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Using Zebrafish to Implement a Course-Based Undergraduate Research Experience to Study Teratogenesis in Two Biology Laboratory Courses

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Abstract

A course-based undergraduate research experience (CURE) spanning three semesters was introduced into freshman and sophomore biology classes, with the hypothesis that participation in a CURE affects skills in research, communication, and collaboration, which may help students persist in science. Student research projects were centered on the hypothesis that nicotine and caffeine exposure during early development affects gastrulation and heart development in zebrafish. First, freshmen generated original data showing distinct effects of embryonic nicotine and caffeine exposure on zebrafish heart development and function. Next, Cell Biology laboratory students continued the CURE studies and identified novel teratogenic effects of nicotine and caffeine during gastrulation. Finally, new freshmen continued the CURE research, examining additional toxicant effects on development. Students designed new protocols, made measurements, presented results, and generated high-quality preliminary data that were studied in successive semesters. By implementing this project, the CURE extended faculty research and provided a scalable model to address national goals to involve more undergraduates in authentic scientific research. In addition, student survey results support the hypothesis that CUREs provide significant gains in student ability to (1) design experiments, (2) analyze data, and (3) make scientific presentations, translating into high student satisfaction and enhanced learning.

Introduction

Recent calls for advances in undergraduate science education have encouraged increased undergraduate participation in authentic research. Undergraduate research increases student retention and persistence in science, and encourages students to pursue graduate education or careers in science.[1,2] The 2011 *Vision and Change in Undergraduate Biology Education* report[3] calls for departments to ''introduce the scientific process to students early, and integrate it into all undergraduate biology courses,'' and the 2012 President's Council of Advisors on Science and Technology (PCAST) report advocates replacing standard laboratory courses with discovery-based research courses.[4]

While many universities find it difficult to accommodate large numbers of undergraduates in a mentored research experience,[5–8] a successful strategy to increase student involvement in research is accomplished through undergraduate course-based research projects related to faculty research interests. This can produce novel results that are of broad interest to the scientific community.[8–11] Course-based or classroom undergraduate research experiences (CUREs) integrate research skills and practices into the course curriculum, thereby providing a large number of students the opportunity to engage in authentic research in the classroom and labs. Introductory-level courses with large enrollment benefit the most from these CUREs and have shown to retain students considering science careers,[7,12–14] positively affecting students' persistence in science career paths.[1,15]

CUREs and undergraduate research experiences within a mentor's laboratory produce many of the same gains.[14,16] Integrating research in undergraduate biology laboratory also extends faculty research by producing novel preliminary data and expands the faculty member's research team to include students in the classroom. With these goals in mind, CUREs were designed to address real world issues with compelling, medically relevant global health themes. Both CUREs focused on the teratogenic effects of various compounds on zebrafish embryos. The project included Introductory Biology and Cell Biology laboratory course students, building continuity between successive semesters in the two subjects.

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It is known that certain environmental factors can cause birth defects. At least 7.9 million people worldwide are born with a birth defect each year, and about 10% of these are due to environmental factors, including maternal smoking, alcohol use, nutrient imbalances (folic acid and vitamin A deficiency), pesticides, food preservatives, plastics, plastics components, solvents, metals, and numerous air pollutants.[17] Alcohol, nicotine, and caffeine are commonly used by pregnant mothers, and are among the most common factors responsible for the global burden of disease worldwide.[17]

Prenatal alcohol exposure can cause fetal alcohol spectrum disorder (FASD), and the authors (J.A.M., P.M., and S.S.) have previously studied a zebrafish model of FASD.[18–21] In animal models and humans, nicotine exposure during embryogenesis can affect behavior, cognition,[22–24] lung development,[25–27] and induce neural[22] and cardiac function defects.[28,29] Caffeine exposure during embryogenesis may increase risk of childhood obesity,[30] affects respiratory control,[31,32] and alters cardiac development.[33,34] Effects of nicotine and caffeine on gastrulation and effects of nicotine on heart development are not known.[35]

Zebrafish have numerous advantages as a model system in scientific research, and have many educational uses for undergraduate and K-12 teaching laboratories.[36–38] Zebrafish are inexpensive. Each breeding pair produces numerous embryos in a time-controlled manner, which provides enough embryos at different times of the day for a large, multisection laboratory course. External development and optical clarity of the embryo allows easy observation using simple dissecting microscopes equipped with inexpensive digital cameras. Recent studies showed[35] that high school students can successfully perform research using zebrafish to examine the effects of environmental toxicants.

