

A laboratory exercise to illustrate increased salivary cortisol in response to three stressful conditions using competitive ELISA

Mark F. Haussmann, Carol M. Vleck, and Eugenia S. Farrar

Department of Ecology, Evolution, and Organismal Biology, Iowa State University, Ames, Iowa

Submitted 29 June 2006; accepted in final form 28 November 2006

Haussmann MF, Vleck CM, Farrar ES. A laboratory exercise to illustrate increased salivary cortisol in response to three stressful conditions using competitive ELISA. *Adv Physiol Educ* 31: 110–115, 2007; doi:10.1152/advan.00058.2006.—Perceived stress activates the hypothalamus-pituitary-adrenal axis, resulting in the release of glucocorticoids into the systemic circulation. Glucocorticoids cause the elevation of blood glucose, providing the necessary energy for the organism to cope with stress. Here, we outline a laboratory exercise that uses a competitive ELISA kit to illustrate the response of salivary cortisol concentrations to three stressful conditions. Twelve undergraduate students in the General and Comparative Endocrinology course at Iowa State University were subjected to presentation stress, fasting stress, and competition stress to determine their effect on salivary cortisol concentrations. Students had elevated salivary cortisol in response to each of these stresses compared with basal conditions. These results reiterate the importance of glucocorticoids as mediators of the stress response. This study also incorporates the use of the ELISA technique, a modern laboratory tool used to determine the amount of endogenous antigens in plasma or saliva. This laboratory exercise can easily be adapted to fit into already existing physiology and endocrinology curriculums.

endocrinology; stress response; glucocorticoid

THE DEFINITION OF STRESS has assumed a ubiquitous connotation in today's society. Stress has come to embody almost any factor that induces physiological or psychological tension. The term "stress" was introduced in 1935 by Hans Selye (28), who explained the phenomenon as nonspecific bodily changes that occurred in response to physically harmful stimuli, or "stressors." More recently, stress has come to embody negative effects on the system in which stressors elicit a bodily response perceived as unrest or one that causes anxiety (22). More appropriately, however, stress is the sum of biological reactions to intrinsic and extrinsic stimuli that results in a perturbation from homeostasis (4). Stress experienced chronically can have widespread negative effects on a number of physiological systems, including reproduction (35), growth (25), metabolism (32), immune function (13, 22), and behavior (4).

The adaptation to physical or psychological stress usually involves the activation of the hypothalamus-pituitary-adrenal (HPA) axis. The resulting release of hypothalamic corticotropin-releasing hormone, anterior pituitary adrenocorticotropic hormone, and adrenal glucocorticoids (cortisol and corticosterone) in anticipation of or during stressful situations is interpreted as a homeostatic response of the body (7). During times of stress, the increased metabolic demands of the body can be met through glucocorticoid release. The bioavailability

and metabolic clearance of glucocorticoids are regulated in part by binding globulins. Free, unbound glucocorticoids can enter cells and allow energy mobilization to fuel fight-or-flight responses (20). The degree and duration of the increase in glucocorticoid concentrations above basal levels are indicative of stress, and greater glucocorticoid concentrations over longer periods of time are interpreted as indicating more stressful situations (3, 5, 7, 17, 31).

The objectives of this laboratory were to measure salivary cortisol concentrations from third- and fourth-year undergraduates with the use of a commercial kit based on competitive ELISA and to correlate the plasma cortisol levels with different stressful situations. These experiments were designed to illustrate how psychological and physiological stressors impact the release of cortisol by the HPA axis in an interactive laboratory setting using current research diagnostic tools.

MATERIALS AND METHODS

This laboratory was exempt from Institutional Review Board human subject approval according to Iowa State University guidelines. In 2004, the Iowa State University comparative endocrinology laboratory had 12 biology and genetics undergraduates (8 women and 4 men), aged between 20 and 22 yr. To assess the effects of stress on salivary cortisol, three experiments were performed during the semester. This design is attractive for a number of reasons.

- It allowed the stress laboratory to be partitioned into three separate experiments that took place throughout the entire semester, lending depth and experimental realism that cannot normally be attained with laboratories that are completed in a single period.
- Two of the three experiments took place in conjunction with other laboratories that effectively tied together different endocrine topics and experiments without overburdening class time.
- Because the experiments were spaced throughout the semester, the students had additional time to explore the subject literature and formulate predictions about what they expected to find.

