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# Monitoring gut acidification in the adult Drosophila intestine

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**TITLE:**

# Monitoring Gut Acidification in the Adult *Drosophila* Intestine

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**KEYWORDS:**

Gut, Acidification, Intestine, Copper cells

**SUMMARY:**

Here, we present a standardized protocol for monitoring gut acidification in *Drosophila melanogaster* with optimal output. We first use this protocol for gut acidification monitoring in *Drosophila melanogaster* and then demonstrate its use in non-model *Drosophila* species.

**ABSTRACT:**

The fruit fly midgut consists of multiple regions, each of which is composed of cells that carry out unique physiological functions required for the proper functioning of the gut. One such region, the copper cell region (CCR), is localized to the middle midgut and consists, in part, of a group of cells known as copper cells. Copper cells are involved in gastric acid secretion, an evolutionarily conserved process whose precise role is poorly understood. This paper describes improvements in the current protocol used to assay for acidification of the adult *Drosophila melanogaster* gut and demonstrates that it can be used on other species of flies. In particular, this paper demonstrates that gut acidification is dependent on the fly's nutritional status and presents a protocol based on this new finding. Overall, this protocol demonstrates the potential usefulness of studying *Drosophila* copper cells to uncover general principles underlying the mechanisms of gut acidification.

**INTRODUCTION:**

In the insect gut, copper cells share cellular and functional similarities with the acid-producing gastric parietal cells (also known as oxyntic) of the mammalian stomach. This group of cells releases acid into the intestinal lumen. The function of acid secretion and anatomy is evolutionarily conserved. The major components of the discharged acid are hydrochloric acid and potassium chloride. The chemical mechanism of acid formation in the cells depends on carbonic anhydrase. This enzyme generates a bicarbonate ion from $CO_2$ and water, which liberates a hydroxyl ion that is then discharged into the lumen through a proton pump in exchange for potassium. Chloride and potassium ions are transported into the lumen by conductance channels

resulting in the formation of hydrochloric acid and potassium chloride, the main component of gastric juice[1-4].

Although the mechanisms of acid formation are well understood, much less is known about the physiological mechanisms that regulate acid secretion. The goal of developing this method is to help better delineate the cellular pathways that coordinate acid formation and secretion and determine the role of acid in mediating intestinal physiology and homeostasis. The rationale behind the development and use of this technique is to provide a consistent and reliable method to study the process of gut acidification in *Drosophila* and non-model organisms. Although a standard protocol for determining *Drosophila* midgut acidification currently exists[2,5,6], significant variability was observed in the extent of acidification in wild-type (WT) flies while using this protocol for studying copper cell function. To understand the basis for this observed variability and obtain consistent results, several aspects of the standard protocol were optimized as described below.

**PROTOCOL:**

NOTE: The standard laboratory line Oregon R was used as a WT control. All flies were reared on standard cornmeal-molasses medium (containing molasses, agar, yeast, cornmeal, tegosept, propionic acid, and water) at room temperature with 12/12 h light/dark circadian rhythm.

**1. Preparing for the assay**

1.1. Collect female flies (0–2 days old, non-virgin) under $CO_2$ anesthesia and allow them to recover on standard cornmeal food for at least 3 days before experiments.

1.2. Starve the flies for ~24 h at room temperature (~23 °C) in vials containing a laboratory wipe tissue soaked with ~2 mL of deionized water.

1.3. Prepare the fly food with bromophenol blue (BPB) as follows:

1.3.1 Melt the fly food in a microwave and then let it cool until it is lukewarm.

1.3.2 Add 1 mL of 4% BPB to 1 mL of lukewarm food and mix well.

1.3.3 Using a pipet, add the fly food containing BPB into a single dot (~200 µL) in the center of a Petri dish.

**2. Gut acidification monitoring assay**

2.1. Transfer starved flies into a Petri dish containing single dots (200 µL) of fly food supplemented with 2% bromophenol blue (BPB). Allow the flies to forage for 4 h at room temperature while exposed to light.