A sustainable, multi-year CURE was developed to study environmental chemicals that induce birth defects, allowing students to test the hypothesis that nicotine and caffeine exposure during early development affects gastrulation and heart development in zebrafish. The authors thus tested the hypothesis that student participation in CUREs will promote research, communication, and collaboration skills.

Our project has many advantages: (1) Similar experimental procedures with zebrafish can be performed in each semester in Introductory Biology laboratories while testing different environmental factors, transforming every semester's laboratory into discovery research; (2) Continuity can be designed into the research project as undergraduates progress through various biology courses and to themed learning communities or capstone research experiences; (3) The subject matter is highly relevant and engaging, especially for students interested in health-related professions and the physiological and societal effects of environmental toxicants on health.

Materials and Methods

Student populations

Introductory Biology at Indiana University-Purdue University Indianapolis (IUPUI) is a large-enrollment, typically freshman, ''gateway'' course required for Biology and other science majors and taken by about 900 students per year. Our study included three honors sections in the course, $\sim$20 students per section, 50 (women: 31 and men: 19) in fall 2013 and 65 (women: 41 and men: 24) in fall 2014. Students attended 1 weekly laboratory section per week (Tuesday midday, Tuesday afternoon, or Thursday afternoon), for 3 h for the duration of the semester (17 weeks), and worked outside of the normal lab time if required. Student research was done in teams of three that were assigned based on their first lecture exam performance (17 teams in fall 2013 and 21 in fall 2014). At least one student from each research group was required to come to the faculty laboratory outside of normal lab time.

Cell Biology laboratory is a required course for biology majors. Sections met twice a week for 1 h and 50 min throughout the semester (17 weeks). In spring 2014 there were 21 junior/senior biology honors students (women: 13 and men: 8). Students either worked independently or in pairs (10 teams in spring 2014). At least one student from each research group was required to complete work outside the lab time in the faculty research laboratory.

Zebrafish husbandry

Zebrafish (*Danio rerio*; Hamilton; TL strain) were raised and housed under standard laboratory conditions[39] in accordance with Indiana University policy on animal care and use. Tank dividers in the breeding tanks allow timed breeding of zebrafish pairs at various times of day for different laboratory sections.

Embryo treatments

For the Introductory Biology laboratory CURE, zebrafish embryos were treated with 150 mM ethanol from 2–48 hours post fertilization (hpf) or placed in normal embryo medium[39] by the instructor. After ethanol treatment, the embryos were grown in normal embryo medium.[39] All embryos were then fixed in 4% paraformaldehyde (PFA) at 3 days post fertilization (dpf). Students treated zebrafish embryos with nicotine (1.3–1.7 mM) (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/zeb) from either 2–24 or 2–48 hpf.

Embryos were washed and placed in normal embryo medium after completion of the treatment. Students scored survival at 2 and 3 dpf. Embryos were fixed in 4% PFA to score pericardial edema. To measure acute effects of nicotine or caffeine on heart function, 3 dpf control embryos were treated with 0.05, 0.1, and 1.2 mM of nicotine or 100 μM, 500 μM, and 1 mM of caffeine and heartbeats per minute was counted under the dissecting microscope. Finally, students designed and executed treatments using various compounds, including pH, $PbCl_2$ and coumarin. Folic acid, retinoic acid, and other compounds were tested for potential rescue effects from teratogenesis (Supplementary Table S2).

In the Cell Biology laboratory CURE, the instructor treated zebrafish embryos with 150 mM ethanol from 2 to 8 hpf and fixed embryos in 4% PFA to allow students image and measure gastrulation/epiboly progression. Students treated zebrafish embryos with nicotine (1.3–1.7 mM), caffeine (1.3–1.5 mM), or nicotine+caffeine at various concentrations (Supplementary Table S1) from 2 to 8 hpf. Embryos were fixed in 4% PFA to measure gastrulation progression (with and without phalloidin staining). Students made phalloidin staining solution (phosphate buffered saline [PBS] containing 1% Tween 20, 1% DMSO, 0.02% NaN_3, and phalloidin stock diluted 1:50)[20] and incubated the embryos in solution at room temperature overnight.