Before the first experiment, the class discussed how a variety of factors (time, gender, age, diet, etc.) can affect the accuracy of hormone measurements. For example, sampling protocols need to account for the circadian cycle of cortisol (17), and treatment groups had to control for gender since it can affect cortisol levels (15). In our class, this allowed for a discussion of how to properly design an experiment followed by the student's active involvement in the design of the three stress experiments. In addition, each day a sample was taken, students completed a survey specifying factors that could have influenced their salivary cortisol levels (food eaten, medication, recent exercise, etc.). Collection of this information allowed us to exclude biased samples from later analysis. Instructors who use this laboratory exercise should be particularly aware of how cortisol's circadian cycle may influence their class's results. Specifically, there is a sharp drop in circulating cortisol in the beginning of the light cycle, and thus morning laboratories should control for a time effect (17).

Address for other correspondence and present address of M. Haussmann: Dept. of Biology, Kenyon College, Gambier, OH 43022 (e-mail: haussmannm@kenyon.edu).

Experiment 1. Presentation stress: does giving a graded presentation in front of your instructors and peers elevate salivary cortisol levels? In our comparative endocrinology laboratory, students perform six indepth laboratory experiments on themselves or study animals that focus on important endocrine systems (hypophysectomy, metamorphosis, glucose tolerance, stress, castration, and mechanisms of hormone action). These laboratory experiments often run for several laboratory periods throughout the semester. During three laboratory sessions at intervals throughout the semester, the students, lecture instructors, and graduate student teaching assistant (TA) attend formal 45-min summary presentations on the experiments given by pairs of students. Preparation for these presentations provides students opportunities to read primary literature and form a cohesive presentation on the subject area as well as analyze and present the experimental data. These classes also provide a chance for the class to have an open discussion about the data, what in the laboratory experiment could have been done differently, what statistical tests were appropriate for proper data analysis, and what current research is being done in the subject area.

Because presentations in a classroom setting are often stressful to the presenting students, we decided that this was an opportune situation to study the effects of a psychological stress on salivary cortisol levels. These presentations were graded by the lecture instructors, TA, and other students, adding to the potentially stressful situation. At the beginning of the presentation period, saliva samples were collected from everyone, resulting in eight nonpresenting students and four presenting students giving a saliva sample. Over two presentation periods, this formed paired samples from eight students, one associated with a presentation situation and one before a nonpresentation situation, allowing for presenting students to act as their own controls. The third presentation period was not included in the analysis because it occurred during the last week of classes, so samples could not be analyzed by the students before the end of the semester.

Experiment 2. Fasting stress: how does a 15-h fast affect salivary cortisol levels? One of the laboratory experiments performed in our comparative endocrinology class examines the glucose tolerance test (GTT), which is used as a diagnostic tool to detect diabetes mellitus. This laboratory requires students to fast for 15 h before the afternoon laboratory session. At the beginning of class, students imbibe a glucose solution (with dose adjusted for body weight) and then using a glucometer to measure their blood glucose levels over the next 2 h to plot their glucose tolerance curve. The 15-h fast allowed for the opportunity to explore whether fasting, both a psychological and a physiological stressor, would affect salivary cortisol levels. The advantage of this experiment was augmented both because of cortisol's role in carbohydrate metabolism and because two experiments could be performed in the same laboratory period (the GTT and stress of fasting). Students in class took a salivary sample at the beginning of the period (fasting sample) and at the end of period (1 h after they had imbibed the glucose solution). This design produced paired samples from 10 of the 12 students in class; one student did not participate because she had diabetes mellitus, and another student's data were dropped from the analysis because his fasting sample did not contain enough saliva for analysis.

Experiment 3. Competition stress: how do in-class competitive activities that have no bearing on class grade affect salivary cortisol levels? We designed a third experiment that would also act as a psychological stressor but without the added consequence of the stressful stimuli having a direct effect on the student's grade. This experiment allowed us to explore whether competitive games could affect student's salivary cortisol levels.

Normally, students are aware of what activities are scheduled for laboratory periods prior to class time; however, for this period, students were only told that it was part of the stress experiment. Upon the initiation of class, all students gave a saliva sample to serve as a baseline, and, after this, students were told about the laboratory

activity for that day. The laboratory period was split into two activities: a stress bowl and a competitive card game.

The stress bowl was similar to a quiz game in which students on two teams had to correctly answer questions to win team points. Stress bowl rules were adapted from the rules of "Sports Day," an in-class activity designed to stimulate student-professor dialogue and reinforce class subject matter in another Iowa State University class (1). Briefly, two team captains were randomly chosen from the class; they then picked their teams one student at a time until no students were left. Each team had six members. The stress bowl was run in three rounds, and each round had six questions, necessitating that each student answer one question per round. Before a question was read, each team sent one representative to the front of the classroom, where they faced the class with the chalkboard behind them. A bell was located on the table in front of the students. When both students indicated that they were ready, the laboratory TA read a question related to endocrinology. At any point during the question, students could ring the bell and attempt to answer the question correctly. Once the bell was rung, however, the TA stopped reading the question and the student who rung the bell had to attempt to answer. If the student was wrong, the TA reread the entire question and the other student was allowed to try to answer the question. The questions in each round became subsequently more difficult. *Rounds 1 and 2* contained questions with spoken answers. *Round 3* contained questions that required that the students to diagram their answer on the board (e.g., outline the organs, hormones, and positive and negative feedback of the HPA axis). *Round 1* questions were worth 1 point, *round 2* questions were worth 2 points, and *round 3* questions were worth 3 points. At the end of the game, the team with the most points won the stress bowl.

