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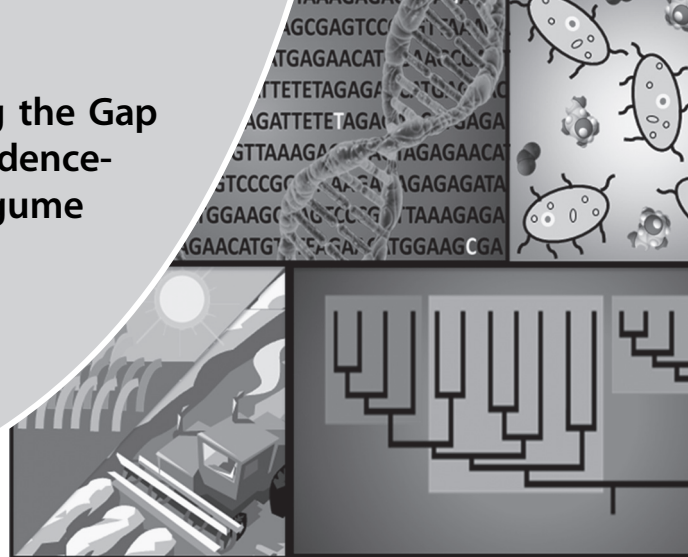
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Nitrogen Fixation: Fixing the Gap between Concept- & Evidence-Based Learning with Legume Biology

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ABSTRACT

Students often struggle to connect concepts with evidence, sometimes because development of research skills has not been emphasized in their science courses. We developed a strategy and protocol to train high school students in research and experimental investigation of questions related to course material on legume biology. The richness of this subject matter allows for adaptations of our framework to address diverse areas of science, including principles in ecology, environmental science, agriculture, microbiology, and evolution. Our framework includes a hands-on classroom inquiry that investigates the symbiotic relationship between nitrogen-fixing rhizobial bacteria and legumes. This student-led, inquiry-based project employs an intellectually demanding, hands-on method of education to build critical research skills using an adaptable model and inexpensive materials. We also report positive student feedback from a post hoc survey to gauge student attitudes toward the activity and the effectiveness of this framework.

Key Words: Legumes; nitrogen cycle; data collection; planting experiment.

○ Introduction

Although a ubiquitous topic in introductory biology and environmental science, the nitrogen cycle is not easily observed by students tasked to understand its importance as a biogeochemical pattern and its implications for plant growth. Generating engaging, student-led, inquiry-based lesson plans involving the nitrogen cycle is difficult and, therefore, students generally learn the nitrogen cycle through memorization of the flow of nitrogen in different forms through the atmosphere, soil, microorganisms, and plants. Later, students may separately learn how nitrogen is an essential component of amino acids, the building blocks of proteins, and is required for all life. Few hands-on activities are available to high school teachers that allow students to engage in this important cornerstone of environmental biology, yet we know that active-learning strategies increase student learning and long-term retention (Ausubel, 2000; Marshall, 2013; Almeida-Gomes et al., 2016). This is why it is an ideal core topic for piloting an inquiry-based lesson plan.

To bridge the gap between concept and evidence, we designed an experiment in which students investigate the implications of the nitrogen cycle for plant growth. Specifically, we use legumes as an effective tool to study the nitrogen cycle because legumes associate with nitrogen-fixing bacteria in a mutualistic symbiotic relationship, which gives them a unique and key role in this cycle (see Supplemental Material S1). While certain species of legumes are important crop plants, legumes are also found in all terrestrial ecosystems, which allows legumes to easily serve as tools to investigate principles in ecology, physiology, environmental science, agriculture, microbiology, and evolution (Nap & Bisseling, 1990; Liebman et al., 2011; Mulalelem et al., 2016; Krieg et al., 2017; Saha et al., 2017).

Here, we share our student-led, inquiry-based lesson plan with detailed instruction (below and in Supplemental Material S2–S6), provide student feedback (Figure 4 and Supplemental Material S7), and offer suggestions for implementing the activity and adapting it to a diverse set of topics. Students build critical research skills while simultaneously learning about the nitrogen cycle through our hands-on, intellectually demanding lesson. Similar to other studies, our framework utilizes legume biology and inexpensive materials as adaptable, widely applicable tools (Hughes, 1994). However, unlike many inquiry-based lesson plans available on the topic, our framework encourages student-generated hypotheses, as opposed to students participating in inquiry-based lessons with a priori hypotheses. We emphasize development of a student's ability to make meaningful observations, think critically about experimental results, and draw evidence-based conclusions.

