CBE—Life Sciences Education
Vol. 6, 332–342, Winter 2007

Article

A Semester-long Student-directed Research Project Involving Enzyme Immunoassay: Appropriate for Immunology, Endocrinology, or Neuroscience Courses

Sharon Ramos Goyette and Jane DeLuca

Biology Department and Neuroscience Program, Stonehill College, Easton, MA 02357-1135

Submitted January 5, 2007; Revised June 20, 2007; Accepted July 10, 2007
Monitoring Editor: Deborah Allen

The following project aimed at promoting integrated and long-lasting learning is described for an Immunology course, but it may be adapted to other disciplines. Students were asked to develop and carry out a research project to examine the relationship between immune function and stress. The experiments were required to include the assessment of salivary cortisol and salivary IgA (sIgA) with enzyme immunoassays. All other aspects of the experiments were developed by student groups with appropriate guidance from the instructor. Data are presented for one group project that assessed the effect of music on cortisol and sIgA. Overall levels of sIgA and cortisol were consistent with reported values. Students found a significant decrease in cortisol over time. Additionally, there was a trend that supported the overall student hypothesis regarding the effect of stress and immune function. Compared with the same Immunology course that included an instructor-designed experiment using enzyme immunoassays for cortisol and sIgA, several assessments (e.g., final grades and comments on student evaluations) show that overall learning seemed to be much better in the course with the student-directed research project.

INTRODUCTION

The primary goals of the course in which this approach was developed were to help students 1) gain a deep understanding of immunology and 2) develop an appreciation for the process of scientific research. To achieve these goals, the following objectives were established: help students take ownership of knowledge, refine critical-thinking skills, increase scientific literacy, experience the ambiguity inherent in science, and relate science to real-life and actively participate in "knowledge integration" (Linn *et al*., 2006). In part, this was accomplished by expecting students to develop and carry out a student-designed group research project. Specifically, students worked in groups to develop a hypothesis regarding the relationship between stress and immunity and to design and carry out an experiment that would test their hypothesis. The only requirement of the experiment was that it must involve analyzing both salivary IgA (sIgA), an antibody, and cortisol, a stress hormone, with enzyme-immunoassays (EIAs).

DOI: 10.1187/cbe.07–01–0001
Address correspondence to: Sharon Ramos Goyette (sramosgoyette@stonehill.edu).

Course Content and Nature of EIA

The content taught in the Immunology courses focuses on cells of the immune system (e.g., leukocytes and other cells), signal transduction, and cell–cell interactions that are necessary for proper immune system function. Early topics include identification of the cells and molecules that play key roles in innate immunity. The relationship between innate and adaptive immunity serves as the foundation to discuss the development and diversity of B cells and T cells. Emphasis is placed on T-cell receptor diversity and then on the function, structure, types, and synthesis of antibodies that are produced by certain types of B cells. Additional time is spent on the genetic mechanisms that increase the specificity of antibodies, which, in the human body, are unique to the immune system. The course also highlights the mechanisms by which antibodies exert their effects, called "effector" functions, and where appropriate, the molecular biology necessary for those effector functions to take place.

Much of the course, therefore, emphasizes the details of the basic molecular, genetic, and cellular aspects of the immune system. That approach is balanced with discussion of broader topics weaved throughout the semester to provide students with an understanding of how the immune system functions as a whole. These topics include autoimmune disease, the possible link between lack of parasites and allergies in developed countries, and the effects of stress on the immune system. The laboratory component of the class consists of several standard 3-h labs and a semester-long research project addressing the relationship between stress and immune function. Combining traditional labs with an open-ended lab project has been shown to be an effective pedagogical approach (Sadler 2004; Halme *et al*., 2006).

The project requires students to use EIAs. These assays are used for several reasons. First, they provide an excellent opportunity to teach certain aspects of immunology, including antibody isotype, subtype, distribution, and function. In healthy individuals, antibodies are immunoglobulins (Igs) that bind noncovalently to certain aspects of nonself molecules (antigens) and elicit a series of events aimed at the recognition and removal of that nonself entity. The recognition of nonself molecules and the "effector" function are carried out by two different regions of the immunoglobulin. The portion of the immunoglobulin that binds the nonself molecule is labeled the "variable region" because it varies according to the particular characteristics of the nonself molecule and stage of infection. The second part of the immunoglobulin may be one of five types or isotypes that are defined by the "constant regions." The constant region denotes the conformation of the immunoglobulin and, therefore, in part, predicts the localization of the isotype (e.g., blood vs. saliva) and determines which effector functions occur after the antibody has become bound to a nonself molecule. For example, IgA is an antibody isotype that plays an important role in immune defense of the tissues that are in contact with the external environment, such as the mouth, gastrointestinal tract, and nose. In that role, IgA is found as a monomer. The constant region of IgA also makes it possible for it to form a dimer. As a dimer IgA may be secreted outside the body (sIgA), and it is found in saliva, sweat, and milk.

In the application used in the Immunology class, sIgA was measured in saliva. A different antibody isotype, IgG, which is small and flexible, is the antibody that is most abundant in the blood. IgG tends to be the isotype that is used in research applications, including immunoassays, partly because it is easily harvested from blood. Explanation of the EIA technique itself, which in this context involves sIgA and IgG, provides an opportunity to underscore several concepts integral to the course, including isotypes, localization, and functions of antibodies. Second, EIAs are appropriate for an undergraduate laboratory course. Kits are easy to use and are commercially available for several hormones and molecules present in saliva. Saliva can be collected easily from student "participants" via passive drool into small tubes. Measuring sIgA levels establishes at least one dependent variable. Levels of cortisol may be used as a dependent variable or as a type of control establishing a baseline, depending on how the students design the experiment. Either way, knowing that two biological indicators will be assessed frees students to think about how stress might affect immune function. A host of student-generated hypotheses can be tested with this simple and straightforward technique.

Relationship between Course Content and Stress and Immunity

The relationship between stress and immunity was chosen as the research topic for three reasons. First, this topic provides ample opportunity to discuss the immune system as a whole. For example, in class, we may discuss that the synthesis of a chemokine, interleukin (IL)-2, necessary for the proliferation of T-cells is inhibited by cortisol, a hormone released during stress (Northrop *et al*., 1992). Consequently, long-term exposure to cortisol can actually diminish the size of the thymus (the anatomical structure of the immune system where T-cells develop) (described in Sapolsky, 1999). This also provides an opportunity to further distinguish between cell-mediated immunity [T cell based immunity] and humoral immunity [B cell based immunity]).

Second, the relationship between stress and immunity was chosen as a topic for this course because students are familiar with the subjective experience of stress. In the course, we build on that experience and provide a biological framework for the common experience of stress. The experiment provides both a biological definition of stress as a physiological state that can be assessed, in part, in terms of cortisol levels and a biological relevance in terms of an effect on the immune system that can be assessed, in part, via sIgA levels. Coupling a common "real-life" experience with an intangible concept can help students understand the concept and increase their appreciation of science (Hobson, 2001; Lynd-Balta, 2006).