2.2. After 4 h, collect the flies and anesthetize them on ice; surgically isolate their guts.

2.2.1. Perform the surgery in 1x phosphate-buffered saline (PBS) with forceps under a stereomicroscope (see the **Table of Materials**). Isolate the gut by holding the thorax with a pair of forceps and pulling down the abdomen with a second pair until the CCR of the gut is visible, taking care to ensure that the intestine remains attached at both ends.

2.3. Determine acidification of the gut by examining the color of the CCR of the gut (**Figure 1C**; yellow indicates acidified, and blue indicates not acidified).

2.4. Count only those flies that show robust BPB staining in their guts.

2.5. Calculate the percentage using the following equation:

$$Percentage\ of\ flies\ with\ acidified\ guts = number\ of\ flies\ acidified\ \times\ 100\ /(number\ of\ flies\ acidified + number\ of\ flies\ non-acidified)$$

NOTE: A percentage of 0 indicates that no flies acidified their gut, whereas a percentage of 100 indicates all flies acidified their gut.

## 3. Mounting and image acquisition

NOTE: This step is additional to acquire and process images for the respective conditions for further analyses as the samples cannot be preserved for longer. These images must not be used for any gut acidity quantification.

3.1. Following dissection, mount the samples in PBS onto a glass slide.

3.2. Acquire the images under a microscope using cellSens Entry software (see the **Table of Materials**).

3.2.1. Place the prepared slide under the microscope and adjust the sample using the eyepiece.

3.2.2. Shut off the eyepiece to open the shutter for the camera.

3.2.3. Open the software on the connected computer.

3.2.4. Choose the correct objective lenses, click the **live** button, and select the **standard setting with exposer time adjustment**.

3.2.5. Focus on the CCR region and take the snapshot.

3.2.6. Right-click on the snapshot image window and save it as a .tif file.

3.3 Align and process the images further using Fiji software.

3.3.1 Import the .tif file in Fiji software and clear the unrelated background.

3.3.2 Adjust the intensity and contrast to optimize the CCR and other gut regions.

3.3.3 Add the scale bar and save as a .tif file.

**RESULTS:**

We starved Oregon R female flies for more than 20 h and then fed them food supplemented with BPB (2%) for ~12 h, as described previously[7-11]. Bromophenol blue (BPB) is a pH-sensing dye. It changes from yellow at pH 3.0 to blue at pH 4.6 and above. Following gut dissection, as previously reported, some flies were found to produce acid as indicated by yellow color in the CCR of the gut (**Figure 1B**). Surprisingly, in contrast to published results, the intestines of some flies were blue, suggesting that they had failed to acidify their guts. These inconsistent results indicated that the protocol needed to be modified to optimize for consistent and interpretable outcomes.

To optimize the BPB protocol, two new modifications were incorporated. First, to better control the onset of feeding, flies were starved and then placed on spots of food with BPB in the center of a plate (**Figure 1A**). Second, we began assaying for gut acidification at time points closer to the onset of feeding. Female flies were starved for >20 h, provided fly food with BPB in a small Petri-plate arena (see **Figure 1A**), and allowed to feed for various time points until 4 h while dissecting guts at 1 h intervals. The number of acidified guts (yellow color) and non-acidified guts (blue color) was determined, and the percentage of flies showing gut acidification was calculated for each time point (**Figure 1B**). Within 30 min, ~20% of flies had acidified their gut. After an hour, ~40% of guts showed evidence of acidification, while after 2 h and 3 h of feeding, the percentage of acidified guts increased to ~60% and ~70%, respectively (**Figure 1B**). This indicates that there is an increase in the percentage of flies showing gut acidification with time. Almost 90–95% of guts were acidified when flies were fed for 4 h (**Figure 1B**). We used this optimized protocol of 4 h feeding for subsequent experiments.