○ Objectives

This extended laboratory experiment connects the theme of “Science Is a Process” from the College Board’s unifying constructs for AP Environmental Science with the topic of the biogeochemical cycle of nitrogen from the course outline. Our objectives in this lesson were threefold. Using an inquiry-based lesson that guided students

to discovery, we aimed to build research skills, improve scientific literacy, and create an understanding of the often difficult-to-grasp concept of the nitrogen cycle. These objectives were achieved through an introductory lecture followed by a long-term research experiment using legumes in a high school AP Environmental Science class.

○ Conceptual Preparation

The introductory lecture about basic legume biology formed a foundation of common, shared knowledge that we feel is imperative to the success of active-learning strategies. Background reading may be assigned as an alternative to the introductory lecture (e.g., textbook passages or the background document we provide; see Supplemental Material S1). Instructors should adapt the introductory material on the basic role of legumes to the context of their class subject (e.g., evolution, agriculture, microbiology). We provided our class an overview of biotic and abiotic players in the nitrogen cycle, then focused on how plants and legumes specifically might play a different role from other plants in the nitrogen cycle. Our lecture introduced students to common legumes, such as the crop plants *Phaseolus vulgaris* (common bean) and *Cajanus cajan* (pigeon pea, which is common in many Hispanic, African, and South Asian communities) and the common weeds *Medicago polymorpha* and *M. lupulina* (a table of common legumes, suited to various climates and cultural contexts, is provided in Supplemental Material S1). In this lecture, we also outlined the constraints that students would have in designing their experiment – mainly time frame and available resources (e.g., scissors, buckets to collect soil, trowels or shovels, plastic bags, Sharpies, and tape for labeling).

Having established an intentionally incomplete but basic understanding of legume biology, we guided small-group discussions to develop research questions and hypotheses surrounding plant growth, soil type, soil treatments, and nitrogen levels. Our role here was to remind students that their hypotheses needed to be testable within the available time frame and using only the available supplies/materials.

By brainstorming research questions and hypotheses in small groups, students learn valuable communication skills and acceptance of other viewpoints that differ from their own (Hess, 2009). This process also fosters critical-thinking skills as students discuss how to connect the appropriate research method to address their specific research questions (Driver et al., 1994). Further, students improve their ability to explain their thoughts and support their ideas with reason when they must communicate their ideas to others (Osborne, 2010). The role of the instructor during this process is not to implant research ideas, but to make sure that students' hypotheses are testable hypotheses that require reasonable research methods. The instructor can help students decide on reasonable hypotheses by asking follow-up questions and reminding students of the time constraints and project supplies. The discussion was concluded by bringing the whole class together to share their hypotheses and research approaches. Together, students agreed upon select hypotheses and research methods to move forward with as a class. Below is a list of the key hypotheses and their connections to class content areas, listed as "Discovery," each supported by "Evidence" from the experiment. Throughout the process – from development of hypotheses to careful scientific observations to interpretation of their

experimental results – students were encouraged to discover deeper aspects of legume biology that were not discussed in the introductory lecture and make connections to the nitrogen cycle (see below).

○ Evidence-Based Reasoning

Key student-driven hypotheses and evidence-based connections to concepts by discovery are as follows:

- **Hypothesis 1:** Legume development is significantly affected by the presence or absence of soil bacteria: legumes that grow in sterile soils (A) have less leaf chlorophyll and (B) are smaller than plants growing in fresh soils with soil bacteria.
- **Evidence:** Data in Figure 3A, B (and Supplemental Material S5).
- **Discovery 1:** Legumes rely on symbiotic bacteria called rhizobia to convert atmospheric dinitrogen (N_2) to ammonia (NH_3), a form that the plants can use to make proteins and other biological molecules (Zahran, 1999). Chlorophyll content is commonly used as a surrogate for leaf nitrogen level because the two are highly correlated. In legumes, leaf nitrogen level is affected by rhizobial association. Thus, higher chlorophyll levels are consistent with more productive rhizobial associations.
- **Hypothesis 2:** Legume development is significantly affected by the presence or absence of soil bacteria: legumes that grow in sterile soils have fewer root nodules than plants growing in fresh soils with soil bacteria.
- **Evidence:** Data in Figure 3C (and Supplemental Material S5).
- **Discovery 2:** To establish symbiosis, rhizobia infect legume roots to form nodules that contain differentiated bacteria; then they begin the mutual exchange of beneficial substrates: the plant provides molecules produced by photosynthesis to the bacteria, while the bacteria provide nitrogen in the form of ammonia to the plant (Okazaki et al., 2013).

