A Research-Oriented Approach to Digestive Physiology to Replace Traditional Enzymatic Laboratories.

Gregory M. Grabowski and Jelena Holt
University of Detroit Mercy
Biology Department
4001 W. McNichols Rd.
Detroit, MI 48219.

ABSTRACT: The current trend in research-oriented education offers an opportunity to revitalize physiology laboratories. Physiology laboratory manuals have traditionally adopted a demonstrative format that typically reinforce concepts describe in lecture. This format proves to be especially limiting in digestive physiology laboratories. The initial goal of the proposed laboratory is to localize digestive enzymes within the digestive tract of cockroaches, and to develop general conclusions about their similarities to mammalian digestion. Students prepare homogenates of digestive tract segments from cockroaches and perform assays for protein, lipid, and carbohydrate digestion. This approach not only demonstrates the practicality of lecture material, but also provides a springboard for independent research opportunities.

KEYWORDS: Cockroach, Digestive Physiology, Invertase, Amylase,

INTRODUCTION
With the current focus on research-based education, physiology laboratories have to go beyond classical demonstrations to inductive approaches to physiology. The focus of laboratories and manuals alike has been on demonstrations of human physiologic concepts described in lecture using “classical” techniques and technology. This demonstrative laboratory format does substantiate lecture material, but leaves students with little more than validation of the physiologic litany from class. Traditional digestive physiology laboratories exemplify the demonstrative format currently found in many manuals. These laboratories typically involve assays of digestive enzymes purchased from a chemical supply company or involve the collection of salivary amylase from students. The former provides a good indictor of a student’s attentiveness in lecture, allowing them to confidently predict that a protease described in lecture digests proteins, amylase digests polysaccharides, and so forth. The latter’s collection of saliva in a test tube provides the same problems associated with the use of other human bodily fluids, such as blood, in a student laboratory. The following laboratory exercise describes a research-oriented approach to digestive physiology through the localization of enzymatic activity within the digestive tract of cockroaches. This laboratory not only provides students with practical application of a knowledge base developed during their undergraduate tenure, but also acts as a springboard into independent-research projects.

Being around since the early Paleozoic Era, insects were the first animals to successfully adapt to life on land (Gordon, 1996). Undergoing adaptive radiation, a plethora of anatomical and physiological
adaptations evolved among insects that occupy copious niches on land. Considered a living fossil, cockroaches first made their evolutionary appearance 340 million years ago, during the Carboniferous Period. During this period cockroaches were suspected to be so numerous that they represented 40% of the insect life. Today there are 3,500 species of cockroaches found on every continent except Antarctica. Truly representing the diversity of insects, the cockroach family provides excellent models for anatomical and physiological investigations.

Facing the same environmental challenges as man, cockroaches share many similar anatomical and physiological features with us, as well as adaptations unique to insects (House, 1965; Barnes, 1980). Comparing the digestive organs of cockroach and mammalian systems, both share salivary glands, liver (fat bodies), esophagus, intestines, and rectum. The crop and proventriculus of the cockroach provide storage and mechanical processing of food similar to the mammalian stomach. Pancreatic contribution to digestion in mammals is shared with the gastric ceca and ventriculus of the cockroach. Enzymatic similarities between humans and cockroaches within the digestive tract include protease, lipase, and carbohydrase activities (House, 1965; Chippendale, 1978). The pH of the gastrointestinal contents of insects ranges from 5 – 8. This range precludes the physiologic activity characterized by the mammalian stomach. Rather than pepsin-like enzymatic activity, insect protease activity is characterized as being trypsin-like. Like protease activity, lipase activity also functions within specific pH ranges. Cockroach lipase has an optimal pH of 8. This optimum is typical of the cockroach midgut, however, regurgitation of gastrointestinal contents from the midgut into the crop with a pH of 5 inactivates lipase activity. Carbohydrase activity in insects is estimated to include up to 30 different enzymes, each specific for a carbohydrate substrate (House, 1965). This estimate arguably may include enzymes that cleave specific glucosidic bonds rather than specific substrates, falsely inflating the number of carbohydrases in insects. Despite years of investigation, it is also unclear where specific enzymes occur in the cockroach digestive tract (Barnes, 1980). Due to the diversity of cockroaches, and insects in general, the location and activity of digestive enzymes vary. The initial goal of the purposed laboratory is to attempt localization of digestive enzymes to specific digestive organs, and to develop general conclusions about their similarities to the functions of the mammalian digestive system. This approach to investigating the physiology of digestion not only demonstrates the application of lecture material to practical research endeavors in a controlled environment, but also provides an opportunity for independent research at the undergraduate level.