Upon completion of the stress bowl, each student took a saliva sample and then each team of six students sat around a separate table to play a competitive card game. We played "Spoons," a fast-moving card game that requires a high degree of vigilance among participants. A group of six students sat around a table with five spoons placed in the middle of the table. Each student was dealt four cards, and, when the dealer was ready she/he began drawing a card from the pile and discarding it to her/his right at a fast rate while always keeping four cards in his/her hand. The student to the right of the dealer would pick up the discarded card and quickly decide whether to keep it or discard to their right. In this way, cards were passed around the table from left to right. The goal of the game is to have four matching cards (e.g., 4 nines or 4 queens), and, upon obtaining this, the student would grab one of five spoons in the middle of the table. Once any student grabbed a spoon, all students were free to grab one of the remaining spoons. Since there are one less spoon than students playing the game, the student who does not have a spoon does not participate in the next game. The five remaining students play a second game with only four spoons in the middle, and this continued until one student was left. The class played two complete rounds of spoons, and a last saliva sample was then collected from the students. The second saliva sample was taken ~1 h after the basal sample, and the third saliva sample was taken ~2 h after the basal sample.

Salivary cortisol analysis. Saliva samples were collected into salivettes according to the manufacturer's instructions (Sarstedt, Newton, NC). The quantitative measurement of cortisol in saliva was performed using a cortisol ELISA according to the manufacturer's instructions (Salimetrics, State College, PA). To carry out the ELISA kit test procedures, the laboratory or department must have the equipment and supplies shown in Table 1. Briefly, an ELISA works by the principles of a competitive binding assay. The ELISA microplate is coated with monoclonal antibodies to cortisol. Cortisol in saliva samples and in a set of standards (termed "cold" cortisol) is added to wells of the microplate. After this, cortisol linked to horseradish peroxidase (termed "hot" cortisol) is added to each well. "Cold" and "hot" cortisol compete for a limited number of antibody-binding sites, and, after an incubation period, unbound components are washed away. Bound "hot" cortisol is measured by the reaction of

Table 1. *Equipment and supplies needed to run ELISA*

Item	Company	Approximate Cost
Salivettes	Sarstedt	\$48 per 100
ELISA: cortisol diagnostic	Salimetrics	\$160 per kit (96 wells)
Pipette (20–200 μ l)	Many options	~\$100
Pipette tips	Many options	~\$10 per 1,000 tips
Vortex	Many options	Starting at ~\$200
Incubator	Many options	Starting at ~\$400
Plate reader	Many options	Starting at ~\$4,000
Centrifuge (15 ml conical)	Many options	Starting at ~\$3000
Statistical software	Many options	\$50 academic version (JMP)

Shown are the companies where supplies were ordered and their approximate costs (in U.S. dollars).

its peroxidase enzyme on a substrate (tetramethylbenzidine). The reaction produces a color that is measured at 450 nm on a plate reader. The intensity of the color reflects the amount of peroxidase detected, which is inversely proportional to the amount of cortisol present.

We determined the intra-assay reproducibility reported as coefficients of variation, which ranged between 0.16% and 1.49%. While the ELISA was carried out by the TA because it is a one-person job, a class period was used to fully discuss the history and principles of competitive binding assays (radioimmunoassay and ELISA). Pipetting variation between students makes full class participation in an ELISA impractical, but students were allowed to pipette their own standards and then used their own data to calculate and plot a standard curve that they could compare to a computer-generated standard curve.

Statistics. We used paired *t*-test analyses for salivary cortisol levels in *experiments 1* and *2* and to compare basal cortisol levels across *experiments 1* and *3*. We used simple linear regression to compare presenting and nonpresenting salivary cortisol levels in *experiment 1*. In *experiment 3*, the cortisol samples measured at three different times were compared with repeated-measures ANOVA, and differences were assessed using post hoc Tukey's highly significant difference test. *P* values of <0.05 were considered significant.

Assessment. At the end of the semester, we assessed the effectiveness of the laboratory by asking students a series of questions. This allowed for a quantitative assessment of how the stress laboratory supplemented student learning. The students were also encouraged to submit a written assessment on how they thought the stress laboratory reinforced their learning. Finally, the TA was able to assess the students' knowledge of the endocrinology of stress by reading and grading their laboratory reports for the stress laboratory.

