EFFECTS OF HIGH URACIL DIET-INDUCED GUT DYSBIOSIS ON COURTSHIP BEHAVIOR IN *DROSOPHILA*

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Abstract.–Past research has established that *Drosophila* gut inflammation via the dual oxidase (DUOX) regulatory pathway is induced by exposure to bacterial-derived uracil, which can be secreted by allochthonous and autochthonous bacteria in the midgut. However, the effects of the inflammatory response and resulting gut dysbiosis on the courtship behavior of the *Drosophila* host have yet to be determined. This work studies the changes in *Drosophila* courtship behavior resulting from diet-based exposure to uracil, a ligand for DUOX-dependent reactive oxygen species (ROS) generation in the epithelia of the midgut. The effects of a high-uracil diet on courtship behavior were determined by comparing courtship index (CI) values of flies treated with 20 nM of uracil for 16-hr (short-term) or 10-d (long-term) exposure to age-matched control flies. Although short-term uracil treatment caused no change in courtship behavior, long-term uracil treatment resulted in a significant decrease in CI. This result suggests that the underlying association for the behavioral change may be influenced by gut inflammation initiated by long-term exposure to uracil. By measuring the effects of an innate immune response such as exposure to a ligand for DUOX-dependent ROS generation on the courtship index of *Drosophila,* the current understanding of the relationship between gut inflammation and behavioral changes in animals can be expanded. Given that prior research has also established that the DUOX-dependent response can be found in animals ranging from *Drosophila* to humans, the behavioral changes observed from induction of the pathway have the potential to expand this connection in the future.

Keywords: inflammation, gut microbiota, courtship

In recent years, the role of microbiology in the field of neuroscience has rapidly expanded as research has focused on the connection between gut microbiota and the brain in animals. More specifically, the impact of gut microbiota on the behavior of organisms has become a topic of interest. An accumulation of past

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experimental data has established a consensus that changes in the composition of gut microbiota influence brain function and behavior via communication with the central nervous system through neural, endocrine, and immune pathways (Cryan & Dinan 2012). Additionally, behavior and mood disorders such as clinical depression have been proven to stem from an inflammatory response to gut dysbiosis. For example, it has been demonstrated that endotoxin infusions to healthy subjects with no history of depressive disorders results in the emergence of classical depressive symptoms, which are attributed to an increase in production of pro-inflammatory cytokines (Clapp et al. 2017).

While the link between gut dysbiosis, inflammation, and the resulting effects on behavior have been observed in mammalian organisms, research exploring whether this connection exists in the classic experimental model fruit fly, *Drosophila melanogaster*, is lacking. With a median lifespan of 35-45 days, the utilization of the fruit fly as a model for gut dysbiosis provides advantages for experimentation (Broughton et al. 2005). First, the short maturation time of the organism allows for new generations to be produced rapidly. This advantage results in a large number of subjects being available for experimental trials in a short time frame. Secondly, the microbial community structure found in the *Drosophila* gut contains only 1-30 taxa (Broderick & Lemaitre 2012). This simple microbiome is a stark contrast to that of vertebrates, whose gut communities contain more than 500 taxa. As a result, the relative simplicity and low variation in *Drosophila* gut microbiota make the organism an advantageous subject for research in dysbiosis (Lee & Lee 2014). In addition to gut microbiota research, *Drosophila* are also useful for observing behavior. The courtship index (CI) is a quantitative measurement of the courtship behavior exhibited by *Drosophila* (Siegel & Hall 1979; Sokolowski 2001). Because there is a stereotypical pattern of courtship behavior in *Drosophila* with defined indexes that have been observed by prior research (Villella et al.

1997), any changes in such behavior due to experimental factors are easily discerned.

Like most animals, *Drosophila* maintain a microbial community in their gut with a majority of the community consisting of non-pathogenic bacteria. However, unlike mammalian organisms, they do not have an adaptive immune system and operate solely on innate immunity to prevent colonization by opportunistic pathogens. Specifically, dual oxidase (DUOX) plays a significant role in controlling opportunistic pathogens in the gut by inducing de novo generation of microbicidal reactive oxygen species (ROS), which is part of the organism's inflammatory response. Past research has discovered that production of ROS by DUOX is activated by bacterial-derived uracil, a nitrogenous base that is secreted in high quantities by pathogenic bacteria and in low quantities by non-pathogenic resident bacteria. These findings demonstrated that the mechanism by which *Drosophila* gut epithelia selectively allows the growth of non-pathogenic bacteria while suppressing pathobiont colonization is mediated by bacterial uracil secretion in a DUOX-dependent inflammatory response (Lee et al. 2013).

