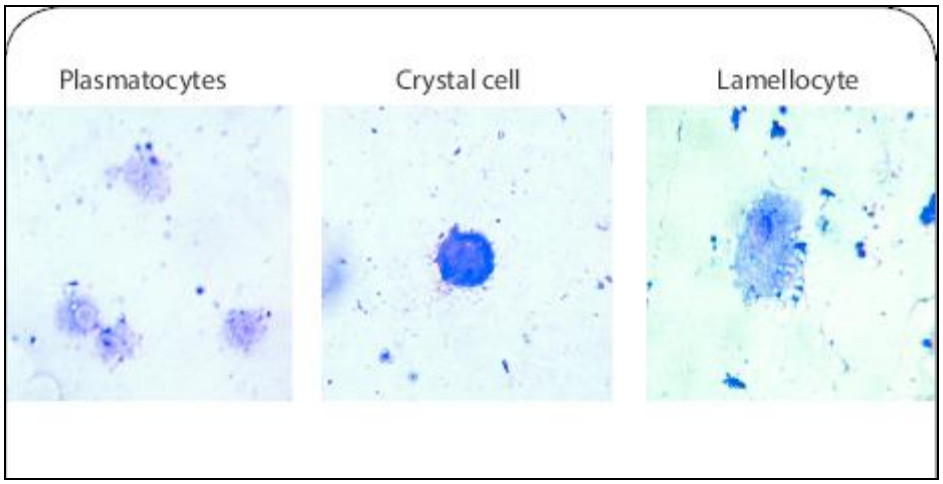

Fig 3: Types of hemocytes stained and viewed under a microscope. Adapted from (Petraki, 2016) [5].

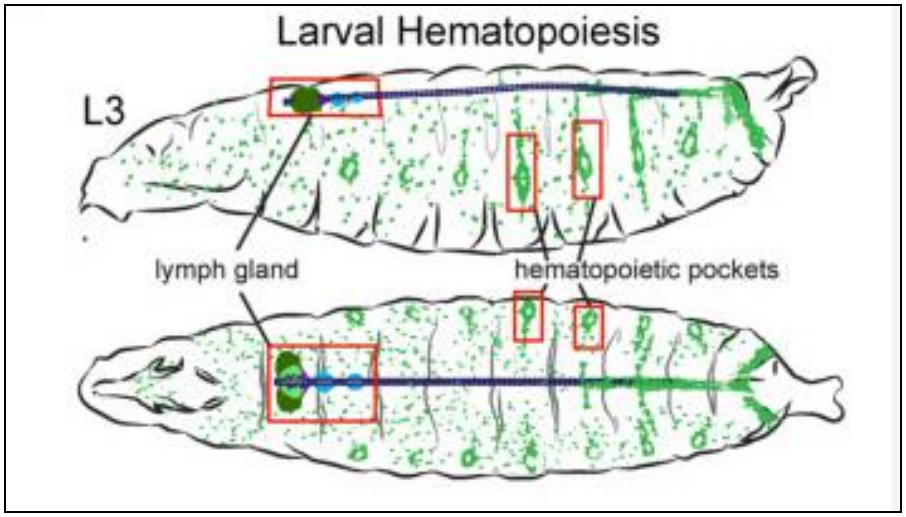

Fig 4: Image of stained hemocytes in a 2nd instar Drosophila larva. Adapted from (Petraki, 2016) [5].

Literature Review

The immune response in Drosophila melanogaster has been extensively studied in recent years. Present research on the relationship between the immune system and metabolism in Drosophila is voluminous and full of general consensus referring to several dietal relationships. (Unckless, 2016) [8] States that the researchers fed two

different diets to two groups of Drosophila different diets, one with high glucose and the other with low glucose; both groups were infected with a bacterium and their survival rate was monitored over a few weeks. The flies that were fed higher glucose diets had lower resistance to the bacterial infection and often ended up dying faster than the flies on the lower glucose diet. The lower resistance resulting from the glucose diets was a very surprising discovery to the researchers, who had hypothesized contrary results. From this study, the researchers found that not only is there is a relationship between metabolism and immune health, but this regulation is mainly controlled by genetic variation and gene expression; the experiment tested five protein-carbohydrate diets (PCs) to determine this, and of the five, the fourth high-glucose PC diet explained 11% of the total gene variance, and as was with the other studies, free glucose levels were positively correlated with severity of infection across genotypes when tested with the high glucose diet.