RESULTS

Salivary cortisol concentrations, ranging from 0.10 to 1.56 μ g/dl, fell within the measurable range of the ELISA. There were no differences between male and female basal salivary cortisol concentrations ($P = 0.65$). Basal salivary cortisol concentrations did not differ between the presentation stress experiment and competition stress experiment ($P = 0.46$), suggesting that assays were repeatable within individuals.

Experiment 1. In the presentation stress experiment, students presenting in front of the class had higher salivary cortisol levels than when they were not presenting ($P = 0.006$). Salivary cortisol concentrations were higher during presentations in six students, not different in one student, and lower in one student compared with nonpresenting levels (Fig. 1). Students presumably could have had other stresses outside of class that could impact their salivary cortisol levels, but even if this were the case, the immediate stressor of the presentation was enough to cause a response. Presentating salivary cortisol

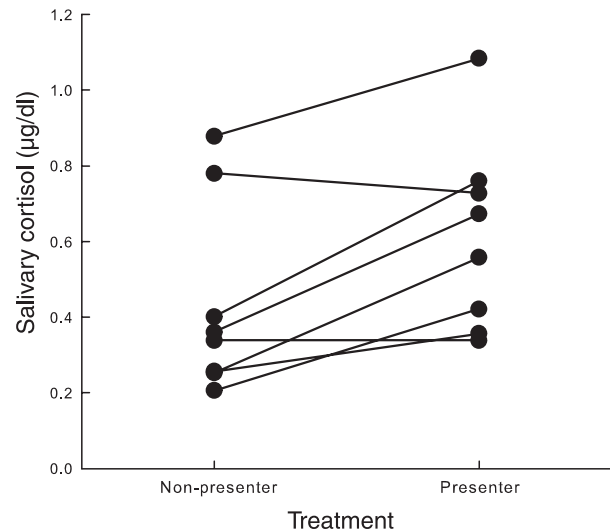


Fig. 1. Salivary cortisol concentrations (in ng/ml) of students sampled before a graded oral presentation and at a time when students were not presenting. Values from individuals are linked by solid lines.

levels were positively correlated with nonpresenting salivary cortisol levels ($P = 0.014$, $r^2 = 0.67$), indicating that those students who have relatively high presentating salivary cortisol levels also have relatively high nonpresenting salivary cortisol levels (Fig. 2).

Experiment 2. In the fasting stress experiment, students had higher salivary cortisol levels in the fasting sample than after the completion of the GTT ($P = 0.023$). In all but one student, salivary cortisol levels were higher in the fasting sample compared with the post-GTT sample (Fig. 3), although there was a variation in the fasting sample. The fasting salivary cortisol concentration did not correlate with the fasting blood glucose concentration ($P = 0.55$, $r^2 = 0.05$; data not shown).

Experiment 3. In the competition stress experiment, salivary cortisol levels were higher after the stress bowl and card game than in basal samples, although there were no differences

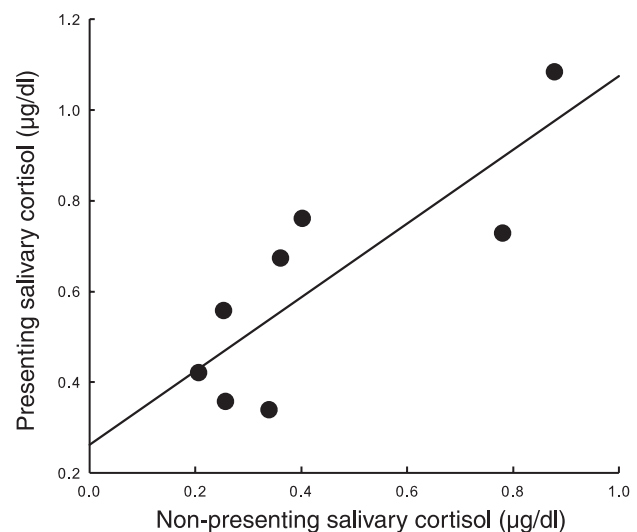


Fig. 2. Relationship between individual salivary cortisol concentrations (in ng/ml) before a graded presentation and when students were not presenting. The line is a best-fit regression through the data.