Third, stress and immune function was chosen as a topic because our understanding of this relationship continues to evolve. The traditional view is that stress has a deleterious effect on immune function. This idea has been modified by Dhabar and McEwen (1996, 1997) who have demonstrated that under moderate stress of a short duration, certain cells of the immune system are actually taken out of the circulation and brought to areas such as the skin, which are likely to be damaged during the kind of "fight-or-flight" situation under which the endocrine and immune systems evolved. The movement of these cells out of the circulation and into the tissues likely to be damaged during a stressful situation has been termed "stress-induced trafficking" (Dhabar and McEwen, 1997; McEwen and Lasley, 2004). The student projects aimed at assessing the relationship between immunity and stress are analyzed within this emerging theoretical framework. Interestingly, many studies (e.g., Pawlow and Jones, 2005, Watanuki and Kim, 2005) but not all (e.g., Ng *et al*., 2003; Park and Watanuki, 2005) detect a reciprocal relationship between cortisol and sIgA. Increases in sIgA have been observed within as little time as 0.5 h (Pawlow and Jones, 2005) to 1 h (Bishop *et al*., 2006). This increase most likely reflects changes in the recruitment of sIgA to saliva, and it does not represent de novo synthesis to a novel antigen. For excellent and accessible summaries of the multilevel, duration- and intensity-dependent dynamics between the immune system and the stress response, please see McEwen and Lasley (2004) and Sapolsky (1999).

Pedagogical Approach

Because students learn in a deeper and longer-lasting way when we shift away from thinking about learning as a fact-based passive event to a concept-based, inquiry-driven active process (Felder, 1995; Huba and Freed, 2000; Handelsman *et al*., 2004; Knight and Wood, 2005; Lawson, 2006), open-ended inquiry is built into this assignment. Students work in groups to generate their own hypotheses, refine a final hypothesis, then design and carry out an experiment to test it. Finally, they interpret and present their results. To carry out this project, students must reason through several ideas, predict possible outcomes, and work out confounding and unintended consequences. The assignment is designed this way because it has been shown that "meaningful and lasting learning" takes place when students "repeatedly engage in the generation and test of their own self-generated ideas" (Lawson, 2006). By asking students to answer—in fact, to figure out how to answer—their own questions, they become complicit in their own teaching (National Research Council [NRC], 2000). Throughout the project, students wrestle with a logical presentation of their findings involving iterations of "increasingly interconnected views about the phenomena" (Linn *et al*., 2006). Organizing information by making connections at multiple levels promotes longer-lasting learning (Leamnson, 1999). This process and the resultant learning that should occur are referred to as "knowledge integration" (Linn *et al*., 2006), and it produces a working conceptual framework (NRC, 2000). To do well, students have to understand not only each new concept in isolation but also how to relate these to other concepts—and not only concepts from immunology class. The conceptual framework begins with lecture, and it gets worked through and integrated into the students' existing schemas during class discussions. Early discussions involve developing hypotheses with subsequent discussions shifting to the design of the experiment. Throughout the process, students identify areas where their own knowledge is lacking (by comparing their own knowledge with that of the other students), which creates an environment that encourages them to take ownership of their own knowledge. During discussion they also apply critical-thinking skills to the suggestions of others and they identify areas where the group as a whole needs more information. They figure out how to obtain the missing pieces (which requires independence and scientific literacy) and they then integrate the new information (again, by taking ownership and thinking critically) within their working conceptual frameworks. These "metacognitive" activities in turn produce a more sophisticated conceptual framework (NRC, 2000). Finally students engage in the active process of synthesizing their results, which requires further knowledge integration and the final iteration of their conceptual framework. It is important for students who plan to continue in any field that requires a biology degree to understand the ambiguity inherent in science. During experimentation, in the interpretation of results and at several levels, it is expected that students will experience and struggle with uncertainty. This is a critical benefit of this assignment, and it is unachievable with more traditional labs.

To determine whether this approach with a student-designed project would meet the objectives outlined above and therefore enhance student learning, the outcomes of this course were compared with a similar course that substituted the student-designed project with an instructor-designed research project. Overall learning was much better in the class with the student-designed project. Described below are the specific details of the Immunology course that included the student-designed project and the data showing the benefit of this approach. Although the details below describe an Immunology course, the project can be incorporated into other courses, such as Endocrinology or Neuroscience, by changing the analytes in the final EIAs.

MATERIALS AND METHODS

Course Outline

According to Stonehill College policy, laboratory grades are part of the overall course grade and not a separate grade. The laboratory component of the course counted for 25% of the overall grade, and final individual laboratory reports were required in both courses. Laboratory reports were weighted more heavily in the course with the student-designed projects (9%) than in the course with the instructor-designed projects (5%), because students in that course played a larger role in developing and carrying out the project.

In the course with the student-designed experiment, students were told on the first day of class that they would be expected to carry out an experiment to assess the relationship between stress and the immune system. The expectation of a student-designed project is also clearly stated in the Immunology laboratory syllabus:

"This course will serve as a venue to demonstrate and integrate the knowledge, skills and other forms of expertise gathered over your college career. Toward this end, we—as a class—will design a study and perform it based on our collective hypotheses. You, the class, will derive the hypothesis and consequently the methods, under my direction. You will each be required to write a methods section, a methods protocol and work on any questionnaire, etc., that will be administered to subjects."

The first assigned reading is a chapter from *Why Zebras Don't Get Ulcers* (Sapolsky, 1999; a new edition came out in 2004 just as this approach was first being adopted). The chapter "Immunity, Stress and Disease" explains in a very accessible way the dynamic relationship between aspects of immune function and stress. Students are told that we have the facilities to analyze sIgA and cortisol levels in saliva with a technique called a competitive EIA (Figure 1) Within several weeks, two student groups were created by dividing the class of 19 into two groups consisting of 9 and 10 students each. The instructor (S.R.G.) felt that overseeing two independent research projects would be manageable.

Planning for the Overarching Goal by Emphasizing the Conceptual Framework over Technique

Before the projects can begin, the students must learn a few concepts in immunology, and they need to be exposed to several laboratory techniques. (A sample lab syllabus is provided in Table 1.) The labs begin with several so-called "cookbook" experiments to provide opportunities for students to practice or acquire skills and become introduced to new techniques in the subject area (Handelsman *et al*., 2004). First, blood typing is used to demonstrate agglutination/hemagglutination and to provide a strong "clumping" or "grainy" visual of the antibody–antigen reaction. Exercises that involve assessing the components of blood, such as examining histological samples of blood smears and running gels to separate blood proteins such as transferrin and albumin in addition to the immunoglobulins, are also carried out. These exercises lay the foundation and provide an opportunity for students to visualize several aspects of the immune system. Students also develop skills including those involved in pipetting and gel electrophoresis.