(Ponton, 2019) [6] Details a positive relationship between protein-carbohydrate diets and Drosophila's immune health; this study hypothesized that a low protein, high carbohydrate diet fed to Drosophila will stimulate the immune system the best. The results supported the hypothesis but contradicted the results of the previous study; there was a significant negative nonlinear relationship between the percentage of dietary protein and the level of expression for six out of nine genes coding for antimicrobial peptides (compounds consisting of amino acids that aid in innate immunity in multicellular organisms), meaning that the peptides are consistently and closely related to the P: C ratio. These results suggest that a carbohydrate-biased diet can maintain a higher constitutive expression of antimicrobial peptide genes that might allow flies to better fight infections and injuries. The paper also supported the conclusion that high glucose diets will increase vulnerability to infection. When fly larvae are infected, their metabolism increases and less glucose is added to the proteins, instead being used to fight off the infection. Therefore, the flies would prefer a diet with a higher portion of carbohydrates instead of proteins. While this study supports the conclusion that sugars and carbohydrates do in fact benefit the immune system, it should be noted that all the P:C ratios tested in this experiment were always greater than 1, meaning there was always more protein than carbohydrates in the diet. This essentially blindsides the study to diets that are not so high in protein, since they never tested P: C ratios that had a higher percentage of carbohydrates.

Materials and methods

Hemocyte Bleed/Scrape Assay (Petraki, 2016)

One glass slide was prepared with a 10 mm square-shaped Pap-pen well and kept in a moist environment to prevent the well from drying out. Using a small stick, the larvae was picked out of the dish and placed on a slide on top of a cold block. Larvae can only be kept for a limited time in water or on a cold block, so the specimens were used within 45 min to avoid death of the larvae.

One larva was put in the first Pap-pen well and two sterile needles were used to make an incision at the posterior and anterior ends of the larva. The larva was held with forceps to make incisions more accurately, and the incisions were made on the front side of the larva. The larva was allowed to bleed for a few seconds on the slide, and it was made sure that the larva was not left on the slide for each step.

The lymph gland was then identified in the larva with the use of a small flashlight to see the gland fluoresce through the larval body wall. A needle was used to pin down the larva close to the lymph gland, and another was used to make a clean cut through the lymph gland. A controlled cut was made so that not too much lymph was released at once.

After waiting for a few seconds for the larva to bleed again, the larva was transferred out of the well with a small stick, and the well was incubated on the slide for further imaging using the Countess.

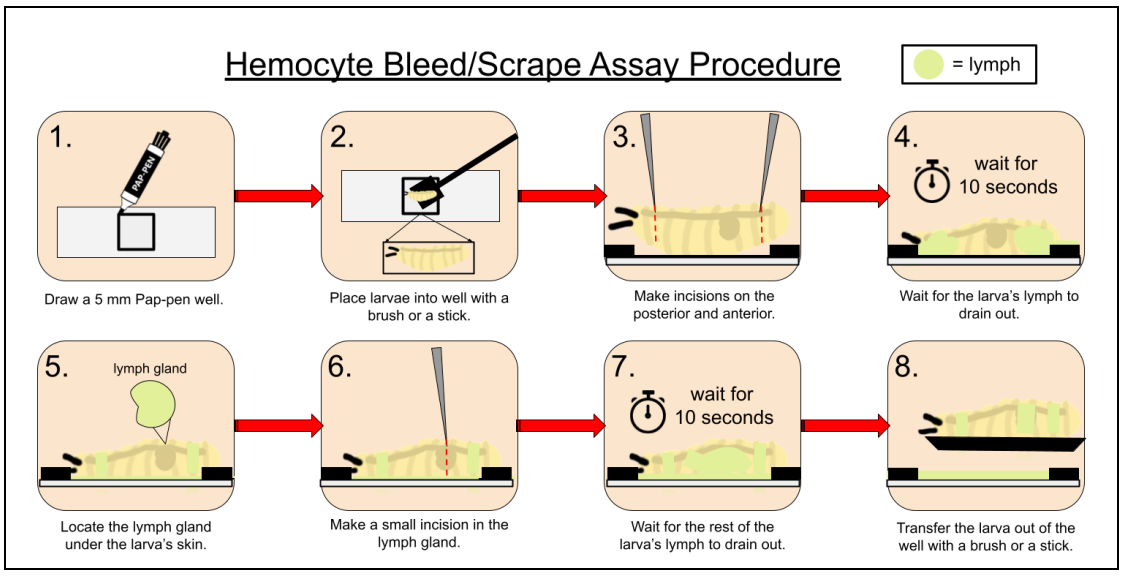

Fig 5: Simplified representation of the Hemocyte Bleed/Scrape Assay. Adapted from (Petraki, 2016).

Countess Quantification Procedure

The hemocytes were put inside a chamber slide and then into the Countess apparatus. The larva's collected hemocytes were then transferred into a cuvette, which was swirled gently using small circular movements for 30 seconds. The chamber slide was positioned carefully, and the hemocytes in the cuvette were pipetted into the half-moon shaped chamber opening labeled A on the side of the chamber slide. The slide was then inserted into the Countess apparatus, with the hemocytes on top of the slide and the A chamber facing the port on the apparatus, and the slide was pushed into the port until there was a soft click.