METHODOLOGY

Investigation of the function of the cockroach digestive tract can be anatomically divided into three regions (House, 1965; Barnes, 1980; Gordon, 1996), the foregut (stomodaeum), the midgut (mesenteron), and the hind gut (proctodeum). The foregut includes the mouth, esophagus, crop, and proventriculus, whereas the midgut includes the gastric ceca and ventriculus. The hindgut consists of the intestine, rectum, and anus. Anatomical sites designating the junctions between the three regions include the proventriculus dividing the foregut and midgut, and pylorus with diverticulating malpighian tubules dividing the midgut from the hindgut. Because entomology is under-represented in most undergraduate curricula, students must first become familiar with the organization of the cockroach’s digestive tract prior to dissection (see Figure 1).

Cockroaches can be anesthetized using an ether or carbon dioxide chamber. Once completely anesthetized, scissors’ points are placed between the junction between the third and second to last tergites. Tergites are the dorsal plates of the chitinous exoskeleton, whereas sternites are the ventral plates. Two incisions are made along each laterally arranged spiracles, continuing through to the thorax. Once the
tergites are freed from the underlying connective tissue they can be removed in one piece. By grabbing the head with a forceps and cutting the surrounding neck chitin, the entire digestive tract can be removed by gently lifting the head and freeing the attached tract moving caudally toward the anus. When the entire digestive tract is removed, it should immediately be submerged in a Petri dish containing a 0.9% NaCl solution.

After each segment of the digestive tract is positively identified, the segments can be sequentially removed for homogenization. Fat bodies (liver) intermingled with the remaining organs can also be removed and homogenized for assay. Beginning with the esophagus and attached salivary glands, the segments are placed in a Dounce homogenizer with 1.0 ml of 0.9% NaCl solution. The homogenized segments are poured into 1.5 ml centrifugation tubes, labeled, and spun down for five minutes. The pellet is discarded and the supernant is retained for the digestive enzyme assays given below (if time does not permit assays, the supernant can be frozen at −70 C without losing activity).

Five sets of centrifugation tubes for each nine digestive tract segments are required. Two sets of tubes will be required for protein digestive assays that consist of ninhydrin and Biuret tests. One set of tubes is necessary for a lipid assay, and two sets are needed for sucrose and starch digestion assays. The segments to be assayed are salivary gland, crop, proventriculus, ceca, ventriculus (red), ventriculus (white), intestine, rectum, and fat bodies. The ventriculus can be divided into red and white segments in Madagascar hissing cockroaches, Gromphadorhina portentosa, based on a clearly demarcated color change midway along the ventriculus.

**Assay Procedures:**

**Proteins** (Woodring and Dietz 1992):

1) Mix 100 µl of tissue solute with 100 ul of a 1.0% egg albumin solution into two sets of tubes.
2) Incubate both sets of tubes for 1.5-2.0 hours at 37C.
3) *To one set of tubes, add 200 µl of 0.1% ninhydrin, and boil for 5-10 minutes.
4) **To the second set of tubes, add 200 ul of a 10.0% NaOH solution, then add an additional 200 µl of a 1.0% CuSO4 solution.

* Ninhydrin tests positive for amino acids when purple.
** Biuret tests positive for protein when blue.

**Lipids** (Tharp 1997):

1) Mix 100 µl of tissue solute with 200 ul of litmus cream* into one set of tubes.
2) Incubate for 1.5-2.0 hours at 37C.

Litmus cream tests positive for lipase when pink-pinkish blue, demonstrating a change in pH.

* Litmus cream is prepared by mixing a 0.1% aqueous litmus solution with heavy cream until a pale blue color is achieved, if too dark additional incubation time is required.