Next, students perform immunodiffusion tests that precipitate soluble antigen in large insoluble antibody–antigen complexes with the Ouchertlony and Mancini tests. This transitions the students from qualitative techniques with hemagglutination and double immunodiffusion (Ouchterlony) to a quantitative technique with radial immunodiffusion (Mancini). This is also a good time to introduce serial dilution. These tests can be carried out early in the semester when students are still in the beginning stages of developing their own experiment. All provide uncomplicated, accessible, and easily visualized results that help reinforce the concept of antibody isotype and antibody–antigen interaction. (A double and single immunodiffusion kit is available from Carolina Biological Supply [Burlington, NC; catalog no. 20-2118; Meat Adulteration Test]). Through lecture and these early labs, examples are provided to help students understand that the antibody–antigen relationship is used in many common laboratory techniques called "immunoassays," which include Western blots and various EIAs, such as the enzyme-linked immunosorbent assay (ELISA). The indispensable utility of immunoassays for research advances and in clinical and diagnostic applications provides a starting point to discuss the merits of various "detection systems." Commercially available Western blot kits are used and carried out in one or two separate lab sessions (available from Modern Biology, West Lafayette IN; catalog no. 801: Serum Proteins and the Western Press Blot and catalog no. IND-4: Development of the Immune System and the Western Press Blot). This would also be an excellent time to introduce immunochemistry if facilities allow. Discussions include the purpose of incubations, the conceptual basis of "blocking" and carrying out washes, and the importance of diminishing and measuring nonspecific binding. Finally, a commercially available "practice ELISA" is performed (Modern Biology; catalog no. IND-3: The ELISA Immunoassay).

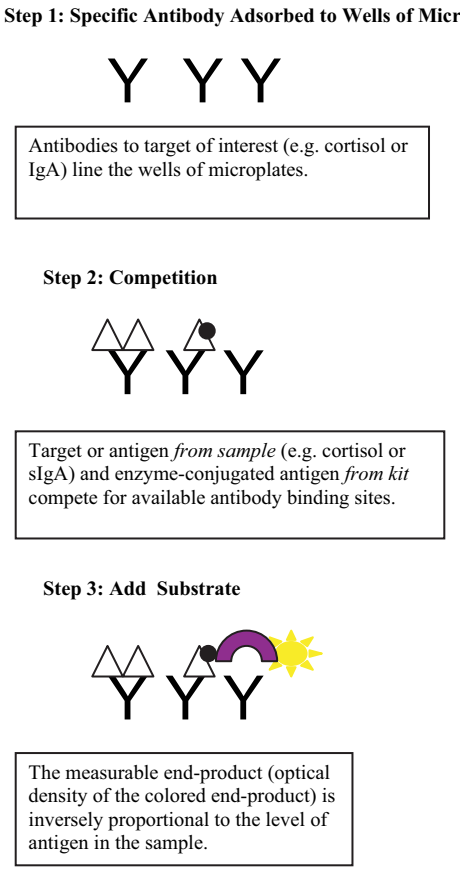

Figure 1. In the competitive EIA, the target molecule competes for available antibody binding sites with target that has been manufactured with enzyme conjugated to it. Only target molecules will bind the specific antibody, which lines the wells of the microplate (Step 1). Target molecules (from the saliva sample and from the kit) will compete for available antibody binding sites (Step 2). Substrate will only bind to enzyme. Enzyme is only present (conjugated) on the manufactured target from the kit. The substrate will be converted into a "colored" end-product (Step 3). Therefore, the amount of target in the sample is inversely proportional to the measurable "end-product." [For example, manufactured (conjugated to enzyme) target can bind high levels of antibody in the plate if there is little target in the sample to compete for the antibody.] The absorbance of the colored end-product is measured with a plate reader. Y, specific antibody; △, target, ●, enzyme; ∩☼, colored end-product.

Assessment of Student Learning

To assess whether this project enhanced student learning, overall grades, quality of laboratory reports, and year-end evaluations were compared between this course with the student-designed project and another course with an instructor-designed project. Both courses were taught by the same instructor (S.R.G.) in subsequent fall semesters. The Immunology course is one of four upper-level elective courses, considered "Capstone" courses, which are part of the overall "Cornerstone Program" at Stonehill College (for more information, please see http://www.stonehill.edu/academics/AcademicLife/Cornerstone.htm). All biology majors are required to take one Capstone course. Both courses were similar, but the instructor designed the EIA experiment (instructor-designed) in the subsequent course. In this course, students provided saliva samples at the beginning of the first laboratory session. The very beginning of the semester was considered to be a time of very low stress for returning upperclassmen. The next saliva sample was provided during the laboratory session that took place during the week in which midterms are typically given. This was considered to be a time of high stress. The hypothesis that stress, as assessed by cortisol levels, would affect immune function, as assessed by sIgA levels, was very similar to the hypotheses generated by the groups in the course with the student-directed projects. In the course with the instructor-designed experiment, the stressor was the time of year; in the student projects, high heat served as potential stressor and "calming" music as a de-stressor.

Table 1. Sample laboratory syllabus in the course with the student-directed project

Course outline	Exercise
Week 1	Cells of immune system/blood typing
Week 2	Blood typing using saliva/gel electrophoresis blood proteins
Week 3	Immunodiffusion/Western blot
Week 4	Western blot/development of the immune system
Week 5	Design studies
Week 6	Midterm exam
Week 7, write-up due	Data collection
Week 8	Data collection
Week 9	Data collection
Week 10	Practice ELISA
Week 11	EIA
Week 12	EIA
Week 13	Computer lab
Week 14	Presentations

There are two other differences between the courses. In the course with the instructor-designed experiment, no time off was needed to design an experiment or to collect data. Data were collected on the students themselves. (The hope was that this would increase student investment/interest in the outcome.) Therefore, students in the class had an additional four cookbook type labs: 1) a lab assessing the susceptibility of bacteria to various conditions, e.g., pH or antibiotics; 2) virus DNA fingerprinting; 3) amylase press blot; and 4) an additional Western blot used to identify differing constant regions of IgGs in different species. Please note that students in this group participated in more labs based on concepts covered in lecture. The final difference between the two courses exists in the nature of the final exam. In both courses, students are required to complete a 10-page research paper. In this paper, students are expected to demonstrate their ability to place a complex issue in the field of their major within a liberal arts context as part of their Capstone requirement. Paper topics have included, "HIV in Africa," "HIV in the United States," "The HPV vaccine," "Malaria and DDT," and "The Relationship between Influenza and Schizophrenia." To research their project, students use a minimum of eight primary literature resources to explore in-depth the immunology issue at hand and two secondary sources to provide the liberal arts context. All students are required to present the results of their research. To ensure attendance at the presentations and to acknowledge the work that goes into such papers, the final exam contains questions that are placed in the context of the student presentations. Below is an example of a multiple-choice question that was on the final exam in the course with the instructor-guided project: In "student x's" presentation on methicillin-resistant *Staphlococcus aureas* (MRSA), he discussed the presence of a protein secreted by MRSA strains that binds to the Fc region of circulating IgG. This would prevent what?

a. complement binding

b. opsonization

c. phagocytosis

d. all of the above

Therefore, although the question is about a very basic concept in immunology, the function of the constant region of IgG, and it is an appropriate final exam question for either course, this specific question was only asked in the course with the student-designed experiment. A direct comparison between the scores on final exams cannot be made for this reason.