○ Details of Activity

Understanding how to design an experiment is an essential skill for science students. Students can have difficulty understanding how to design an experiment with the appropriate methods to effectively test their hypothesis. Following the classroom discussion, we created a summarizing PowerPoint presentation that clarified the conclusions of the previous classroom discussion and reviewed the steps in the experiment (see Supplemental Material S2). During this explanatory process, the teacher should emphasize the importance of randomization, sampling techniques, replication, and potential sources of error. In order to create a more robust experiment, we made slight changes to the students' selected research methods that reflected these concepts (it is also worth mentioning that any experiment can be expanded or reduced in size by changing the number of species, soils, and replicates, in order to fit the needs and capability of each class). We continually emphasized to students why this research design was selected and how our changes helped contribute to answering the question. During data collection, students were exposed to process-based learning by following step-by-step instructions (see Supplemental Material S2). Our general procedure in conducting the experiment was as follows.

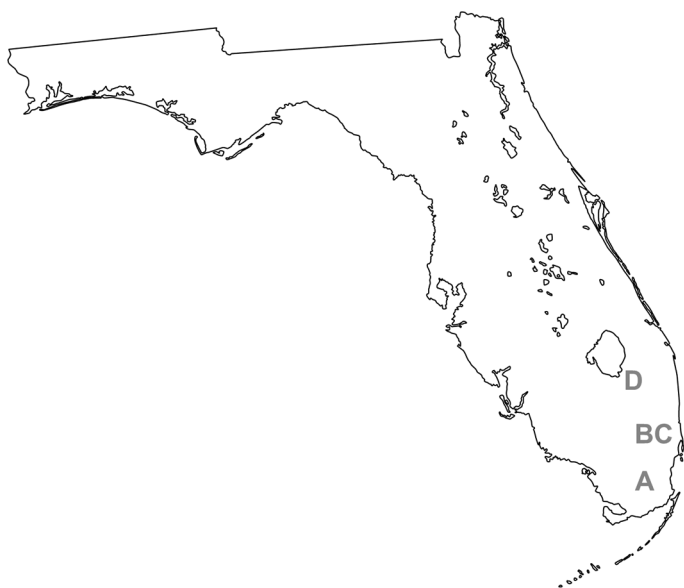


Figure 1. Soil collection sites in southwest Florida: (A) Homestead farm soil, (B) field soil near Pine Crest School, (C) lake soil near Pine Crest School, and (D) Canal Point USDA soil.

Ten gallons of soil were collected from each of four sites in south Florida, on the criteria of proximity and soil type: the Redlands agricultural area, the Canal Point sugarcane area, a lakeshore near the school, and a field near the school (Figure 1). To contrast the effects of soil bacteria on plant traits, half of the 10 gallons of soil from each site was sterilized by autoclaving, killing all soil microbes. Soil collection and preparation was completed by laboratory assistants and volunteers from a nearby university (Florida International University in Miami). The project could be adapted so that students collect soil on their own from backyards, farms, or natural areas; the goal is to capture soils different in soil bacteria, and choosing different soils from different habitats is the most likely way to achieve this. By comparing plant growth and rhizobial association on soils from different sites, one can remove the need to autoclave soil if no autoclave is available. However, when available, we feel this is an excellent opportunity to teach aseptic technique. Alternatively, simple but effective techniques have been developed using a household microwave (Ferriss, 1984; Eichenberger, 1991; Nelson and Trabelsi, 2016).

In the classroom discussions, students identified several common legumes and then chose five leguminous species to be planted in each of the four soil types. We ended up selecting legumes that perform well in our subtropical conditions and are popular food crops with our large Hispanic community. However, in more temperate climates, plants such as garden peas, soybeans, or peanuts may be more suitable (see Supplemental Material S1). Students potted and planted each plant species in each soil type in 160 mL cone-shaped containers (Cone-tainers; SC10-R, Stuewe & Sons, Tangent, Oregon). Cone-tainers are not essential; pots or other containers could be used instead.