**Carbohydrates** (Welsh and Smith 1960):

1) Mix 100 µl of tissue solute to 100 µl of a 2.0% sucrose solution and place into 1 set of tubes.
2) Mix 100 µl of tissue solute to 100 µl of a 1.0% starch solution (sufficiently cooled after boiling) to the second set of tubes.
3) Incubate both sets of tubes for 1.5-2.0 hours at 37C.
4) To the sucrose set of tubes add 4 drops of water, one of Fehling A solution*, one of Fehling B solution**, and then boil for 5-10 minutes.
5) To the starch set of tubes add two drops of iodine solution.

Sucrose digestion by invertase is indicated by an orange precipitate. Starch digestion by amylase is indicated by a brown color and lack of purple precipitation.

*Fehlig A solution: Copper sulfate, 17.3 grams, in 250 ml of distilled water.
**Fehlig B solution: Sodium potassium tartrate (Rochelle salts), 86.5 grams, in 125 ml of distilled water.

**Controls:**

1. Ninhydrin assay: An aqueous solution of glycine mixed with the ninhydrin solution
2. Biuret assay: The egg albumin solution mixed with the 10.0% NaOH and 1.0% CuSO4 solutions.
3. Lipid assay: Litmus cream solution diluted with distilled water to replace the tissue solute.
4. Sucrose digestion assay: Replacement of tissue solute with a glucose solution.
5. Starch digestion assay: Substitution of distilled water for tissue solute.

**RESULTS**

Overall, the data collected from students reflect general trends in insect digestion (Table 1). Statistical contrasts of data collected over a two-year period tested the following null hypothesizes using a Chi square test ($\chi^2$):

1) Digestion of protein, sucrose, and starch did not occur, therefore representative polymers were present in each gastrointestinal segment.
2) Protein and lipid digestion occurred in all gastrointestinal segments, which is indicated by the presence of amino acids and pH change, respectively.
Both null hypotheses were rejected, with the most striking contrast occurring between tests for amino acid and protein in the salivary gland, proventriculus, rectum, and fat bodies. Each of the mentioned gastrointestinal segments demonstrated a high incidence of protein and low incidence of amino acids in tested samples. This indicates little to no protein digestion occurred in these segments, however, the crop, ceca, ventriculus (red and white), and intestine equally exhibited both amino acids and proteins. This latter trend indicates active protein digestion in those segments.

Similar to mammalian salivary glands, those in the cockroach begin the process of carbohydrate digestion. Unlike mammals, the salivary glands of the cockroach appear to also have lipase activity. This phenomenon is not uncommon amongst many insect families, as well as amongst different species within insect families (House 1965). The ceca and ventriculus demonstrate the pancreatic ability described above, contributing to the break down of protein, lipids, and carbohydrates. Significant histological features have not as yet been identified to explain the color difference between the red and white regions of the ventriculus. Although trials that are more experimental are required, the present trend suggests a tapering in enzymatic digestion in the white segment of the ventriculus that continues with the proceeding intestinal and rectal segments. This is indicated by the declining activity noted in the amino acid, lipid, sucrose, and starch assays (Table 1).

Table 1. Summary of results from 23 cockroach (Madagascar hissing cockroach, Gromphadorhina portentosa) experiments performed over a two-year period. The ninhydrin and Biuret tests were used to detect the presence of amino acids and protein, respectively. Lipid, sucrose, and starch digestion were detected via pH change, precipitation of monosaccharides, and color change, respectively. Chi square test \((\chi^2_{0.05,8})\) performed on data reject null hypothesizes: 1) Digestion of protein, sucrose, and starch did not occur, and 2) Protein and lipid digestion occurred in all gastrointestinal segments.