In addition to the effect of feeding, the effect of temperature at which flies were raised on gut acidification was examined. Flies were reared at 23 °C and 30 °C, and female flies were starved for ~20 h. Flies were then fed fly food supplemented with BPB for 4 h, and the percent of gut acidification was determined as described above. We observed no difference in gut acidification for these two temperatures (**Figure 1C**), suggesting that temperature, unlike feeding, does not affect gut acidification.

**Gut acidification protocol demonstration for non-model organisms**

*Drosophilae* species are phylogenetically separated over millions of years (see **Figure 2A**). Over this vast period, they have adapted to different habitats and diets[12], raising the possibility that some species may not acidify their gut in the same manner as *D. melanogaster*. We used *D. melanogaster* (fruit), *D. sechecllia* (morinda fruit), *D. erecta* (pandanus fruit), *D. pseudoosubcura* & *D. virilis* (plant sap), and *D. mojavensis* (cactus fruits) (**Figure 2B**). To demonstrate that this

protocol could be used for other *Drosophila* species, these species were fed fly foods supplemented with BPB for 4 h, and the percent of gut acidification was determined as described above. Robust gut acidification was observed for all species tested (**Figure 2B**). This result suggests that acidification of the gut is evolutionarily conserved among diverse *Drosophila* species and that this protocol can easily be implemented for other organisms.

**FIGURE AND TABLE LEGENDS:**

**Figure 1: Gut acidification monitoring.** (**A**) Schematic drawing of feeding arena. The blue dot represents fly food with bromophenol blue (a pH-indicating dye). Other spots represent fruit flies. (**B**) Graphical representation of percentage of flies showing gut acidification fed for different durations over 4 h. Representative gut images of an acidified gut and a non-acidified gut. The red arrow indicates acidic release in the copper cell region of the midgut. *n* = 4 experiments, 25–30 female flies per experiment. Scale bar = 500 µm each. Asterisks indicate significant differences from the control group (one-way ANOVA, followed by a Bonferroni test) *P < 0.05; **P < 0.01; ****P < 0.0001. (**C**) Flies were fed fly food with BPB for 4 h at 23 °C or 30 °C. Percentage (*%*) of flies showing gut acidification. *n* = 4 experiments, 25–30 female flies per experiment (unpaired *t*-test followed by non-parametric Mann-Whitney U test and Wilcoxon rank-sum test. Abbreviation: ns = not significant.

**Figure 2: Phylogeny of gut acidification phenomenon.** (**A**) Phylogenetic relationship of *Drosophila* species along with their feeding habit and habitat. 1 mm bar indicates 1 million years. (**B**) Percentage of flies (*Drosophila* species) showing gut acidification, fed fly food with BPB for 4 h. *n* = 4 experiments, 25–30 female flies per experiment (one-way ANOVA, followed by a Bonferroni test). Abbreviation: ns = not significant.

**DISCUSSION:**

A critical step in this protocol is the proper dissection of the gut to visualize the CCR for the acidification phenotype. The acid released from the copper cells is confined to the CCR when the gut is intact. However, during dissection, leakage caused by rupture of the intestine can lead to diffusion of acid from the CCR and result in a gut mistakenly scored as a negative for acidification. In addition, the yellow color indicative of acidification fades within 5–10 min after dissection, underscoring the importance of scoring intestines for the acidification phenotype soon after isolation. Finally, current protocols[7-11] that assay the state of acidification in the fly gut rely on supplementation of fly food with BPB, without consideration of the feeding status of the animals. However, during our studies, we found that acidification of the gut was not constitutive but rather dependent on feeding following prior starvation. As such, accurate evaluation of the acid state of the gut using BPB as an indicator of gut pH requires consideration of the fly's nutritional status along with any other variables being considered.