Microscopy, image analysis, and heart rate counting

Fixed embryos were mounted individually ($n = 10$–20 from control and compound-treated groups) in glycerol on a depression slide and imaged with classroom dissecting microscopes fitted with a Celestron microscope camera (Torrance, CA) that mounts on the eyepiece. To examine pericardial edema, embryos were mounted laterally and imaged at higher magnification. For confocal microscopy, the instructor acquired images using a Zeiss (Thornwood, NY) Observer Z1 LSM 700 confocal microscope (40×1.1 NA W or 20×0.8 NA objectives).

ImageJ, a free image software from National Institutes of Health (Bethesda, MD) was used for image analysis and measurements. Epiboly progression (spreading of blastomeres over the yolk cell), body length, and eye diameter were analyzed. Students analyzed images outside the lab. Figures were assembled in Adobe Photoshop (San Jose, CA). Tutorials were provided for the software packages. In the phalloidin images, only epiboly progression was examined.

To measure the heart rate, 3 dpf control embryos were treated with nicotine or caffeine and immediately mounted in 2% methylcellulose on a depression slide. Heartbeats per min were counted from 5 to 15 min after exposure. Every group member counted the heart rate for three embryos from each treatment condition. Pooled data were used to calculate average heart rate per treatment.

Statistics

During the course, students' individual project measurements (control and compound-treated groups) were compared by Student's *t*-test using freely available statistical calculator, GraphPad software (La Jolla, CA).

After the course, the complete data set (all control groups and all treated groups) was compiled and analyzed by the IU Biostatistics core service to access overall significance of the entire project from each Introductory Biology section. To compare body length and eye diameter from the control and ethanol groups, paired *t*-tests were used. To assess whether an association exists between dose versus survival percent or dose versus heart defect, a Spearman's correlation coefficient was used. Because the students reported only the mean and standard deviation, an ANOVA could not be performed. Dose group comparisons were made using logistic and ordinal logistic regression for survival and heart defect, respectively. Experiments were accounted for as random cluster effects in these logistic regression models.

Assessment methods

Introductory Biology laboratory course evaluation. Our study was reviewed by the Indiana University Office for Research Compliance and was found to be exempt from Institutional Review Board review for human participants. To evaluate the outcomes of this course, a CURE survey developed by David Lopatto[40] (Supplementary CURE survey document) was administered in fall 2014 (pre- and post-CURE) at the beginning and end of the semester. The CURE survey instrument can be used to compare our students' responses with the similar data collected from other CURE projects nationwide.[41,42]

The CURE survey collected students' demographic data, academic information, reasons for taking the course, and the survey measures the level of experience with various course elements, science attitudes, and learning style. This survey asked questions about science attitudes before and after the course, and, in the post course survey, estimated learning gains and perceptions of benefits from the course. IUPUI student survey data were compared to ''all student data'' that represent data from CURE survey obtained from June 1, 2014 to January 6, 2015 by David Lopatto's group. Learning gains were also compared with the results of Summer Undergraduate Research Experience (SURE) survey,[43] developed by David Lopatto's group to assess one-on-one mentored summer research experiences.

Cell Biology laboratory course evaluation. Questionnaires were created by instructors to determine student satisfaction and gather student feedback anonymously at end of the Cell Biology course using online tool SurveyMonkey (Palo Alto, CA).

Results

CURE design

The CURE was developed using parameters recommended by Kloser *et al.*[44] and Gardner *et al.*,[45] including straightforward protocols with limited prior laboratory expertise required, reproducibility, sufficient variables for developing hypotheses, a shared database for students, assessment reflecting authentic scientific communication, and expertise from faculty researchers.

The integration of CUREs into the Introductory Biology and Cell Biology laboratory courses emphasized the following principles of undergraduate research experiences: laboratory skill development, enhancing student's engagement in class, students critical thinking, integration of biological concepts, application of knowledge, and generation of new knowledge.[44,45]

The Introductory Biology laboratory course was roughly divided into three parts (Table 1). The introductory 2–3 weeks were devoted to building students' knowledge of scientific literature, laboratory skills, and introducing the zebrafish model system (e.g., handling embryos, zebrafish developmental biology, and embryo treatments). In the next 3 weeks, students selected from instructor designed research projects. In the remaining 8–10 weeks, students developed their own research questions, writing research proposals, performing research experiments, analyzing results, and presenting their results in both scientific paper and poster formats.