Student Populations

Another component of the Cornerstone Program is a Learning Community, which ties together two courses with an integrative seminar beginning sophomore year. Several are offered, and students must take one to graduate. The integrative component of a popular Learning Community for Biology majors, Organic Chemistry of the Cell, involves analyzing primary literature (Almeida and Liotta, 2005). Ten students in the course with the student-directed research projects took the Organic Chemistry of the Cell Integrative Seminar before taking the Immunology course in their junior or senior year. The following year, a different group of 10 sophomores took another section of the Organic Chemistry of the Cell Integrative Seminar and then took the Immunology course with the instructor-designed experiment in their junior or senior year. The final grades in the Organic Chemistry of the Cell Integrative Seminar did not differ between these two groups. The final grade average in the Integrative Seminar for students who took the Immunology course with the student-directed experiment (87.25 $\pm$ 3.63) was not significantly different from that of the students who took the Immunology course with the instructor-designed experiment (85.39 $\pm$ 3.90). The student populations in each class were very similar. Of the class with the student-directed research project, five were male and 14 were female. There were 16 biology majors, two biochemistry majors, and one multidisciplinary studies major. Seventeen students were seniors and two students were juniors. In the class in which the instructor designed the experiment, three students were male and 16 were female. Two students were biochemistry majors and 16 students were biology majors. In this class, there was one student (a junior and psychology major) who withdrew from the class. With the exception of the student who withdrew, all other students in the course with the instructor-designed experiment were seniors.

Student-designed Experiments

Experimental Design Lab. Once we reach the point in the course when discussion of antibodies takes place, about 3 to 4 wk into the semester, class time and at least one lab session are devoted to helping students develop and design a feasible experiment. A portion of class time is dedicated to discussion of the relationship between stress and immune function [based on the Sapolsky (1999) reading] and this germinates the discussion of possible research questions. One lab is dedicated to experimental design. Students are expected to bring literature that supports some aspect of their working hypothesis to lab. (Informal discussion after class and throughout other labs and during office hours before this lab session greatly facilitates this step.) This lab is best carried out in a space where students have access to PubMed (www.pubmed.gov) so that they can further explore directions that may arise as they develop the actual experiment. At the end of this lab session, each group should have developed a completed protocol flow chart for their specific experiment. In this case, one group (Music) decided to investigate the effects of music on stress and immune function. The other group (Heat) decided to examine the effect of very high temperature on stress and immune function. The participants in both experiments were students in laboratory sections of the BI 101 Biological Principles course at Stonehill College, and they received extra credit for their participation. Participants gave informed consent and provided saliva via passive drool according to the directions of the manufacturer of the competitive EIA kits (Salimetrics, State College, PA). Saliva samples were kept on ice and then transferred to -20°C where they remained until the competitive EIA was run.

Music. The Music student researchers hypothesized that calming music would decrease cortisol levels and increase sIgA levels. In this experiment, participants worked on complex biology questions for ~45 min while listening to either calming music or silence. Student researchers developed a cover story that involved gathering data on freshmen biology knowledge but did not mention the "background" music or silence. Although participants were told that sIgA and cortisol would be assessed in saliva, student researchers did not divulge the hypothesis until the conclusion of the session.

Heat. The Heat student researchers hypothesized that high temperature would be a source of stress and increase cortisol levels and decrease sIgA levels. In this experiment, subjects worked on simple 550-piece puzzles while being exposed to high temperatures or a temperature of ~25°C. The puzzle was used simply as a distracter, and was not intended to have any effect on stress or immune function. Again, participants did not know the expectations of the researchers, but they were debriefed when the session ended.

Most of the data were collected at a time other than the designated laboratory period. This was anticipated, and at the outset students knew that "lab" time would be substituted for times when data collection would be optimal. Student researchers were given three labs off to collect data. The instructor felt that the time trade-off for collecting data was fair.

Analysis

The following analysis was the same for both courses. Students in both classes had already performed the "practice ELISA" (which does not include the long incubations necessary for a research grade EIA), so they had been through the steps and conceptual basis

underlying each step. However, because students performed an actual experiment and not a cookbook experiment designed to fit into 3 h, the instructor had carried out several steps involved in the EIA before the designated lab time began for the sIgA and cortisol EIA labs. Students were invited to participate during these steps. The reading of the EIA plates was planned to coincide within the last 20 min of the specific lab period.

sIgA. For this protocol (sIgA, catalog no. 1-1602 Salimetrics), on the day the EIA is to be run, the instructor thaws all saliva samples. Samples had been frozen to precipitate the mucins in saliva. Once thawed, samples are vortexed, and then centrifuged. Twenty-five microliters of clear supernatant is removed and placed into a new tube. Standards, "zeros," and nonspecific binding tubes are also prepared according to the manufacturer's instructions. Diluent is added to all tubes. Then, the antibody–enzyme conjugate is prepared and added to all tubes. The conjugate then incubates with the sample, standard, or control tubes for 90 min. The process up to this point takes ~2.5 h. The student lab is planned to begin just after this incubation. The next step in the protocol is the plating of all tubes. The instructor does the plating while students are arriving in lab. Students are given a template with the planned layout of the plate. If the actual plating is different from the template, students note this on their sheets. Another 90 min incubation ensues. During this time, a traditional prelab lecture takes place, and students are brought up to date on the steps that have already been carried out. The next step requires six washes. These washes are done manually, and each student takes a turn washing wells. (This can take ~45 min, depending on the students' abilities.) This step constitutes 2 h and 15 min of a traditional 3-h lab, and the next incubation, which is with substrate, takes 40 min. Students add the substrate. Finally, stop solution is added, and a 3-min incubation takes place. The absorbance of each well is determined using a microplate reader, which generates the raw data.

Cortisol. This protocol (Salivary Cortisol Enzyme Immunoassay kit, catalog no. 1-0102/1-0112, 96-well kit; Salimetrics) is much shorter than that for the detection of the sIgA. In this case, the instructor still thaws, vortexes, and centrifuges all samples. Samples, zeros, nonspecific binding wells, and standards are all plated. Assay diluent and then enzyme conjugate is added to all wells. This begins an hour-long incubation during which target from sample and target from the kit compete for binding sites. The lab is scheduled to begin during this incubation when a prelab lecture takes place. After this, students are given the plate layout, and they begin manually washing the wells. This is done four times. The next step is to add the substrate, and this begins a half-hour incubation. Stop solution is then added, and plates are incubated for another 3 min. The absorbance of each well is determined using a microplate reader.