Pre-sterilized Cone-tainers were filled with soil and gently compacted to within 1 inch of the Cone-tainer top. Two seeds of the same type were planted less than 1 inch from the soil surface and gently covered with soil. Students should be instructed to not plant seeds too deep in the soil column, which would reduce germination

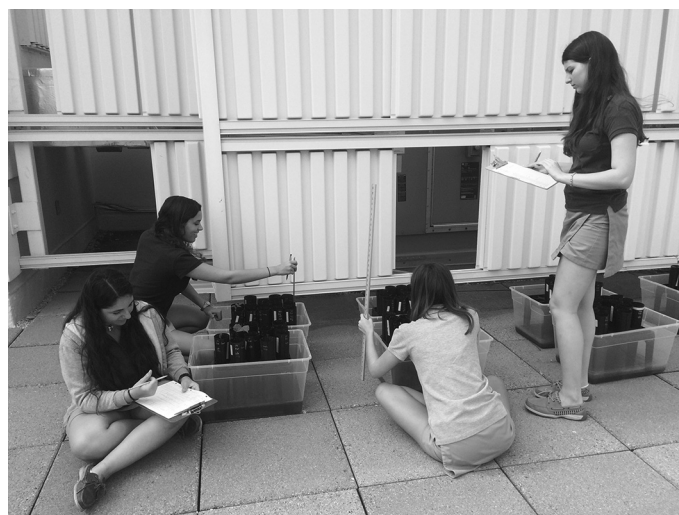


Figure 2. Image of students and experimental design.

greatly. This is particularly true with small-seeded species, such as weeds like *Medicago lupulina* or clovers like *Trifolium repens*.

Seeds of each plant species were planted with five replicates in each type of soil: five legume species $\times$ five replicates $\times$ eight soil types (half sterilized and the other half unsterilized) = 200 Cone-tainers (Figure 2). Cone-tainers were arranged within Cone-tainer racks (RL98, Stuewe & Sons). The Cone-tainers were arranged in several large plastic tubs with a few inches of water. Each tub contained Cone-tainers with a single soil type (and sterility status) to avoid cross-contamination of soils from different areas or different sterility. Each tub contained all plant species: five Cone-tainers of each legume species. Thus, each plastic tub contained 25 Cone-tainers, which in total contained all plant species, randomly assorted in the tub, with the same soil type (Figure 2).

Students watered plants daily until seedlings developed, after which plants were watered weekly. The plastic tubs containing the plants were placed in a semi-shaded area, in ambient temperature and light.

Students determined the effects of soil type and bacterial symbionts on plant development and functional traits by measuring stem height, number of leaves, flower or pod formation, and chlorophyll content (SPAD502 chlorophyll meter, Konica Minolta, Japan), every two weeks for the duration of the experiment (see Supplemental Material S3). We allowed the plants to grow for five months, although shorter durations of just a few weeks will work. Fully developed nodules can usually be found on legumes by two to three weeks of age.

Root length, stem length, and fresh weight of the plant were also recorded on fully mature plants at harvest (see Supplemental Material S4). The time to maturation varied among species and soil types, but plants generally matured about 16 weeks after planting. This works well with the moderate winters of south Florida and the large legumes like pigeon pea. However, nodules typically develop in two to three weeks, and some legumes with short life spans, such as black medic (*Medicago lupulina*, a common temperate forage crop and weed), can mature in five to six weeks.

Root nodules were counted, then removed for storage on a drying agent (Drierite) and refrigerated at 4°C. This allows downstream analysis of the nodules, for teachers who want to train students to

grow bacteria on Petri dishes (see the American Society of Microbiologists web page for K–12 lesson plans at <https://www.asm.org>).

As a suggested adaptation, students could measure nitrogen levels in the soil prior to planting and again after harvesting the legumes to measure changes in nitrogen levels for each legume species in each soil type.

Data organization and analysis is an important part of research and is therefore emphasized in our learning strategy. Data can be collected initially on paper or the provided worksheet (Supplemental Material S3 and S4), then sent as a Microsoft Excel spreadsheet to the instructor for final aggregation. We recommend assigning basic data-analysis tasks such as averages, minimum and maximum, and standard deviation (see, e.g., Supplemental Material S5). Simple graphs displaying the means between soil treatments can be easily made in Excel (e.g., Figure 3).

If the class is capable, basic statistical analyses to determine whether the relationship between the growth of the legume and the proxies used for nitrogen levels (chlorophyll levels and number of nodules) was statistically significant can be performed in Excel or with free software programs such as the R statistical environment or MYSTAT. If nitrogen levels of the soil were measured pre-planting and again post-harvest, students could also look at their statistical correlation with plant growth. We have included example data for any data analysis exercise (see Supplemental Material S5). While we do not provide further statistical advice beyond basic summary statistics (see, e.g., Supplemental Material S6), we encourage instructors to explore statistical skills with their classrooms more than we did. One strategy may be to share these data with instructors of mathematics courses to integrate this work across classrooms.