Acidification of the gut is conserved from lower multicellular to higher organisms. However, little is known about its function in most animals and the full extent of the molecular and cellular pathways that regulate it. In humans, lack of gut acidification is associated with the malabsorption of nutrients, while excess acid in the gut can result in intestinal ulcers[13]. Thus, insights gained from research on gut acidification are likely to provide new insights into the

treatment and cure of intestinal diseases caused by defects in the regulation of acid secretion.

*Drosophila* has recently emerged as a powerful model for the study of gut acidification[2,5,6]. Genetic studies have identified genes required for the establishment of acid-secreting cells and the machinery involved in the production of acid. Drug studies have also been carried out. For example, acidification of the gut is prevented when flies are fed acetazolamide, a carbonic anhydrase (CAH) inhibitor[7], consistent with the central role that CAH plays in the production of protons necessary for acid production. We expect this protocol to help researchers rapidly and cost-effectively discover drug inhibitors or activators of gut acidity. In addition, the application of this method in combination with genetic and biochemical approaches will help uncover the cellular pathways involved in acid secretion and pinpoint the role of gut acidification in intestinal and organismal homeostasis.

**ACKNOWLEDGMENTS:**

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**DISCLOSURES:**

The authors have no conflicts of interest to disclose.

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Figure 1



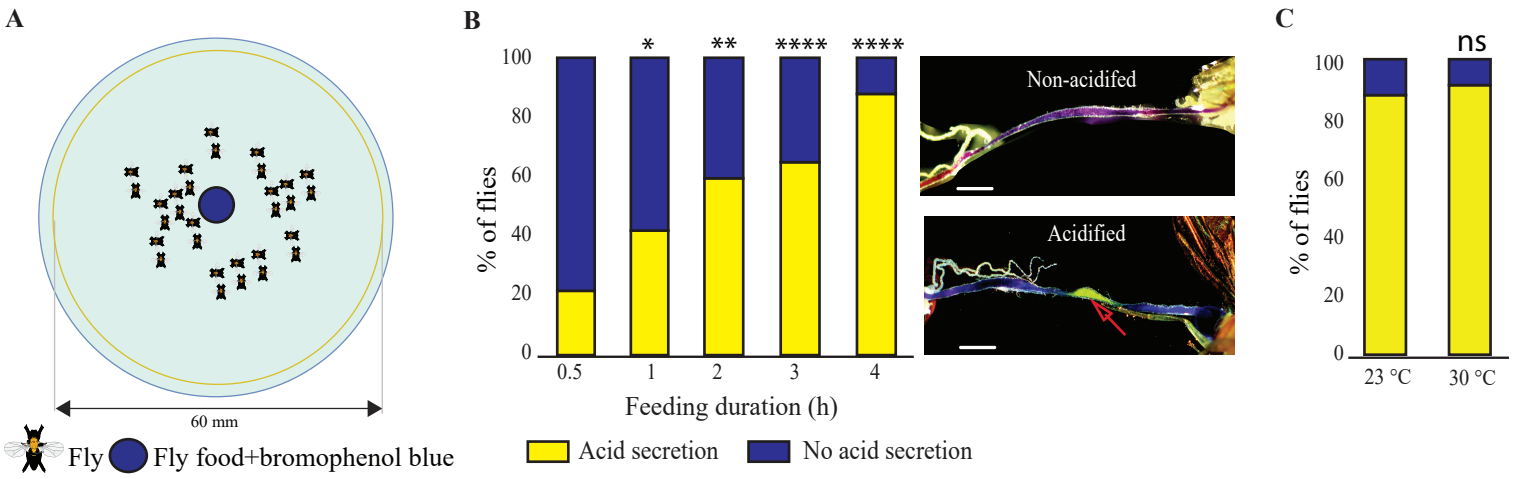

Figure 2