ANALYSIS

EIA plates are read on an ELx808IU microplate reader (Bio-Tek Instruments, Winooski, VT). The microplate reader is purposely set up to output only raw data. Having the raw numbers available is absolutely critical in case there is any mixup of nonspecific binding wells, zeros, or samples. In that case, a new template would need to be programmed into the microplate reader, and the process of entering the new template takes valuable time. Therefore, only the raw data are output. The instructor carries out the data manipulations necessary to generate concentrations according to the manufacturer's instructions. The steps involved in transforming the data are explained during the prior "practice ELISA" lab and again at the next lab meeting. The instructor carries out this portion of the data transformation because unknown values are plotted onto a standard curve generated as a four-parameter sigmoid curve, as suggested by the EIA kit manufacturer. This is best done via software (KC Junior; Bio-Tek Instruments) designed to work with the microplate reader. Students are familiar with best-fit lines and standard curves from introductory courses. Typically, students generate standard curves that are straight "best-fit" lines drawn on graph paper with a ruler to encompass as many points derived from the standards as possible. The line is defined by the formula $y = mx + b$, where m is slope and b is the y-intercept. The four-parameter sigmoid curve is defined as $y = (A - D)/(1 - x/C^{\wedge}B) + D)$. For the purpose of this course, it is important that the students understand the concept of a standard curve; it is not important that they understand the involved calculations used to generate a 4-parameter sigmoid curve. Transformed, the data can be emailed to students, posted to a course website, or made available in another way. Hard copies of the transformed data, which include the standards, unknowns, and a plot of the four-parameter sigmoid curve derived from the data are distributed in class. The next laboratory session takes place in a computer lab so that students can apply statistics to their data. Because biology students rarely, if ever, have the opportunity to apply simple statistics to novel data, a basic tutorial on SPSS is provided at this time. Students derive the means and then perform statistics to determine whether their hypotheses are supported.

Results from Data Analysis

The data for the instructor-designed experiment was consistent with the hypothesis. There were not enough data points from the Heat group to perform meaningful statistics for both sIgA and cortisol. In several cases, there was not enough saliva to carry out both the cortisol and sIgA EIAs. In that case, the sample was subjected to the sIgA EIA. There were also fewer participants in this study overall. (These issues are addressed in *Discussion*.) The data and statistical analysis from the Music experiment are presented. Individual values that exceeded the range of detection were discarded. For sIgA analysis of the possible 44 values (22 matched data points), five values were dropped because they exceeded the highest standard concentration used in the EIA by a factor of 2, five values were dropped because they yielded "zero" scores, and three samples could not be run. This left a total of nine matched data points for sIgA baseline and treatment values. Data are presented as mean ± STE. For both groups, the average level of secreted IgA at baseline was 105.56 ± 45.6 μg/ml, and after treatment it was 183.33 ± 65.3 μg/ml. These values are consistent with manufacturer's guidelines and published data (Bishop *et al*., 2006; Strazdins *et al*., 2005) providing internal validation of the method.

The cortisol protocol and calculations are much simpler than those for sIgA. Unlike the cortisol protocol, the sIgA protocol involves multiplying all transformed data by a factor of 5. It may be for this reason that all of the cortisol values fell within the appropriate range. The mean level of cortisol for both conditions (n = 22) at baseline was 0.31 ± 0.038 μg/dl and after treatment was 0.24 ± 0.03 μg/dl. These are consistent with the manufacturer's guidelines and within the range found in the literature (e.g., Bishop *et al*., 2006; Butovskaya *et al*., 2005; Strazdins *et al*., 2005; Cahill *et al*., 2003), providing internal validation of the method. An overall repeated measures test showed that cortisol values were significantly decreased from baseline regardless of treatment condition ($F_{1,7} = 6.861$; $p < 0.05$). No other main effects were detected and no interactions (sIgA × cortisol or treatment × cortisol or treatment × sIgA) were significant. When data were treated separately as a one-way dependent *t* test or paired samples *t* test, cortisol (n = 22) levels were significantly decreased [$t(21) = 2.67$, $p < 0.01$] and sIgA (n = 9) levels showed a trend toward an increase [$t(8) = -1.609$, p = 0.073] (see Figures 2 and 3). Even though the repeated measures test showed no significant interaction of IgA and cortisol ($F_{1,7} = 2.28$; $p = 0.175$), the relationship between sIgA and cortisol is in the predicted direction. Because the number of data points available for the cortisol data were greater than that available for the sIgA data, a separate analysis of the cortisol data alone to determine whether condition significantly decreased cortisol was also done. An overall repeated measures test of the cortisol data alone based on the available matched 22 samples also showed a significant decrease in

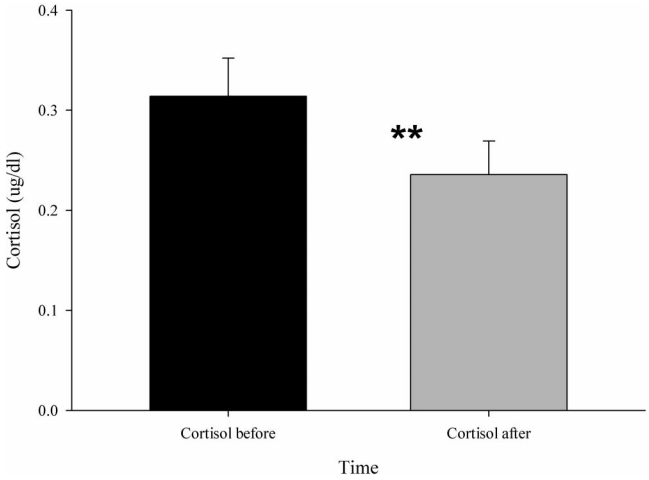

Figure 2. A paired samples *t* test shows that cortisol (n = 22) levels were significantly decreased [$t(21) = 2.67$, $p < 0.01$] from baseline (0.31 ± 0.038 μg/dl) compared with after treatment (0.24 ± 0.03 μg/dl).

cortisol levels after treatment ($F_{1,20} = 7.68$; $p < 0.05$). The interaction of treatment and cortisol, as above, was not significant.

RESULTS FOR STUDENT LEARNING

Reaching the Objectives

Although the instructor-designed experiment did generate significant results and the students seemed to understand EIA, the overall learning experience was much weaker. Figure 4 is a grade comparison showing the significantly higher overall grades achieved by students in the course with the student-designed research projects compared with the grades achieved by the students in the course with the instructor-designed experiment (Mann–Whitney *U* test; $p < 0.05$). In addition to this data, qualitative data in terms of answers to year-end evaluations are also presented to provide a more complete representation of student learning (Sundberg, 2002). In the course with the student-designed project, 9 of 19 students made highly positive comments about the project specifically or about the interdisciplinary nature of the course (Table 2). In contrast, in the class with the instructor-designed project, only 4 of 18 students wrote something positive in the provided space. A selection of questions presented below from the year-end student evaluations suggests that students worked more, enjoyed the class more, and gained a better understanding of immunology in the class with the student-directed research project than the students who participated in the instructor-designed experiment (Table 3). If the answers from the questions to the survey were plotted, it would look as though the class with the student-directed project is "right-shifted" toward the positive compared with the student responses from the class with the instructor-directed project.