The Cell Biology laboratory course was divided into two parts (Table 1) and integrated into the traditional Cell Biology laboratory. At first, students were introduced to the zebrafish model system, microscopy, and image analysis by letting students examine the effects of ethanol on epiboly progression. In subsequent 3 weeks, students worked individually on zebrafish gene expression research projects using bioinformatics tools (S.S., K.A.M., and J.A.M.; article in preparation). In the remaining 3 weeks, students performed research experiments with nicotine and caffeine.

Students were grouped into research teams of two. Multiple experiments (Supplementary Table S1) were designed by the instructor. Coordination of the project was facilitated using an experiment planner on a cloud computing (Google) drive. Students signed up for experiments within this experiment planner. Students analyzed data and made figures. Students presented their posters, and final reports were due in the final week.

Table 1. Introductory Biology and Cell Biology CUREs Organization

	Introductory biology CURE	*Cell biology CURE*	*Course design*	*Scholarly activities*	*Assessment*
Introduction	+	+	Development of laboratory skills and knowledge on scientific literature for instructor designed research project of known outcome	Becoming familiar with scientific literature search, imaging, documentation of experimental work, interpretation of data	Peer-review of alcohol results Lab report (data and conclusions)
Instructor designed research	+	+	Instructor designed research projects of unknown outcome to generate new knowledge	Article search on research topics, documentation of experimental work, result presentation, data interpretation, comparing results with peer groups, scientific writing	Lab report (data and conclusions)
Student designed research	+	±	Discovery research projects to test student generated hypotheses using student designed experiments	Scientific literature search, research proposal writing, documentation of experimental work, result interpretation, poster presentation	Five-page lab report written in scientific paper format Poster presentation rubric Pre- and post-CURE assessment

Introductory biology CURE was divided into three parts, and cell biology CURE was divided into two parts, which were integrated into the traditional cell biology laboratory.
+, Everything in the course design, scholarly activities, and assessment was covered.
±, Discovery research was discussed but not executed.
CUREs, course-based undergraduate research experiences.

The zebrafish CURE built initial skill sets and a research mindset

Introductory Biology laboratory. Lab skills and research habits were developed by having students analyze effects of ethanol on body length and eye size. Students imaged control and ethanol-treated embryos using dissecting microscope, measured body length and eye diameter using ImageJ, and performed statistical *t*-test analysis using the GraphPad calculator software. Students reviewed their peer's results, which encouraged critical thinking. Student data showed that exposure to 150 mM ethanol reduced body length to 2.77 ± 0.28 mm from 3.09 ± 0.172 mm in control embryos ($p < 0.001$). Average eye diameter was reduced in 150 mM ethanol-treated embryos to 0.223 ± 0.03 mm from 0.2678 ± 0.02 mm in control embryos ($p < 0.001$).

Effects of ethanol on the body length and eye size were previously published from our lab,[18,19,21] but these results were unknown to students, helping to develop student curiosity and confidence in their skills. Instructors also saw that students could replicate prior results with accuracy. Students also developed laboratory skills used by all biology students, including proper micropipetting technique, making molar solutions, keeping a lab notebook, conducting PubMed searches, and performing serial dilutions. These activities were designed to integrate within the CURE and achieve later learning goals (Table 2). Students also took the precourse CURE survey (described below) in the 1st week.

Cell Biology laboratory. Like in the Introductory Biology laboratory, research project concepts were introduced in Cell Biology laboratory, starting with an experiment of known outcome. Students compared epiboly progression in the control and ethanol-treated (150 mM) embryos. Epiboly percent progression is an indirect measurement of gastrulation progression,[46] and gastrulation defects produce epiboly defects in early embryos. Student investigation confirmed that at 8 hpf control embryos reached an average of 80% epiboly, whereas ethanol-treated embryos reached an average of only 65% epiboly ($p < 0.01$). Ability of students to reproduce known results[20] built confidence to move to the next phase of the semester.