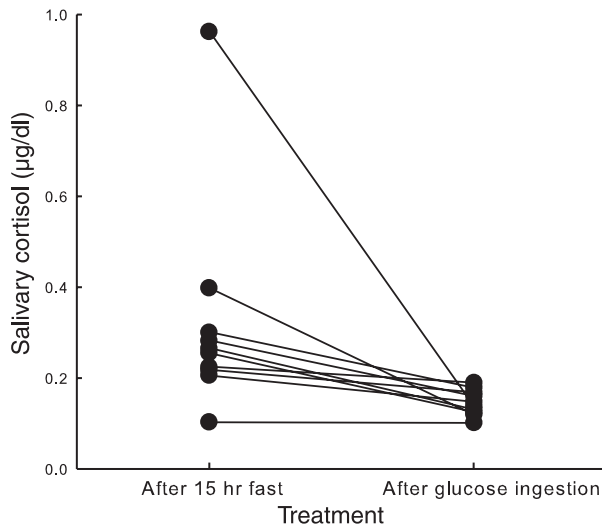


Fig. 3. Salivary cortisol concentrations (in ng/ml) of students after a 15-h fast and 2 h after the ingestion of a glucose solution. Values from individuals are linked by solid lines.

between the stress bowl and card game samples ($P = 0.039$; Fig. 4). The outcome of either competitive activity (being a winner or loser) did not correlate with the salivary cortisol concentration ($P > 0.30$; data not shown).

DISCUSSION

Students in the 2004 Iowa State University General and Comparative Endocrinology laboratory had higher levels of salivary cortisol prior to a formal graded presentation given to their instructors and peers than when they were simply part of the audience watching. This experiment combined two potential stressors, both of which have been found to cause an increase in cortisol: public speaking (18) and examinations (19). Written examinations can cause increases in cortisol (19), but some studies (23, 30) have shown no cortisol response, suggesting that written examinations are not perceived as uniformly stressful to students. Oral examinations, however, routinely elicit a cortisol response and feelings of stress (18), and they have been shown to cause greater cortisol release and feelings of anxiety than written examinations (19). A recent meta-analysis found that the Trier social stress test, consisting of a 5-min speech and a 5-min mental arithmetic problem in front of an audience, provoked a more robust cortisol stress response than several other types of stress test (8).

The presentation stress experiment clearly illustrated the variability in the cortisol response to presentation stress. One student showed no difference in presenting versus nonpresenting salivary cortisol levels, and another student actually showed a decrease. Stress is perceived differently by different individuals, and what is perceived as disturbing or unnerving to one may be exciting and stimulating to another (12). Interestingly, the students in the endocrinology laboratory evaluated their perceived level of stress on a 10-point scale every time a cortisol sample was taken, but there were no correlations between self-perceived stress and cortisol levels in any of the sample periods (data not shown). The presentation stress experiment also illustrated that individuals with higher basal cortisol concentrations also had higher cortisol concentrations

during acute stress and vice versa. Chronic stress can cause an upregulation of the HPA axis, resulting in a greater release of cortisol during acute stresses (6, 13, 24). We attempted to evaluate chronic stress during the first presentation period using a Holmes stress scale, which assesses chronic stress through a series of questions, each with an associated number of points. Students with a higher Holmes score are more likely to be chronically stressed (14), but we did not detect any relationship between the Holmes stress scale and basal cortisol level or any other cortisol measure.

In the fasting stress experiment, students had higher salivary cortisol concentrations after fasting for 15 h than they did after the completion of the GTT. Even short-term fasting is sufficiently stressful to cause activation of the HPA axis and a rise in cortisol (2). Once fasting has ended, cortisol levels drop to basal values quickly (2, 26, 27). The cortisol decline during a GTT has been suggested to reflect a circadian fall in circulating plasma cortisol (27). Basal cortisol has a precipitous circadian drop in the first 4 h of the light cycle (17). While our class met after this time, our data do not refute the possibility of a circadian effect as there were no differences in cortisol concentrations between the fasting samples in this experiment and basal samples from either the presentation stress or competition stress experiments ($P > 0.05$). This suggests that the decrease in cortisol concentration may be due to a circadian rhythm or the combination of a circadian decline in cortisol and the end of the fasting stress. Another possibility is that glucose ingestion directly effects cortisol release through an unknown mechanism (27).

In the competition stress experiment, students played two competitive games that had no bearing on their grade in the course. Salivary cortisol levels were significantly elevated after the first game (stress bowl) compared with basal samples at the beginning of class. In another class, where playing competitive games was associated with earning extra credit points, cortisol also increased significantly over the course of the games (D. Silverthorn, personal communication). A number of studies have shown increases in cortisol after both strenuous (10, 11,