Ownership. Students in the course in which they designed their own projects indicated that they spent more hours on this class than the comparison group, which may indicate an awareness that gaining this knowledge was their responsibility. An independent *t* test indicated a trend in this direction ($t = -2.010$, $p = 0.053$).

Critical-thinking Skills. The students in the course with the student-designed experiment strongly agreed that the course had enhanced their critical-thinking skills ($t = -3.488$, $p < 0.01$).

Experience the Ambiguity Inherent in Science. Two students in the course with the student-designed projects complained that the lab was "disorganized" (Table 2), which was interpreted as an indication of ambiguity.

Scientific Literacy. Year-end evaluations did not suggest any difference between these groups.

Relate Science to Real-life. Ten of the students in the class with the student-directed project strongly agreed that the course increased their interest in the subject matter com-

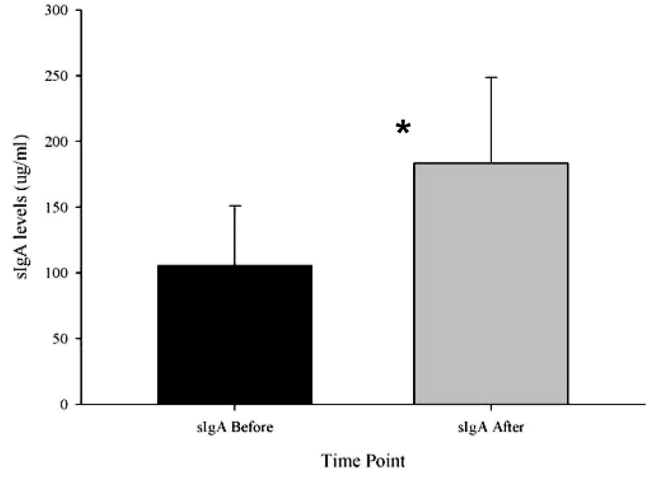

Figure 3. A paired samples *t* test revealed that sIgA (n = 9) levels showed a trend toward an increase [$t(8) = -1.609$, $p = 0.073$] from baseline (105.56 ± 45.6 μg/ml) compared with after treatment (183.33 ± 65.3 μg/ml).

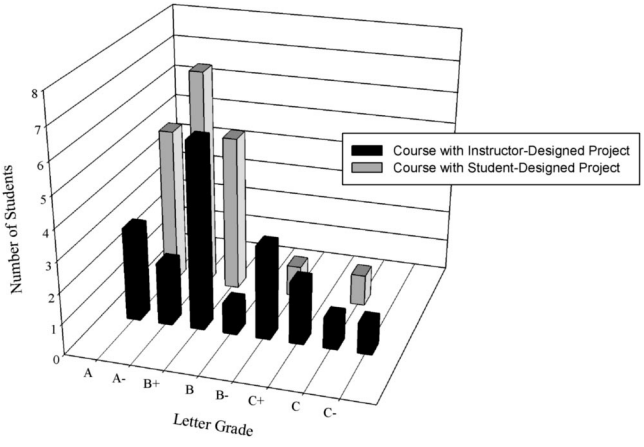

Figure 4. Overall learning was much better in the course with the student-directed experiments as assessed by final letter grade.

Table 2. Comments on student evaluations from the class with the student-directed project (of 19 responses)

Positive comments
Knowledge integration
"capstone experience really tied together multiple disciplines"
"The course material was very complex and challenging. It was also interesting."
"it made connections between previous biology courses"
"I really enjoyed this course and especially our research projects and presentations because I feel as though it increased my awareness of many issues."
Real-life application
"liked integration of cellular/molecular biology with real-life processes"
General comments
"lab was good"
"enjoyed our research projects"
"keeps things interesting and kept us involved in class"
"cortisol and IgA labs were very interesting in that we got the chance to use class/lab time to create and perform an actual experiment - not just some out of the book lab"
Negative comments
Ambiguity
"disorganized in lab"
"I liked the class but lab was very disorganized."
General comments
"if the experiment was started at the beginning of the semester because it seemed rushed with everything else"

pared with only four in the class with the instructor-directed project ($t = -2.771$, $p < 0.05$). Students in the class with the student-directed research projects were also very curious about whether additional classes would build on their work, and one student comment in year-end evaluations indicated an appreciation of the "integration of cellular/molecular biology with real-life processes" (Table 2).

Actively Participate in Knowledge Integration. Overall, students in the student-designed experiment group strongly agreed that class assignments increased their content knowledge compared with students in the instructor-designed course ($t = 2.404$, $p < 0.05$), and four students in this class made specific comments in this regard (Table 2).

DISCUSSION

Student Data

In the Heat experiment, students were able to provide context and write lab reports with their sIgA data. They were working with minimal data; but the lack of a complete data set provided students with an opportunity to understand and explain how issues associated with research can significantly impact results. In the Music experiment, there was an overall decrease in cortisol levels despite condition. The data suggest that cortisol levels decreased regardless of condition and may indicate that anxiety about participation in the experiment subsided as the experiment went on or that simply sitting for 0.5 h to 45 min is enough to lower cortisol levels for freshman biology majors. In either case, the calming music did not lower cortisol levels below that induced over time. The secondary hypothesis that levels of cortisol would affect sIgA levels was somewhat supported. There was a trend that approached significance toward an increase in sIgA levels that mirrored a decrease in cortisol levels. The finding that these data did not fully support their hypothesis provided three additional teaching opportunities: 1) the opportunity to discuss that data does not always turn out as expected; 2) the necessity to go back to the literature and rethink the experiment to explain the data; or 3) the possible result with more time to run additional subjects; in this case, it looks like cortisol/sIgA would have been shown to have a strong significant reciprocal relationship. It is the rare scientist who receives exactly the expected results, so early training in reexamining the data and design is important. This is something to which students are rarely exposed in the "cookbook" labs, in which the experiments come out pretty nearly as expected or do not work at all.

Student Learning

Ownership/Responsibility. The extent to which students took responsibility for the project varied. The fact that the Heat group could only generate enough data to perform meaningful statistics on sIgA speaks to this. A downfall of allowing the students to take a more active role in their research is that they may not. The reason(s) for the dichotomous outcome between the Heat and Music students research groups is not clear. Perhaps an "information sheet" with specifics including the volume of saliva necessary, as well as emergency contact information and supply locations, may have prevented this pitfall for the Heat group. Additionally, it seemed that the Heat researchers were unable to recruit as many participants as they would have liked, perhaps due to the time constraints imposed by student researcher availability. One student complaint was that the project was begun too late in the semester (Table 2). In contrast, both groups of students in the class with the student-designed project did seem to take greater responsibility for acquisition of his or her own knowledge compared with those in the course with the instructor-designed experiment. Year-end evaluations, lab reports, group discussions, and informal questions bore this out.