True to a student-centered learning environment, we administered a post-activity survey to each student to assess student attitudes, learning effectiveness, and self-described skill acquisition (Figure 4 and Supplemental Material S5). When implementing this lesson plan, we suggest administering a pre- and post-lesson survey and assessment to better determine the effects of this lesson plan.

○ Assessments

One of the strengths of this framework is the emphasis on furthering a student's ability to develop testable hypotheses, make meaningful observations, think critically about experimental results, and draw significant conclusions supported by evidence. Because one of our goals is to build research skills and scientific literacy in high school students, we suggest finalizing the project with exercises in scientific writing in which students explain a question of interest, create a formal hypothesis, describe a method to test that hypothesis, interpret their results, and ask further questions. This formative assessment integrates several learning goals and evaluates the student's comprehension of conceptual knowledge and the integration of practical approaches to initiate, design, execute, and report on research. The ability to think critically and link conceptual ideas with practical methods and the evidence obtained was the backbone of our teaching framework. Therefore, we recommend that the ultimate goal should be a student-written scientific paper (we think a great goal would be to encourage motivated students to pursue publishing their work in a high school or undergraduate science journal).

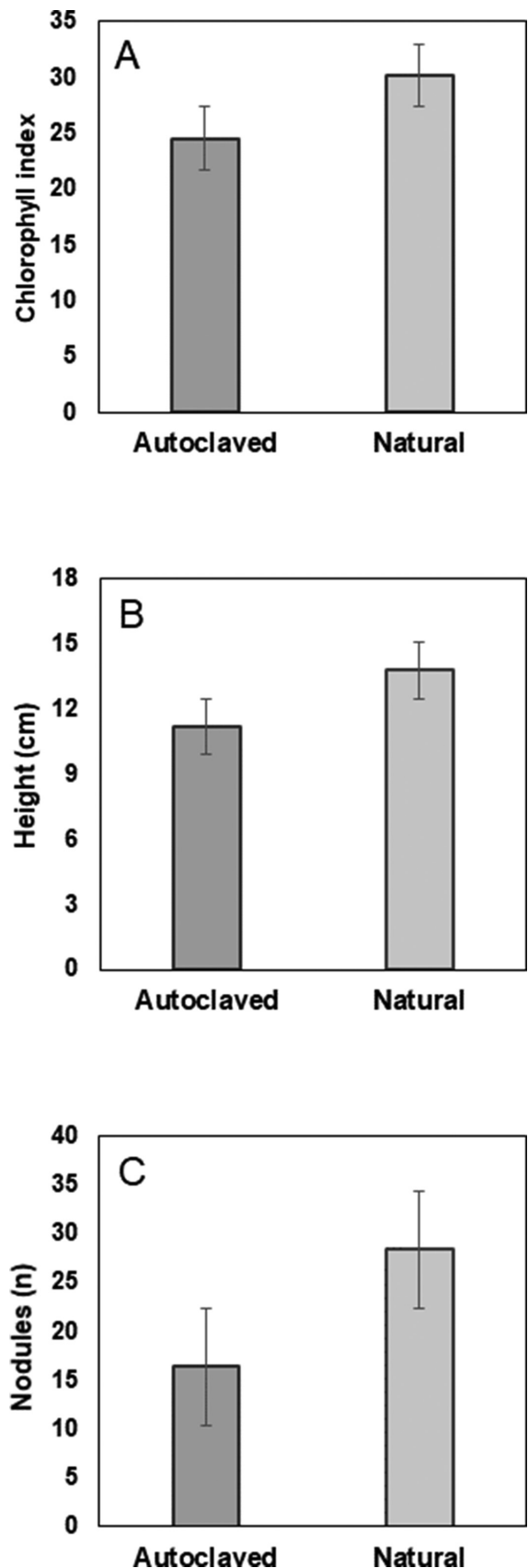


Figure 3. Results (means $\pm$ SE) from the class experiment, showing plant responses to soil treatment (autoclaved = sterile soil, natural = nonsterile soil): (A) chlorophyll index, (B) plant height, and (C) number of nodules.