<table>
<thead>
<tr>
<th>Region</th>
<th>Presence</th>
<th>Protein</th>
<th>Digestion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amino acid</td>
<td>Protein</td>
<td>Lipid</td>
</tr>
<tr>
<td>Salivary Gland</td>
<td>0</td>
<td>21</td>
<td>11</td>
</tr>
<tr>
<td>Crop</td>
<td>16</td>
<td>16</td>
<td>14</td>
</tr>
<tr>
<td>Proventriculus</td>
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<td>17</td>
<td>7</td>
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<tr>
<td>Ceca</td>
<td>14</td>
<td>18</td>
<td>9</td>
</tr>
<tr>
<td>Ventriculus (red)</td>
<td>19</td>
<td>14</td>
<td>11</td>
</tr>
<tr>
<td>Ventriculus (white)</td>
<td>17</td>
<td>20</td>
<td>11</td>
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<tr>
<td>Intestine</td>
<td>10</td>
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<td>2</td>
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<tr>
<td>Rectum</td>
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<td>13</td>
<td>6</td>
</tr>
<tr>
<td>Fat Body</td>
<td>5</td>
<td>15</td>
<td>8</td>
</tr>
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Further contrast amongst carbohydrates and its monomers is necessary before definitive statements about carbohydrate digestion can be made. Future laboratory experiments will include Benedict’s test on aliquots taken from the starch homogenates. This will allow contrast between starch and its monomer glucose, in a similar fashion to that made between amino acid and protein assays above. Since the lipase activity is indicated by a pH change, direct assay of the breakdown of lipid into glycerol and fatty acids is not practical, nor cost effective.

Pooling of data from students enrolled in the physiology laboratory introduces a certain degree of error into the cockroach digestive laboratory. Student’s dissection skills, ability to aliquot samples, laboratory experience, and degree of ongoing socializing all contribute to error in reporting precise results. Likewise, the cockroach’s digestive physiology also contributes to this error. Chyme from the ventriculus can be regurgitated through the proventriculus into the crop (House 1965, Barnes 1980). Regurgitated chyme and its accompanying ventricular enzymes stored in the crop not only permits more time for digestion to take place, but may also account for the digestive activity demonstrated by the assays performed on the crop. Variability of digestive activity in the crop, as well as other organs, may be accounted for by the students thoroughness in washing away potentially regurgitated chyme, reproductive status of the roach, or the roach’s diet. Enzymatic activity along insect digestive tracts has been noted to change in response to altered metabolic demands of gamete production, as well as substrate availability based on the composition of the roach’s current food.

CONCLUSION
The digestive protocol discussed above was incorporated into the physiology laboratory (Bio 464) at the University of Detroit Mercy for the last two years. Comparison of grades from students
participating in the cockroach laboratory was on average slightly higher than those of students participating in the traditional digestive laboratory or not enrolled in the laboratory. The slight difference in students’ percentage grades on lecture exams made the difference between a B and B- for overall class averages for the research-based laboratory student group and traditional laboratory/ no laboratory student group, respectively. This trend, although not statistically significant, may be attributed to the additional effort required of students to search the library and internet for information on insect and mammalian digestive physiology necessary for comparative analysis for their laboratory reports. The guideline for the students’ laboratory reports is taken from the American Journal of Physiology’s author’s guide. Exposure to research-based laboratories and report format has not only provided an impetus for undergraduate students to develop independent research projects, but has also aided our graduates in their professional pursuits. Anecdotes from graduates pursuing careers in medicine, dentistry, and research claim that this format prepared them on a professional level for the research expectations required of them. The trend toward research-based laboratory is not only replacing the traditional demonstrations of systemic physiology, but offering a variety of opportunities for students that is limited only by ingenuity.

QUESTIONS TO ASK:
1. How does the pH of the digestive tract’s regional contents vary between humans and cockroaches?
2. What does the variety of digestive enzymes discovered indicate about the diet of the cockroach species studied?
3. Are there similarities in the sequence of enzymatic activity along the digestive tract between humans and cockroaches?
4. Is there a correlation of the functions of the mammalian GI tract organs and glands with those of the cockroach?

FURTHER RESEARCH:
1. Determination of different pH optima of localized enzymes or homogenates.
2. Contrast homogenate enzymatic activity with gut content (chyme) activity.
3. Tract motility with carmine or carbon powder, altering it with various parasympathetic/sympathetic agonists and antagonists to time passage rate.
4. Starved versus fed (altering diet content) contrast.

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