Critical-thinking Skills. The laboratory reports on the project were better in the self-directed cohort, although there was no specific rubric used to grade these reports. A simple point system for each section was given in the syllabus. The *Discussion* sections from the self-directed reports were more thoughtful and contained more references to the primary literature. The instructor-designed experiment led to the same sorts of cookbook lab reports with discussion sections that are either modifications of the introduction or contain comments that do not require much thought such as "perhaps the reagents were mixed incorrectly" and so on. When students commented on the "next experiment" in the *Discussion* sections of papers based on the instructor-designed

project they often suggested something that would build on the expected conclusion—something almost linear. In the reports on their own research, the "next experiment" was one that addressed a real question in their own research—maybe to test an assumption they did not realize they had made. This represented a real shift in how the students understand science as a process. The higher quality of lab reports and better overall grades complement the students' own appreciation that they had enhanced their critical-thinking skills as indicated on year-end evaluations.

Science Literacy. Although there is no direct data that suggest that the students in the course with the student-designed project became more proficient with scientific literacy, indirect evidence as assessed with class assignments indicates that there was a difference. In both classes, students wrote a final Capstone paper that contained primary references on a separate topic. Students with the instructor-designed experiment were still having issues with the use and acquisition of articles from PubMed and what constituted a primary research article toward the end of the semester. In the class with the student-directed research project, these issues were fewer and handled much earlier in the semester. The students in that class may have learned from each other while they were researching their original hypothesis. This difference seems to be attributable to the different nature of the two projects, with the student-designed project requiring more group interaction. As mentioned earlier, 10 students in each section had previously participated in a course that ties together concepts from organic chemistry and cell biology with primary literature; so, clearly a subset in each course was familiar with accessing and distinguishing primary sources. Perhaps students in the course with the student-designed group who had this knowledge shared it early on with other members of the group. There was no need for students to meet outside of class time in the course with the instructor-designed project. Finally, through their use of primary literature in lab reports and to develop their projects, students in the class with the student-directed research projects seemed to have developed a more sophisticated understanding of the role that primary literature plays in the scientific process.

Ambiguity. In the instructor-directed research project students dealt with ambiguity while they were considering the results. However, up until that point, it was the instructor

Table 3. Course evaluation questions for both classes

Approximately how many hours per week did you spend on this course outside of class?	Instructor-directed project	Student-directed project
1–2	3	4
3–4	12	3
5–6	3	10
7–8		2
>8		
This course was intellectually challenging and helped me develop my analytical or creative skills?[b]	**Instructor-directed project[a]**	**Student-directed project**
Strongly disagree		
Disagree	3	1
Neutral	3	
Agree	10	5
Strongly agree	2	13
The course increased my interest in the subject matter.	**Instructor-directed project[a]**	**Student-directed project[c]**
Strongly disagree		
Disagree	3	
Neutral	3	1
Agree	8	7
Strongly agree	4	10
Homework assignments and readings contributed to my understanding of course content.	**Instructor-directed project[a]**	**Student-directed project**
Strongly disagree		
Disagree	1	1
Neutral	3	1
Agree	13	7
Strongly agree	1	10

Students in both classes were asked to select a response that best describes the extent to which they agreed with each statement. Numbers in the table represent the number of students who selected each response.

[a] Indicates that the student responses between the two courses were significantly different ($p < 0.05$).

[b] A new course evaluation was used in the class with the "instructor-directed project." The exact wording of the comparison question is, "This course helped me become a more critical thinker." The other statements presented here appeared in both versions of the course evaluations.

[c] One student did not fill out a response to this statement but did answer the other questions on the evaluation.

who assumed all the ambiguity in the design and execution of the experiment. In the student-designed experiment, students experienced uncertainty throughout their projects. During each step, student researchers were faced with the reality that science can only account for a limited number of variables at any one time. Students who have not experienced an actual research project may report that they dislike the disorganized, open-ended, or ambiguous nature of this type of project (Felder and Brent, 1996; Felder, 1997). The negative comments regarding "disorganization" made by students in the course with the student-designed project were interpreted as an indication that the project did instill a sense of ambiguity.

Relating Science to Real-life. Because relating science to real-life can increase appreciation for the scientific process in general and help a student understand a specific concept (Hobson 2001; Lynd-Balta 2006), stress was chosen as the research topic in both courses. In the course with the instructor-guided experiment, the students were the participants. In that course, the students' own saliva samples were analyzed for sIgA and cortisol; therefore, one might expect that this would enhance their ability to connect Immunology to real-life. Surprisingly, general comments as well as year-end evaluations indicate that the students who designed their own experiment could more easily relate immunology to real-life compared with those in the course with the instructor-designed experiment. Again, the course comparison shows that overall learning and appreciation of the immune system was positively impacted when students had to make their own connections between the immune system and real-life by developing and testing their own hypothesis.

Actively Participate in Knowledge Integration. From the comments on student evaluations, the quality of lab reports, and the overall grade comparison, it is clear that students had a more integrated conceptual framework regarding immunology in the course with the student-designed project. This is likely due to the nature of the project, which allowed the students to practice with several iterations of their developing conceptual frameworks (NRC, 2000). It is this process that is key to overall deeper and integrated understanding (Linn *et al*., 2006). Allowing students to develop their own hypothesis, design an experiment to test it, carry it out, synthesize the results, and present the data in the context of what is known about the phenomenon requires practice with many iterations of the concept (Lawson, 2006; Linn *et al*., 2006). It encourages the students to make connections at multiple levels (Leamnson, 1999), which generates iterations of their working conceptual frameworks (NRC, 2000), and it leads to an overall deeper understanding of the topic at hand.

FUTURE DIRECTIONS

The success of this approach merits further use, but there are several ways to improve on the overall impact of this approach. Inclusion of an information sheet that would contain information appropriate for all student-designed experiments, such as the total volume of saliva necessary, may have prevented the collection of a limited amount of usable data by the Heat student researchers. A more detailed rubric for grading the final laboratory reports, including assigning points for the importance of critical analysis and the proper use of primary literature, would have provided additional guidance for the students and a more direct assessment of student proficiency in these areas. A peer-assessment tool would help ensure accountability and fairness. Finally, before adopting this approach, an instructor should appreciate that although the time commitment is approximately equivalent, the time is spent differently. Instead of spending a continuous 3 h in lab per week, time is delved out in innumerable small chunks that can be disruptive. The overall enhancement of student learning and overwhelmingly positive year-end evaluations compensate for loss of efficiency in other areas.