The Countess was powered on, the image of the hemocytes was shown on the Countess's screen, and the zoom button and the large black knob (focus knob) were turned counterclockwise and clockwise to focus the image of the hemocytes in chamber A. A location was pressed on the grid below the "Count Cells" button in order to navigate around the chamber and ensure that all of the hemocytes were properly visible. Live hemocytes have bright centers and dark edges, while dead hemocytes will have a uniform dark blue color; dead hemocytes were not counted in the final total for each larva. The smaller black knob on the side of the apparatus was turned clockwise to lock the focus for easier viewing of all the hemocytes.

When it was clear that the hemocytes are visible, the "Count Cells" button above the grid was pressed in order to count all of the hemocytes in Chamber A. Some hemocytes were not included in the counted cells, so the "More Data" button was pressed to check the cell gating to 0-20 micrometers and the quantification procedure was repeated to ensure that all of the hemocytes were counted. If all the hemocytes were still not included, then the upper micrometer limit would need to be incrementally increased until all of the hemocytes are counted.

The final hemocyte count in Chamber A was recorded. If both chambers needed to be filled with one larva's hemocytes due to the hemocytes not fitting in only one, the total hemocytes in Chambers A and B were added together when recording the data. If needed, the quantification steps were repeated for Chamber B by removing the slide, turning it around, and reinserting the slide, Chamber B first, into the apparatus; the counts for both chambers were added together to get the final hemocyte count for that larva.

Results and Discussion

Tables and Graphs

Table 1: Average hemocyte count for both groups.

	No-Honey Larvae	Honey Supplemented Larvae
Avg Number of Hemocytes Per Larva	2060.7375	1318.825

Table 2: Significant values for two-sample t-test.

	N	Mean	Std. Dev	SE Mean
No-Honey Larvae	80	2060.7375	580.4	64.891
Honey-Supplemented Larvae	80	1318.825	304.8	38.098

Observed Difference: 741.913
Standard Deviation of Difference: 75.2482
Unequal Variances
DF: 127
95% Confidence Interval: [593.0119, 890.8141]
Test Statistic: t = 9.8595
No Honey Larvae ≠ Honey-Supplemented Larvae: P-Value < 0.00001

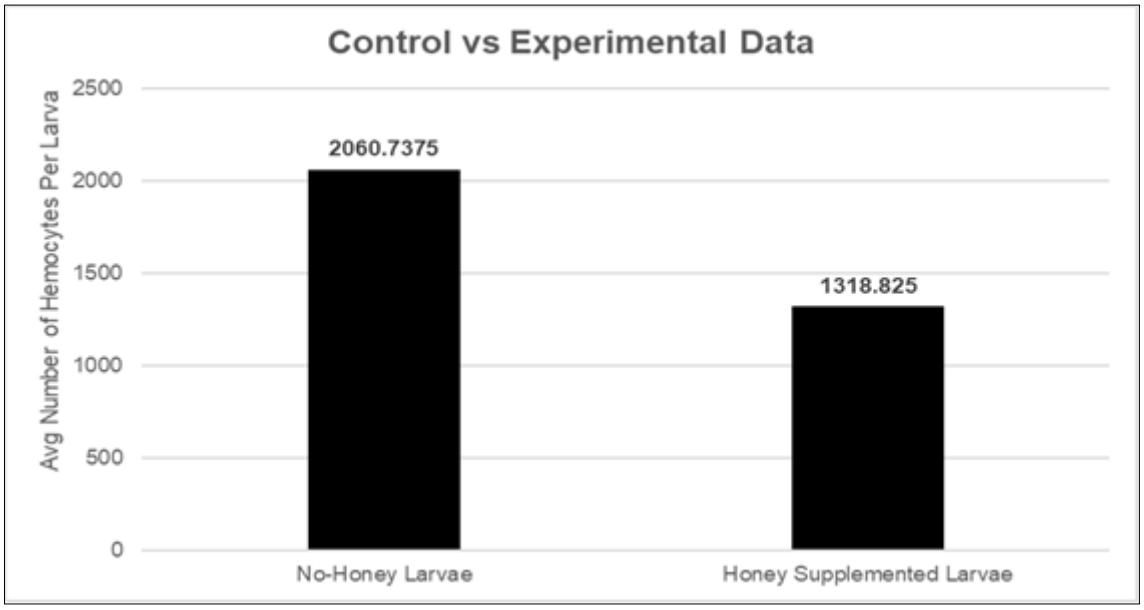

Fig 6: Graphical comparison of average hemocyte counts for both groups.