While research has already discovered a mechanism by which DUOX-dependent inflammation is induced, the effects of such inflammation on brain function, behavior, and memory have yet to be determined. Here we study if a high uracil diet, intended to induce a DUOX-dependent response of reactive oxygen species production, causes a decrease in the courtship behavior of *Drosophila*. This hypothesis will be tested by measuring courtship index. The study will illustrate the contrast in courtship behavior of subjects that have undergone short and long-term uracil exposure. Additionally, the courtship behavior of uracil-fed flies will be compared to flies fed a standard diet. By measuring the effects of diet-based exposure to uracil on the courtship index of *Drosophila,* the current understanding of the relationship between epithelial DUOX-dependent reactive oxygen species generation and behavioral changes in animals can be expanded.

Experimental data regarding the existence of a gut-behavior connection in *Drosophila* will be valuable towards the broader understanding of the gut-brain axis as it could provide insight on how the connection can be attributed to a more primitive immune response.

MATERIALS & METHODS

In order to determine the effects of a short term high-uracil diet on *Drosophila* courtship behavior, virgin male flies were fed uracil solution for 16-hr, and their behavior was analyzed using a courtship assay. Additionally, the effects of long-term exposure to uracil were observed using the same method after a 10-d feeding period. The courtship index (CI) values of uracil-fed flies were compared to age-matched, sucrose-fed flies to determine whether acute or chronic DUOX activation has an effect on the courtship behavior of male *Drosophila*.

Fly Strains and Rearing.–Canton S. fly stocks were maintained at 22º C in 95 by 25 mm polypropylene *Drosophila* vials with standard cornmeal agar feeding media. After each four-week period, fly stocks were transferred to new vials. To collect virgin subjects, flies were anesthetized using carbon dioxide and examined under a light microscope for a meconium, which is indicative of a virgin fly. Male virgin flies were isolated in individual vials to prevent courting of other flies before the courtship assay. Since the behavior of female virgin flies was not measured by the courtship assay, they were kept in groups of 10 (or less) per vial.

Sucrose Feeding Solution.–A 5% sucrose solution was prepared by stirring. After the sucrose was fully dissolved, blue food dye was added. This dye is used to confirm feeding solution consumption immediately prior to behavioral assays by examining the presence of the dye in each fly's abdomen.

Uracil Feeding Solution.–A 20 nM uracil, 5% sucrose solution was prepared for oral uracil feeding based on a previous dose-dependent analysis showing that uracil induces intestinal ROS generation most effectively in a range of 1-20 nM (Lee et al. 2013). For preparation, uracil crystals ≥ 98.0% (TCI America) were dissolved in a 5% sucrose stock solution (20 mM uracil). The 20 mM solution was then serially diluted to 200 μM, to 2 μM, to 20 nM, with stirring at 95º C for 20 min at each step of the dilution. Blue food dye was added and the solution was stored at 1.5º C.

Short-Term Cornmeal-Agar Feeding Experiment.–Virgin males were collected from stock vials and placed in individual cornmeal-agar feeding vials for three days. On the third day, the virgin males were allowed to continue feeding on cornmeal-agar media for an additional 16 hours, after which the subjects were placed in a courtship assay for 10 minutes. This feeding experiment served as a control to provide data on the standard courtship behavior of *Drosophila*.

Short-Term Sucrose and Uracil Feeding Experiment.–Feeding solutions were offered to flies by soaking filter paper in the respective solution, then placing the soaked filter paper in an empty vial. Before sucrose feeding, virgin males were collected from stock vials and placed in individual cornmeal-agar feeding vials for three days. After the third day of cornmeal-agar feeding, flies were assigned to one of two feeding treatments. One group was fed only sucrose, and the second was fed a sucrose-uracil mixture. Feeding was allowed for 16 hours, after which the subjects were placed in a courtship assay for 10 min. After the courtship assay, consumption of solution was confirmed by identifying blue dye in the male abdomen. This feeding experiment served to determine any changes in courtship behavior that may result from sucrose solution feeding as opposed to cornmeal-agar feeding.

Long-Term Sucrose and Uracil Feeding Experiment.–For the long-term sucrose solution feeding experiment, virgin males were

individually placed in an empty vial containing sucrose-soaked filter paper immediately after collection. The flies were allowed to feed in the vial for 10 d. Because the filter paper dried after 48 h, the filter paper was replaced with newly soaked filter paper every 24 h within the 10-d feeding period. On the tenth day of feeding, flies were placed in a courtship assay for 10 min. After completing the courtship assay, consumption of sucrose solution was confirmed by identifying blue dye in the male abdomen. This feeding experiment served as the control. For the uracil feeding experiment, the procedure was replicated using 20 nM uracil, 5% sucrose solution.