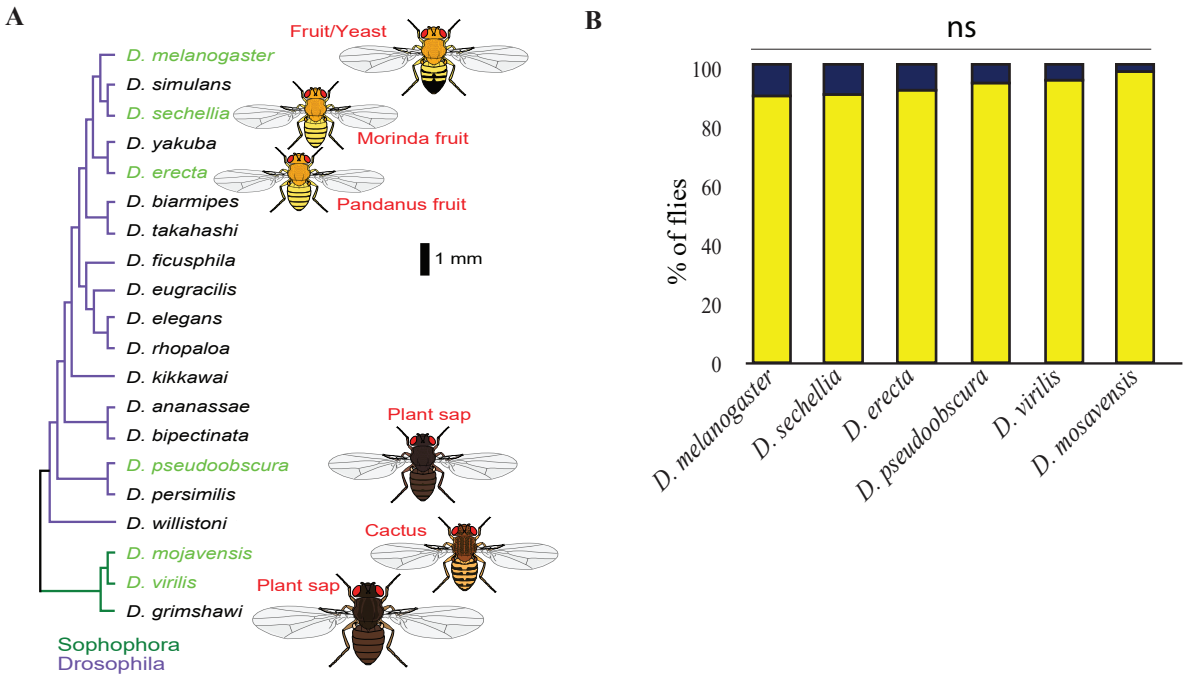

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**Table of Materials**

List of Material (3).xlsx

Dear review Editor,

We thank for the consideration of our work for review under your JoVE. Also, we thankful for the reviewers to contributing their precious time and constructive suggestions.

We take care point to point all the suggestions by reviewers as below. We hope our work will be a good article to go under the “toolkits for non-model organisms” and provide this method to uncover further unanswered questions.

With regards

Benjamin Ohlstein

**Reviewer #1:**
Manuscript Summary:
The manuscript by Abu and Ohlstein describes the method of documenting pH in the gut of drosophilids that is probably transferable to other insects. This method, which was described some time ago, is a very simple form of physiological characterization of the gut. However, this method showed weaknesses in reproducibility that led to some frustration for many researchers. Optimizations have simplified the assay and made it much more robust at the same time.
This method is very well suited for a Jove article.

Minor Concerns:
However, more care should be taken in the wording of the method.

**Reviewer #2:**
Manuscript summary:
Abu and Ohlstein describe a protocol to monitor gut acidification using ingested bromophenol blue (BPB) as a pH indicator in various fly species. The manuscript and protocol and clearly written, and the authors have provided some optimization of the timing for the various steps. I have only minor comments that should be easily addressed by revising the text. However, I've also suggested some experiments that would help validate and broaden the use of the described technique.