Group Results and Comparison

The results gathered from the procedure are shown above in Table 1. The no-honey larvae had an average of 2060.7375 hemocytes per larva tested. This is actually around the average amount of hemocytes for normal 2nd instar larvae as (Petraki, 2016) [5] states. The honey supplemented larvae had an average of 1318.825 hemocytes per larva tested, which is a full 741.913 hemocytes lower on average, according to Table 2. As seen in Figure 6, by pure observation, the average hemocytes for the honey supplemented larvae appear significantly lower in amount than the average hemocytes of the no-honey larvae; the statistical analysis, shown below, does not dispute either.

Statistical Analysis

Table 2 details the statistics of the no-honey and honey supplemented fly groups and the results of the t-test to measure the difference between the means of the two groups. The null hypothesis was that there was no difference between the two groups, and the alternate hypothesis was that there was a statistically significant difference between the two groups. There were each 80 trials and therefore 80 values (N = 80) in each of the two different sets. The analysis software Fathom 2 was used to run the t-test. Unequal variances were assumed, and the standard deviation of the difference between the two groups came out to be 75.2482. The degrees of freedom for the t-test were set to 127, and the t-test yielded a 95% confidence interval between 593.0119 and 890.8141. The t-test statistic was 9.8595, which was far outside the t-test boundaries of the 95% confidence interval. The p-value, indeed, was found to be less than 0.00001, which asserts there was a landslide in support that there was a statistically significant difference between the two groups. Since the honey supplemented flies have a lower average hemocyte count than the non-honey flies, the relationship can instead be described as the supplementation of honey has a statistically significant negative relationship with hemocyte count.

Conclusions

Resolution

The study successfully carried out its short-term purpose to investigate the effect of honey on the immune system. The Hemocyte/Bleed Scrape Assay was integral in assuring that each larva had a representative hemocyte count that could affect the average. The Countess was also crucial in cutting down time by counting the cells per well at swift speeds, so the samples did not lose much integrity, if any. All in all, the two methods offered a feasible way to measure the dependent variable of hemocyte count in a fast and reliable way by incorporating technology into the parts of the experiment that require time to perform. It also provides a definite count of hemocytes per well, which is a certainty very much needed in studies centered around immune health. A currently unsolved problem with current Drosophila immune health research is that age-related problems in the flies tested prevent researchers from making concrete conclusions, especially in longer-term studies. This phenomenon can invalidate a large portion of the data that immune health researchers collect in their experiments with flies; as of today, the only way to minimize this data inaccuracy is to minimize procedure time and to avoid experimenting with the same flies, if possible. This study not only tests each of the flies for an extremely short amount of time, but also avoids using the same flies over and over again per the methods. Thus, this study is able to minimize procedure time immensely and avoids experimenting with the same flies, effectively nullifying the age problem that plagues so many other projects. The results of the study support the assertion that honey has a significant negative impact on Drosophila's immune system, proxied by hemocyte count.

Future Work

Dosage of Honey: Although this development may be expected to yield an increasingly larger hemocyte count the lower the dosage of honey in the diet (based on this study), this may be an entirely different case. As is with a common pattern of ingestion of substances, most substances vital to the human body are dangerous if they are consumed in extremely large or extremely miniscule amounts. This variation in the dosage of honey in diets could provide a sense of what amounts of honey are appropriate to be ingested in order for the immune system to continue to perform at peak function.

Rate of Infection: A factor that should be considered as another measure of effectivity could be a study of the rate of infection as it propagates through the fly's body; it is important to not only know the status of the immune response (as this study explores) but also that of the pathogen. Perhaps honey may degrade the immune response, but its anti-microbial effects, specifically on bacteria, could warrant a different use for honey. A slower rate of infection corresponds with but is not always caused by increased immune system effectiveness; therefore it will be crucial to the outcome of this study to also explore how the spread of disease-causing pathogens is affected within the body.

Viral Infections: It is a well-known issue in the field of biomedicine that viral infections cannot be treated with the same means as bacterial infections; indeed, antibiotics and other bacteria-specific treatments often contribute zilch or even hinder the immune system's counteraction of viral infections. While honey may stunt the immune system's ability to produce a coherent immune response for infection in the form of decreasing amounts of hemocytes, it is still worth looking at the effects of honey on viral infections in order to establish a difference between the effectiveness of honey being used to treat bacterial and viral infections, or if there is a difference at all.

Ages of Larvae: Progressively aging cells in both flies and humans lose copious amounts of innate immunity and core function over time according to (Agrawal, 2019) through a process known as senescence. A comparison of honey's effects on older flies would be extremely helpful in determining how honey affects the aging immune system, and whether its negative effects are exacerbated as the larvae get older or younger.

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