Courtship Index.–Behavioral changes in *Drosophila* were measured quantitatively by a courtship index (CI), a numerical output of a 10-min courtship assay (Siegal & Hall 1979). In the courtship assay, a male virgin was placed with a female virgin in a shallow, 5 mm diameter circular chamber and was recorded using a rear-facing camera for 10 min. All flies were placed in the courtship chamber using a non-anesthetizing fly transfer apparatus. The video was analyzed to measure the amount of time that the male subject exhibited courtship behavior (orienting, chasing, tapping, singing, licking, and attempting copulation) within the 10-min period or until copulation. After determining the time in which the male exhibited courtship behavior, the CI value was determined using the following formula:

$$CI = \frac{seconds\ courting}{total\ seconds}\ X\ 100$$

The courtship index was calculated for all trials in each of the three short-term feeding experiments as well as for all trials in each of the two long-term feeding experiments.

Statistical Analysis.–All data were analyzed using Prism 7 statistical software (GraphPad). Data were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons tests or by unpaired *t*-tests. All data are presented as the mean + standard error of the mean (SEM).

RESULTS

To determine the effects of short-term uracil feeding on the courtship behavior of *Drosophila*, we measured the courtship index (CI) after 16 hours of uracil treatment in male subjects. After experimentation, it was found that the mean CI was 78% (2.9 SEM, n = 14) for the standard fly media control, 85% (2.5 SEM, n = 14) for the sucrose solution control, and 83% (2.2 SEM, n = 14) for the uracil treatment subjects (Figure 1). Data comparison of both controls and the uracil treatment shows no statistical significance ($F = 2.178$, $df = 2, 39$, $P = 0.13$). It is important to note that while there is an increase in the mean CI values of both the sucrose control and uracil treatment flies in comparison to the standard fly food control, this deviation is not significant. The deviation can be attributed to the scent of sucrose on the male fly, which may cause an increase in the female fly's participation in the courtship process due to olfactory attraction.

To determine the effects of long-term uracil feeding on courtship behavior, we measured CI values of 10-d uracil treated male subjects. After experimentation, it was found that the mean CI value was 83% (2.9 SEM, n = 16) for the sucrose control, and 69% (4.2 SEM, n = 17) for the uracil treatment flies (Figure 2). Data comparison of CI indexes of sucrose control and uracil treated flies shows a statistically significant decrease on CI in uracil treated flies ($P = 0.0125$). Therefore, the results of the experiment demonstrated that long-term treatment of *Drosophila* males with uracil is associated with a decrease in normal courtship activity.

DISCUSSION

While there are multiple immune components that influence gut dysbiosis and its resulting inflammation in *Drosophila,* one component is a DUOX-dependent response to the secretion of bacterial uracil from allochthonous and pathogenic autochthonous microbiota (Lee et al. 2013). In the current study, it was demonstrated that long-term treatment of male flies with uracil via solution feeding

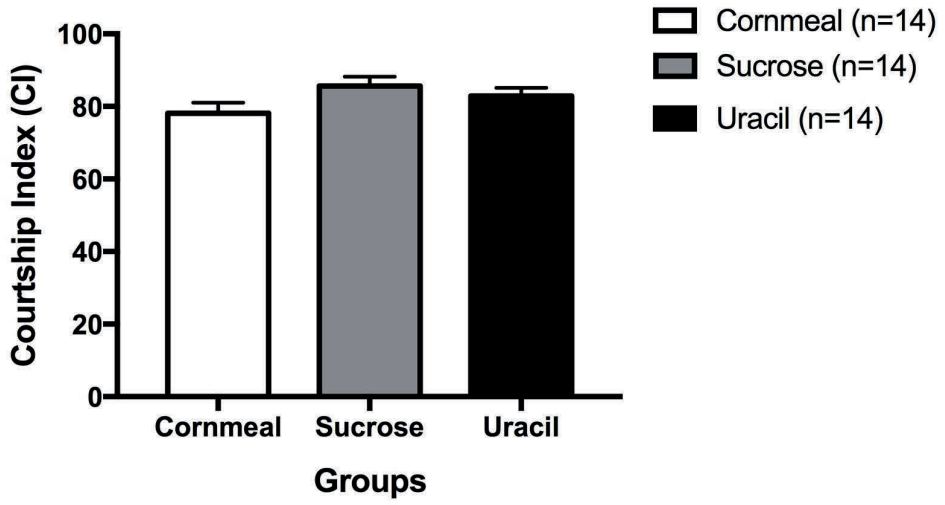

Figure 1. Courtship index values following an acute (16-hr) cornmeal treatment, sucrose treatment, and uracil treatment. No significant differences in courtship behavior was observed among treatments.