It's not entirely clear to me what the assay is specifically measuring. When the flies ingest food and BPB, is this clearing out the gut contents and so you're now getting a readout of how quickly the fly gut can generate acid and re-establish an acidic pH?

***When the flies are fed the flies have been starved for over 20 hours. As such, their guts are empty. Furthermore, 80% of guts remain non-acidified even after 30 minutes of food availability. Only after a significant time after food availability (2-3 hours) do we see***

***evidence of gut acidification in over half of the flies suggesting that it is the presence of food that triggers acidification. This argues that acidification is not a default state as the reviewer suggests, but rather a regulated response to diet.***

If so, then I think you're measuring a gut acidification rate, the kinetics of which will be difficult to resolve given that feeding is not well restricted (i.e. the flies can be ingesting BPB any time during the 4 hour feeding period). That doesn't negate the value of the assay, but it might be helpful to better describe what's going on, or add additional studies that show what's happening during the time-course of the assay.

***As mentioned in the previous response, our data argues that we are not measuring an acidification rate after gut clearing, but rather demonstrating that acidification is triggered by the presence of food. We do agree as the reviewer points out, that feeding is not well restricted. As such we do not attempt to make any conclusions regarding the exact time it takes for food to trigger acid release.***

Showing that this assay, as optimized, can resolve differences would be beneficial too. The current results only show that a measurement can be made, but don't compare the control results to a mutant or other gut acidification manipulation.

***Our current results include measurement of gut acidification of wildtype animals. We expect however following the publication of our manuscript that research labs will use our new approach to not only characterize newly identified mutants that may play a role in gut acidification, but to also reevaluate previously characterized mutants.***

Is fasting required to get flies to ingest sufficient BPB, or to establish the timing for gut acidification by getting (most) animals to eat at the same time?

***As the reviewer suggests, fasting is required to establish the timing for gut acidification by getting (most) animals to eat at the same time.***

There are many studies where fasting animals would not be preferable, and it might be useful to know if there are different ways to do this experiment to test non-fasted animals.

***Eating is pre-requisite for this phenomenon. At this point the only approach we know of to synchronize food ingestion is by starving flies for an extended period.***

Lines 83-88 imply that the initial method didn't work that well, and that's why the authors optimized the timing of the protocol (Figure 1B). However, the optimization suggested that a higher percentage of gut acidification is observed with longer feeding periods, up to 4 hours. So are longer times even better? The initial method uses a 12 hour feeding period, so are the authors implying that there's an optimum between 4 and 12 hours? Why did the authors only test up to 4 hours if their initial method used 12?

***The main point of our work is the demonstration that acidification is dependent on diet. We therefore tested for gut acidification up to 4 hours because its highly likely that within the first 4 hours of exposure to food most if not all the previously starved flies will be consuming food and as result, actively acidifying their guts. With increased exposure to food it's hard to know if a non-acidified gut is due to a satiated fly that has ceased eating or attenuation of the acidification process.***

The authors should clarify the motivation for how these studies were designed and/or consider characterizing the kinetics of the assay more thoroughly. If some of the ideas mentioned above are correct (i.e. that the timing of BPB ingestion is important), a pulse-chase type of study might work better (e.g., brief access to food containing BPB, followed by a chase period in empty vials).

***A chase period in empty vials will result in clearance of food and BPB from the gut. Because measurement of acidification is dependent on the presence of BPB, the proposed experiment by the reviewer is not possible.***

Does diet affect the results? The diet might affect how much BPB is ingested,

***BPB added to food did not affect the result as we only counted flies which showed robust BPB staining in their guts.***

but the components of the diet itself might also affect gut pH or the rate of acidification. According to Deshpande et al. (J. Nutr. 2015; https://doi.org/10.3945/jn.115.222380), yeast is the major component of the fly medium that has any buffering capacity. Thus, different yeast concentrations might change the kinetics of the assay. The authors could mention this, or better yet replicate the study on a range of diets.