TRANSFERABILITY: THE BENEFIT OF THIS APPROACH AND ADAPTING IT TO OTHER COURSES

One unanticipated benefit of the student-directed research project was that several students included this research experience in their curricula vitae, applications to graduate programs and medical schools, or on resumes to gain research experiences in academic and commercial research laboratories. The success and ease of this approach make it an attractive project to incorporate into other courses with a laboratory component, such as endocrinology, neuroscience, or physiology. Commercially available EIA kits measure additional hormones and other biological factors associated with different systems. For example, in an endocrinology, neuroscience, or physiology course, a sex hormone such as estrogen or testosterone in addition to cortisol may be assessed (e.g., Do men and women differ in how stress affects cognition/vision/pain perception/exercise/etc.?). The labs that take place before the actual EIA may also be modified or deleted to make the lab component consistent with the content of the specific class. The only two labs that are integral for understanding the antibody–antigen interaction are the double immunodiffusion and radial immunodiffusion exercises, and these are simple to do and require very little time. Finally, this is an excellent option for nonmajor labs as well, because the questions have real-life relevance, and the project fosters an understanding of the scientific process.

CONCLUSION

The student-directed research project provided a much richer understanding of immunology and science in general for students as assessed indirectly with class discussions and the higher quality of student lab reports and directly with final grades, student comments, and year-end evaluations. To promote deep learning, it was essential that the students themselves derived and tested their hypotheses. The approach described above provides a straightforward and simple technique transferable to several disciplines that allows students to move through several working conceptual frameworks to measurably enhance overall learning.

ACKNOWLEDGMENTS

S.R.G. gratefully acknowledges the Faculty Leaders Program supported by the Davis Educational Foundation. The authors thank the

reviewers and Dr. Craig Almeida for insightful comments on the manuscript.

REFERENCES

Almeida, C. A., and Liotta, L. J. (2005). Organic chemistry of the cell: an interdisciplinary approach to learning with a focus on reading, analyzing and critiquing primary literature. J. Chem. Educ. *82*, 1794–1799.

Bishop, N. C., Walker, G. J., Scanlon, G. A., Richards S., and Rogers, E. (2006). Salivary IgA responses to prolonged intensive exercise following caffeine ingestion. Med. Sci. Sports Exerc. *38*, 513–519.

Butovskaya, M. L., Boyko, E. Y., Selverova, N. B., and Ermakova, I. V. (2005). The hormonal basis of reconciliation in humans. J. Physiol. Anthropol. Appl. Hum. Sci. *24*, 333–337.

Cahill, L., Gorski, L., and Le, K. (2003). Enhanced human memory consolidation with post-learning stress: interaction with the degree of arousal at encoding. Learn. Mem. *10*, 270–274.

Dhabar, F.S., and McEwen, B. S. (1996) Stress-induced enhancement of antigen-specific cell mediated immunity. J. Immunol. *156*, 2608–2615.

Dhabar, F. S., and McEwen, B. S. (1997). Acute stress enhances while chronic stress suppresses cell-mediated immunity in vivo: a potential role for leukocyte trafficking. Brain Behav. Immun. *11*, 286–306.

Felder, R. M. (1995). A longitudinal study of engineering student performance and retention. IV. Instructional methods and student responses to them. J. Eng. Educ. *84*, 361–367.

Felder, R. M. (1997). Beating the numbers game: effective teaching in large classes. http://www.succeed.ufl.edu/papers/97/01230.pdf (accessed 13 September 2006).

Felder, R. M., and Brent, R. (1996). Navigating the bumpy road to student-centered instruction. Coll. Teach. *44*, 43–47.

Halme, D. G., Khodor, J., Mitchell, R., and Walker, G. C. (2006). A small-scale concept-based laboratory component: the best of both worlds. CBE Life Sci. Educ. *5*, 41–51.

Handelsman, J. *et al*. (2004). Scientific teaching. Science *304*, 521–522.

Hobson, A. (2001). Teaching relevant science for scientific literacy. J. Coll. Sci. Teach. *30*, 238–243.

Huba, M. E., and Freed J. E. (2000). Learner-centered assessment on college campuses: shifting the focus from teaching to learning, Boston, MA: Allyn and Bacon, 35.

Knight, J. K., and Wood, W. B. (2005). Teaching more by lecturing less. Cell Biol. Educ. *4*, 298–310.

Lawson, A. E. (2006). Points of view: on the implications of neuroscience research for science teaching and learning: are there any? CBE Life Sci. Educ. *5*, 111–117.

Leamnson, R. (1999). Thinking about Teaching and Learning: Developing Habits of Learning with First Year College and University Students, Sterling, VA: Stylus Publishing.

Linn, M. C., Lee Hee-Sun, Tinker, R., Husic, F., and Chiu, J. L. (2006). Teaching and assessing knowledge in science. Science *313*, 1049–1050.

Lynd-Balta, E. (2006). Using literature and innovative assessments to ignite interest and cultivate critical thinking skills in an undergraduate neuroscience course. CBE Life Sci. Educ. *5*, 167–174.

McEwen, B. S., and Lasley, E. N. (2004). The End of Stress As We Know It, Washington, DC: Joseph Henry Press.

National Research Council (2000) How People Learn: Brain, Mind, Experience and School, Washington, DC: National Academy Press.

Ng, V., Koh, D., Mok, B. Y., Chia, S., and Lim, L. (2003). Salivary biomarkers associated with academic assessment stress among dental undergraduates. J. Dent. Educ. *67*, 1091–1094.

Northrop, J. P., Crabtree, G. R., and Mattila, P. S. (1992). Negative regulation of interleukin-2 transcription by the glucocorticoid receptor. J. Exp. Med, *175*, 637–646.

Park, M., and Watanuki, S. (2005). Specific physiological responses in women with severe primary dysmenorrhea during the menstrual cycle. J. Physiol. Anthropol. Appl. Hum. Sci. *24*, 601–609.

Pawlow, L. A., and Jones, G. E. (2005). The impact of abbreviated progressive muscle relaxation on salivary cortisol and salivary Immunoglobulin A (sIgA). Appl. Psychophysiol. Biofeedback *30*, 375–387.

Sadler, P. M. (2004). Factors influencing college science success. A briefing on a national study of undergraduates in introductory college science courses. National Academy of Sciences Workshop Investigating Introductory Science Courses in the Undergraduate Context: A Systems Approach, Washington, DC, 22–23 June 2004. http://www7.nationalacademies.org/base/Phillip_Sadler_presentation_7_12_04.pdf.

Sapolsky, R. M. (1999). Why Zebras Don't Get Ulcers: An Updated Guide to Stress, Stress-related Diseases and Coping, New York: W. H. Freeman and Company.

Strazdins, L., Meyerkort, S., Brent, V., D'Souza, R. M., Broom, D. H., and Kyd, J. M. (2005). Impact of saliva collection methods on sIgA and cortisol assays and acceptability to participants. J. Immunol. Methods *307*, 167–171.

Sundberg, M. D. (2002). Assessing student learning. Cell Biol. Educ. *1*, 11–15.

Watanuki, S., and Kim, Y. (2005). Physiological responses induced by pleasant stimuli. J. Physiol. Anthropol. Appl. Hum. Sci. *2*, 135–138.