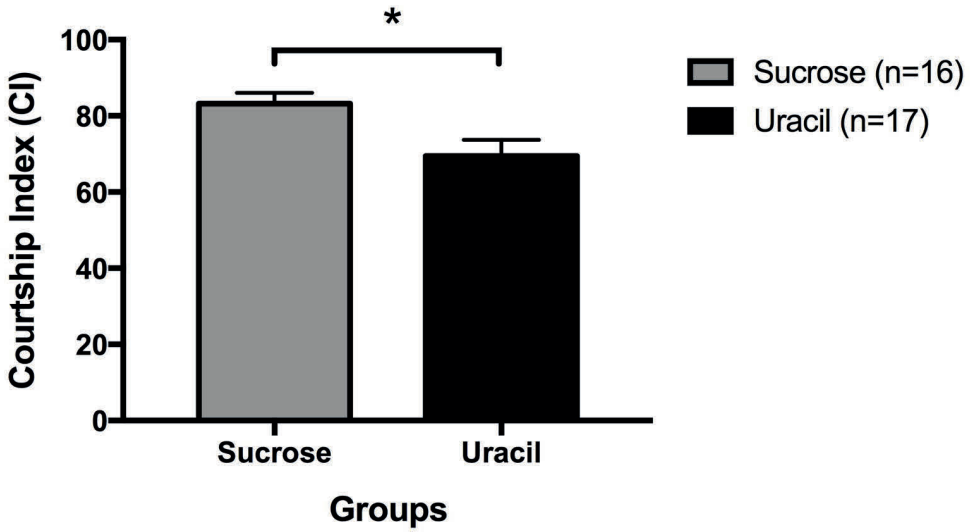

Figure 2. Courtship index values following a chronic (10-d) sucrose treatment and uracil treatment. A decrease in courtship behavior was observed in the uracil treatment compared to the sucrose control ($P = 0.0125$).

results in a decrease in courtship behavior. Sucrose solution alone was not associated with changes in courtship behavior, and uracil is a known ligand for an inflammatory response in *Drosophila* gut epithelia. Therefore, the decline in courtship behavior is potentially associated with chronic gut inflammation induced by uracil.

Treatment of male *Drosophila* with uracil for 16 hours was not associated with any courtship behavior changes. This may be explained by the transient ROS generation induced by short-term uracil exposure. Although ROS may have been produced by the gut epithelia when exposed to uracil, the amount of time that this production was activated by the DUOX pathway was limited. Therefore, there was not adequate ROS production to cause significant damage to the tissues of the fly gut. This result is analogous to the *Drosophila* gut epithelia coming into contact with an allochthonous bacteria that do not colonize. In this situation, the inflammatory response is transient and enough microbicidal ROS is produced to neutralize the foreign bacteria as it passes through the gut. After the bacterial organism has been neutralized and leaves the gut, the ROS response stops, and gut homeostasis is maintained.

Contrary to the short-term response result was the discovery that treatment of male *Drosophila* with uracil for ten days led to a decrease in courtship behavior. This finding can be explained by long-term ROS generation as a result of constant exposure to uracil over a ten-day feeding period. In the same manner as the transient response, an initial detection of uracil by the gut epithelia results in DUOX-dependent ROS production. However, as the fly continues to feed from the solution containing uracil, the gut continues to be exposed to the ligand. This results in constant production of ROS over a ten-day period, which damages the gut tissue and causes a disease phenotype similar to inflammatory bowel disease (Mazmanian et al. 2008). This scenario is analogous to the colonization of a pathogenic, autochthonous bacterial organism in the gut. Bacterial species such as *G. morbifer* are known opportunistic pathobionts that do not invoke an immune response in low levels of growth, but can expand in

population during dysbiosis conditions and cause chronic inflammation via uracil secretion (Lee et al. 2013).

While the results of the experiment demonstrate that long-term exposure to uracil is associated with decreases in the courtship behavior of male *Drosophila*, the determination that chronic uracil-induced inflammation affected the organism's behavior has not been made. As mentioned previously, research regarding the gut-brain axis in *Drosophila* is lacking. Nonetheless, studies that examine the effect of inflammation on behavior in other animal models can be referred to in order to generate a new hypothesis. One such study examined the effects of chronic inflammation on the behavior of mice, and found that proinflammatory cytokines, tryptophan metabolism, and altered serum proteins played a critical role in the complex mechanisms that facilitate gut-brain signaling (Bercik et al. 2010). When considering that *Drosophila* rely on all three of these factors as a part of immunity and metabolism, the change in the subjects' behavior after uracil treatment may be explained by related signaling mechanisms.

In order to better understand the underlying mechanism behind the observed decrease in courtship behavior after uracil treatment, further research is required. First, it is important to confirm that the decrease in courtship behavior that was exhibited is a direct result of uracil-induced gut inflammation, and not due to malaise effect. In order to make this confirmation, it is necessary to assess the locomotor activity of flies exposed to uracil and compare the results to the locomotor activity of control flies. Secondly, recognition of inflammation in the gut epithelia via histological analysis would provide significant confirmation for the current project's data. By determining morphological changes immediately following uracil treatment, changes in courtship behavior can be more strongly attributed to gut inflammation.

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