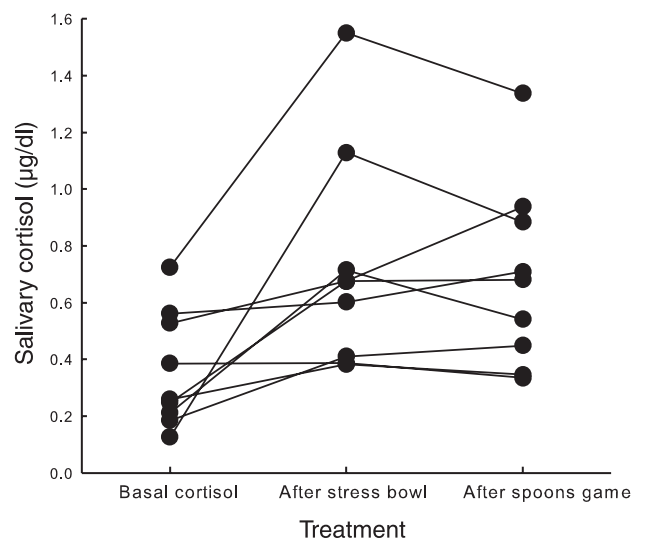


Fig. 4. Salivary cortisol concentrations (in ng/ml) of students before competitive games (basal cortisol), after 1 h of participation in a competitive quiz game (stress bowl), and after 1 h of participation in a competitive card game (spoons game). Individuals are linked by solid lines.

33) and nonstrenuous competitive games (21, 29). The psychological, but not physiological, component of the competition is thought to be responsible for the rise in cortisol. Elloumi and others (10) showed that cortisol levels were significantly increased after a rugby match, but when the competition component was removed by simulating similar strenuous activity in the laboratory, cortisol levels did not rise to the same extent.

Suay et al. (33) reported that winners of competitive sports contests (e.g., judo) had higher cortisol concentrations both before and after the competition than those in losers (33), although others have found no significant differences between winners and losers (9–11, 16). We did not detect any differences in salivary cortisol levels between winners and losers at any of the three sampling periods in our competition stress experiment. This could have been because the outcome of the stress bowl was decided by the last question, and saliva samples were taken immediately afterward. Cortisol released by the adrenals can take up to 10 min before being detectable in the saliva (34). We also did not detect a difference in salivary cortisol sampled after the stress bowl and after the card game. Cortisol levels can remain elevated for 60–120 min after onset of an acute stressor (7), and it is likely that there was not enough time between the two competitive games to determine whether both were perceived as stressful to the participants.

Postexperiment assessment indicated that students significantly increased their understanding of the endocrinology of stress, individual variation in how stress is perceived, the functioning HPA axis, and how hormones can be measured in the blood and saliva (Fig. 5). All the students were involved in the actual experimentation, data interpretation, and graph construction. While students did not perform the entire ELISA, a laboratory period was spent discussing how it functioned, and students were able to pipette their own standards and calculate

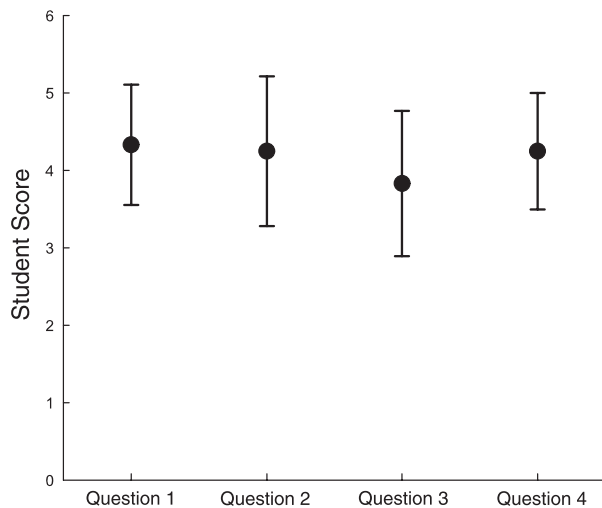


Fig. 5. Mean student scores (\pm SD) to 4 questions assessing laboratory effectiveness. A score of 5 indicated that the student learned a great deal and a score of 0 indicated the student learned very little. *Question 1* was “In general, how much did the stress laboratory reinforce your learning about the endocrinology of stress?” *Question 2* was “How much did the stress laboratory reinforce your learning about individual variation in how stress is perceived?” *Question 3* was “How much did the stress laboratory reinforce your learning about the hypothalamus-pituitary-adrenal axis?” *Question 4* was “How much did the stress laboratory reinforce your learning about how hormones can be measured in the blood or plasma?”

a standard curve. Feedback information indicated that this hands-on experimental exercise was beneficial and useful to the students in the following ways:

- To demonstrate the collection of saliva and the use of it as a specimen for testing the concentration of an endogenous molecule.
- To understand the scientific and technical basis of competitive ELISA.
- To practice pipetting techniques, plot a standard curve for the ELISA, and determine unknown samples read from the curve.
- To practice and perform statistical tests (repeated-measures *t*-test, ANOVA, and linear regression) on experimental variables.
- To know their own salivary cortisol level and how it responds to different stressful conditions.
- To specifically highlight the role of the important stress hormone cortisol and understand more about the complex endocrinology of stress.
- To illustrate how an experiment is properly designed to determine the relationship between different variables of interest.