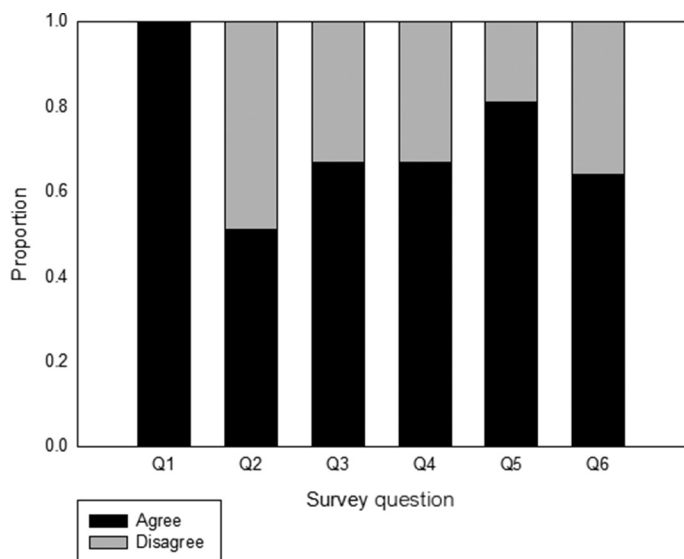


Figure 4. Student survey results. Students were asked to reflect on six statements aimed to gauge their outcomes and attitudes: (Q1) I participated in the experiment. (Q2) The experiment was helpful in understanding the course material. (Q3) I learned new skills like data collection and organization. (Q4) I found this project more engaging than a lecture. (Q5) I think hands-on projects like this are effective and I learned a lot. (Q6) I would like to do something like this again.

○ Conclusions

This activity is readily performed in a variety of classroom settings, and instructors may choose a different subject of focus, such as evolution, agriculture, or microbiology, while still utilizing some of our materials and our general pedagogical framework. In our classroom, students felt more invested in projects involving their own ideas, and a greater sense of accomplishment when the project was concluded (Figure 4). Our student survey revealed that the majority of students found this activity beneficial and reported acquisition of new skills like data organization and experimentation (Figure 4). However, the survey may also demonstrate a well-known bias in the benefits of active learning. Haak et al. (2011) showed that active-learning activities benefit lower-performing students significantly more than high-performing students. This may explain why some students did not perceive this activity as superior to lecture methods.

○ List of Supplemental Material with the Online Version of This Article

- S1: Background information on legume biology
- S2: Harvesting and measuring instructions
- S3: Bi-weekly data sheet
- S4: Final harvest data sheet
- S5: Example data
- S6: Statistical data analysis
- S7: Student survey

○ Acknowledgments

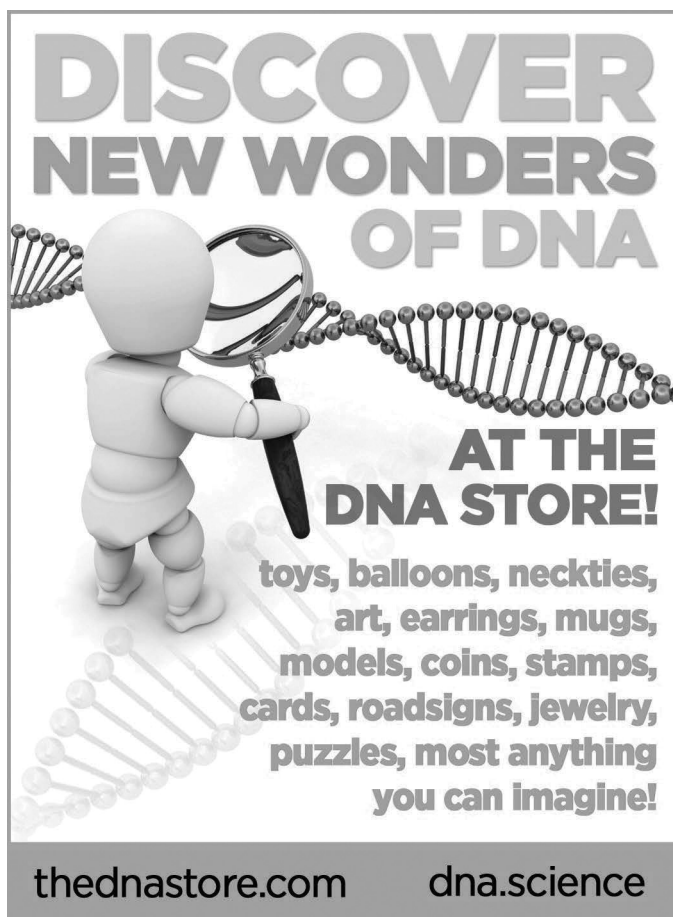
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