***In all our experiments we used the same food source and formula. We agree that the components and of the diet and their concentrations itself might also affect gut pH or the rate of acidification and is currently under investigation in our lab.***

Figure 1B: The first column is described as a 30 minute time point in the main text. If that's correct, the figure axis should be corrected to 0.5 hr.

***The requested change has been made.***

**Reviewer #3:**
Manuscript summary:
Lines 34-35: This sentence needs to be rewritten to convey the message that insect copper cells share cellular and functional similarities with the acid-producing gastric parietal cells (also known as oxyntic) of the mammalian stomach.

***The requested change has been made.***

Line 45: correct grammar.

***The requested change has been made.***

Line 49: There are many Oregon R lines and every lab may have its own. Every line changes over the years, so the authors are using an Oregon R originating line as a wildtype (WT) control obtained from the Bloomington Drosophila Stock Center. BSC# should be mentioned.

***The requested change has been made.***

Lines 51-53: These other Drosophila species lines are unique and they may not represent the

average trait performance of their species. BSC# should be mentioned. See Evangelou et al (PMID: 31540975), where fecal pH of more than a hundred WT D. melanogaster was measured.

***The requested change has been made.***

Lines 53-54: The specific (rather than so called standard) cornmeal molasses medium needs to be described. Diet can directly affect microbiota and overall physiology.

***The requested change has been made.***

Lines 86-87: BPB is a pH-sensitive dye, its useful range lies between pH 3.0 and 4.6. It changes from yellow at pH 3.0 to blue at pH 4.6.

***The requested change has been made.***

Figure 1C: How come all fly guts (unlike in other figures) are acidified?

***While assembling the figure in Photoshop Illustrator, the non-acidified part of the graph was removed by mistake. The figure has been corrected.***

Lines 164-165: Change "but rather dependent on feeding" to convey the following message: "but rather dependent on feeding response upon prior starvation".

***The requested change has been made.***

**Reviewer #4:**
Manuscript Summary:
In this paper, authors optimized the protocol for monitoring gut acidification in Drosophila. In addition, this protocol can be also used in other Drosophila species. I believe that the new protocol will help us understand the mechanism of mediating intestinal homeostasis better.

Major Concerns:
1. In the protocol, authors did not mention whether the female flies used in the study were virgins or not.

***The females used were not virgins***. ***The requested changes has been made.***

2. Authors only claimed that intestines of "some" flies were blue by using the "traditional" protocol. What is the percentage of the "blue gut"? It would be better to quantify the percentage of non-acidification.

***Using the "traditional protocol" we found that the percentage of acidified guts varied widely from 40-70%.***

3. Authors should provide more details methods, for example, the dissection of gut, culture condition of flies. In addition, based on the "traditional" protocol, remain some of the tissues/organs (like brain) instead of the complete dissection. From Figure 1C, authors seemed to follow the "traditional" protocol.

***The requested changes have been made.***

4. Authors should have tried to a longer feeding hour (more than 4 hours), will there be a higher or lower percentage of "Acidified"?

***The main point of our work is the demonstration that acidification is dependent on diet. We therefore tested for gut acidification up to 4 hours because its highly likely that within the first 4 hours of exposure to food most if not all the previously starved flies will be consuming food and as result, actively acidifying their guts. With increased exposure times to food it's hard to know if a non-acidified gut is due to a satiated fly that has ceased eating or attenuation of the acidification process.***

5. Regarding the temperature experiment (Figure 1C), the percentage of acidification is around 100%, which is not consistent with Figure 1B. What is the reason for this?

***While assembling the figure in Photoshop Illustrator, the non-acidified part of the graph was removed by mistake. The figure has been corrected.***

Minor Concerns:
1. Scale bars are missing in Figure 1C.

***A scale bar has been placed in the upper panel of the figure, which is now in Figure 1B.***

2. The number "2" of CO2 should be in subscript (line 38; line 56).

***The requested changes have been made.***