The student learning process may be assessed on the basis of practical aspects and knowledge acquisition. Practical aspects include the following:

- How to perform an ELISA (e.g., the addition of the serum specimen, antibody, and enzyme; use of a fluorescent plate reader to measure absorbance; and the importance of controls).
- How to determine the cortisol concentration by plotting a standard curve from reference standards.
- How to collate data and employ statistical tests to analyze variables to determine their significance and relationships.

Knowledge acquisition includes the following:

- Principles of competitive ELISA.
- Different statistical tests and their appropriate applications.
- The importance and role of cortisol in stress biology.

We evaluated students’ performance through written laboratory reports that included proper statistical analysis of the data and a discussion of their results with appropriate references to primary literature. We also evaluated students’ retention of the practical and knowledge aspects of the laboratory through a formal quiz.

This laboratory exercise was successfully performed in the 2004 spring semester and has been added to the regular curriculum within this General and Comparative Endocrinology laboratory course at Iowa State University. We think that this laboratory is suitable for illustrating the physiology of cortisol release in response to a variety of stressful situations. These experiments may be further modified by adding components that explain and demonstrate other facets of the endocrinology of stress biology (e.g., catecholamine levels, strenuous activities, and formal questionnaires to assess perceived stress). Because each of the three experiments is independent of the others, some could be dropped to fit this laboratory into an already existing curriculum. In conclusion, this laboratory

emphasizes the molecular mechanisms underling the endocrine response to stressful situations while reinforcing the biochemical principles of the ELISA technique. When coupled with appropriate data analysis, this laboratory exercise serves to teach students proper experimental design and technique, thereby helping to teach students endocrinological concepts through an experimental paradigm.

ACKNOWLEDGMENTS

The authors give many thanks to the students in General and Comparative Endocrinology for collecting the data and offering feedback. We also thank Dee Silverthorn for helpful comments.

REFERENCES

- Ashlock D. *Sports Day Rules* [online]. <http://orion.math.iastate.edu/danwell/sdrules.txt> [13 December 2006].
- Bergendahl M, Vance ML, Iranmanesh A, Thorner MO, Veldhuis JD. Fasting as a metabolic stress paradigm selectively amplifies cortisol secretory burst mass and delays the time of maximal nyctohemeral cortisol concentrations in healthy men. *J Clin Endocrinol Metab* 81: 692–699, 1996.
- Breazile JE. Physiologic basis and consequences of distress in animals. *J Am Vet Med Assoc* 191: 1212–1215, 1987.
- Chrousos GP, Gold PW. The concepts of stress and stress system disorders. *JAMA* 267: 1244–1252, 1992.
- Chrousos GP, Gold PW. A healthy body in a healthy mind—and vice versa—the damaging power of “uncontrollable” stress. *J Clin Endocrinol Metab* 83: 1842–1845, 1998.
- Dallman MF. Stress update: adaptation of the hypothalamic-pituitary-adrenal axis to chronic stress. *Trends Endocrinol Metab* 4: 62–69, 1993.
- Dallman MF, Akana SF, Levin N, Walker CD, Bradbury MJ, Suenmaru S, Scribner KS. Corticosteroids and the control of function in the hypothalamo-pituitary-adrenal (HPA) axis. *Brain Corticosteroid Receptors* 746: 22–32, 1994.
- Dickerson SS, Kemeny ME. Acute stressors and cortisol reactivity: a meta-analytic review. *Psychosom Med* 64: 105, 2002.
- Donzella B, Gunnar MR, Krueger WK, Alwin J. Cortisol and vagal tone responses to competitive challenge in preschoolers: associations with temperament. *Dev Psychobiol* 37: 209–220, 2000.
- Elloumi M, Maso F, Michaux O, Robert A, Lac G. Behaviour of saliva cortisol [C], testosterone [T] and the T/C ratio during a rugby match and during the post-competition recovery days. *Eur J Appl Physiol* 90: 23–28, 2003.
- Filaire E, Le Scanff C, Duche F, Lac G. The relationship between salivary adrenocortical hormones changes and personality in elite female athletes during handball and volleyball competition. *Res Q Exerc Sport* 70: 297–302, 1999.
- Grier JW, Burk T. *Biology of Animal Behavior*. St. Louis, MO: Mosby, 1992.
- Harbuz MS, Lightman SL. Stress and the hypothalamo-pituitary-adrenal axis: acute, chronic and immunological activation. *J Endocrinol* 134: 327–339, 1992.
- Holmes TH, Rahe RH. Social readjustment rating scale. *J Psychosom Res* 11: 213–218, 1967.
- Kirschbaum C, Kudielka BM, Gaab J, Schommer NC, Hellhammer DH. Impact of gender, menstrual cycle phase, and oral contraceptives on the activity of the hypothalamus-pituitary-adrenal axis. *Psychosom Med* 61: 154–162, 1999.
- Kraemer WJ, French DN, Paxton NJ, Hakkinen K, Volek JS, Sebastianelli WJ, Putukian M, Newton RU, Rubin MR, Gomez AL, Vescovi JD, Ratamess NA, Fleck SJ, Lynch JM, Knuttgen HG. Changes in exercise performance and hormonal concentrations over a big ten soccer season in starters and nonstarters. *J Strength Conditioning Res* 18: 121–128, 2004.
- Kudielka BM, Schommer NC, Hellhammer DH, Kirschbaum C. Acute HPA axis responses, heart rate, and mood changes to psychosocial stress (TSST) in humans at different times of day. *Psychoneuroendocrinology* 29: 983–992, 2004.
- Lacey K, Zaharia MD, Griffiths J, Ravindran AV, Merali Z, Anisman H. A prospective study of neuroendocrine and immune alterations associated with the stress of an oral academic examination among graduate students. *Psychoneuroendocrinology* 25: 339–356, 2000.
- Martinek L, Oberascher-Holzinger K, Weishuhn S, Klimesch W, Kerschbaum HH. Anticipated academic examinations induce distinct cortisol responses in adolescent pupils. *Neuroendocrinol Lett* 24: 449–453, 2003.
- Mendel CM. The free hormone hypothesis—a physiologically based mathematical-model. *Endocr Rev* 10: 232–274, 1989.
- Meyer G, Hauffa BP, Schedlowski M, Pawlak C, Stadler MA, Exton MS. Casino gambling increases heart rate and salivary cortisol in regular gamblers. *Biol Psychiatry* 48: 948–953, 2000.
- Morley JE, Benton D, Solomon GF. The role of stress and opioids as regulators of the immune response. In: *Stress, Neuropeptides, and Systemic Disease*, edited by McCubbin JA, Kaufmann PG, Nemeroff CB. San Diego, CA: Academic, 1991, p. 221–231.
- Ng V, Koh D, Mok BY, Chia SE, Lim LP. Salivary biomarkers associated with academic assessment stress among dental undergraduates. *J Dent Educ* 67: 1091–1094, 2003.
- Pignatelli D, Magalhaes MM, Magalhaes MC. Direct effects of stress on adrenocortical function. *Horm Metab Res* 30: 464–474, 1998.
- Powell GF, Brasel JA, Raiti S, Blizzard RM. Emotional deprivation and growth retardation simulating idiopathic hypopituitarism. *N Engl J Med* 267: 1279–1283, 1967.
- Reynolds RM, Syddall HE, Walker BR, Wood PJ, Phillips DIW. Predicting cardiovascular risk factors from plasma cortisol measured during oral glucose tolerance tests. *Metab Clin Experimental* 52: 524–527, 2003.
- Reynolds RM, Walker BR, Syddall HE, Whorwood CB, Wood PJ, Phillips DIW. Elevated plasma cortisol in glucose-intolerant men: differences in responses to glucose and habituation to venepuncture. *J Clin Endocrinol Metab* 86: 1149–1153, 2001.
- Selye H. A syndrome produced by diverse nocuous agents. *Nature* 138: 32, 1936.
- Skosnik PD, Chatterton RT, Swisher T, Park S. Modulation of attentional inhibition by norepinephrine and cortisol after psychological stress. *Int J Psychophysiol* 36: 59–68, 2000.
- Spangler G. Psychological and physiological responses during an exam and their relation to personality characteristics. *Psychoneuroendocrinology* 22: 423–441, 1997.
- Stott GH. What is animal stress and how is it measured? *J Anim Sci* 52: 150–153, 1981.
- Stratakis CA, Chrousos GP. Neuroendocrinology and pathophysiology of the stress system. *Ann NY Acad Sci* 771: 1–18, 1995.
- Suay F, Salvador A, Gonzalez-Bono E, Sanchis C, Martinez M, Martinez-Sanchis S, Simon V, Montoro JB. Effects of competition and its outcome on serum testosterone, cortisol and prolactin. *Psychoneuroendocrinology* 24: 551–566, 1999.
- Vining RF, Meginley RA, Maksvytis JJ, Ho KY. Salivary cortisol—a better measure of adrenal cortical function than serum cortisol. *Ann Clin Biochem* 20: 329–335, 1983.
- Wingfield JC, Sapsolsky RM. Reproduction and resistance to stress: when and how. *J Neuroendocrinol* 15: 711–724, 2003.