Effects of nicotine and caffeine on zebrafish development: new knowledge generated by students in Introductory Biology and Cell Biology courses

In both courses, discussion of known environmental toxicants that produce birth defects in humans and animal models

Table 2. Techniques and Skills Acquired in Research-Based Laboratories

Techniques	*Research related skills*
Microscopic slide preparation	Keeping a lab note book
Imaging	Writing research proposal and lab reports in a scientific paper format
Zebrafish handling	Reading and critical analysis of research article
Statistical analysis	Team work and collaboration
Medline search	Design and execution of research experiments
Making molar solutions	Drawing conclusions and analysis of results
Embryo treatment	Presentation of research result in scientific paper and poster format
Tissue fixation	
Embryo dechorionation	

formed a basis for the next experiments in the CURE. Students were given the opportunity to choose an experiment from a list of instructor-designed experiments. Experiments varied concentrations of either nicotine or caffeine in zebrafish embryo treatments (Supplementary Table S1). Neither instructors nor students knew the outcomes. Students were responsible for performing, analyzing, and comparing results with peer teams, and they were also asked to provide possible explanations if contradictory results were found. At the end of the project, all class data was compiled, which allowed students to see the overall outcomes of each treatment and dose.

Nicotine exposure during early development affects survival rate and produces malformed heart. In Introductory Biology, the following hypothesis was introduced: *Nicotine exposure during early development causes developmental and functional defects in zebrafish heart*. Students were introduced to heart development in zebrafish and human with a short presentation, and students read two published research articles chosen by instructors.[23,35] These articles were discussed in the recitation portion of the course.

Students picked one treatment condition (each condition was tested by at least two groups) from the planner posted on Google drive (Supplementary Table S1) and treated the embryos in class. Each group had their own control embryo set. Students came to the faculty lab outside of the normal meeting time for 10–15 min to change embryo medium and to add fixative to embryos. Embryos were imaged in the next class period.

Students performed a *t*-test comparing the control group with the treated group, and reported embryo survival, pericardial edema, and *t*-test results. In the subsequent 2 weeks, students repeated their experiments, documented defects, and analyzed results. Students testing similar nicotine concentrations compared their results. Finally, the whole class discussed their results with peers and instructors, and students compiled data to generate graphs. This allowed students to appreciate the correlation between doses and defects. Adobe Photoshop was introduced to help students make figures. Students uploaded their data including figures to a common Google drive.

Survival rate and heart morphology was observed in embryos after nicotine exposure (concentrations of 1.3–1.7 mM for exposure periods 4–24 or 4–48 hpf). Nicotine exposure from 4 to 24 hpf did not affect the survival rate, even at the highest concentration (data not shown), but exposure for an extra day (4–48 hpf) reduced embryo survival in a dose-dependent manner (Fig. 1A). After the course was completed, statistical analysis on all students' data (Supplementary Table S3) showed that survival percent was negatively correlated with nicotine dose (Spearman's correlation = –0.62, $p < 0.0001$). Pairwise comparisons of each dose level were all significant (with the exception of 1.3 mM vs. 1.5 mM).

Nicotine-exposed embryos showed pericardial edema and had various degrees of heart defects at 3 dpf. Students sorted the embryos into four classes based on the severity of the pericardial edema: control morphology, mild, moderate, and severe edema (Fig. 1B–F). As the concentration of nicotine increased, numbers of severely affected embryos increased (Fig. 1G). Additional statistical analysis found a strong positive correlation between nicotine dose and heart defect severity (Spearman's correlation = 0.80, $p < 0.0001$; Supplementary Table S4). Pairwise odds ratio was estimated from the ordinal logistic regression on heart defect level. Overall effect of dose was significant ($p < 0.0001$).

An example of student generated image data and pericardial scoring data for the entire class is shown in Figure 1B–G. Students in both semesters obtained similar results confirming a teratogenic effect of nicotine on heart development. Students also examined the effect of nicotine on heart function, showing that the heartbeat of zebrafish embryos increased when exposed to lower concentrations of nicotine, but higher concentrations reduced the heartbeat (Fig. 1H). These novel results, generated by students, show clear effects of nicotine on survival of embryos and early development effects on the heart.

Exposure to nicotine and/or caffeine during early development reduced epiboly progression. The following hypothesis was tested in the Cell Biology laboratory course: *Nicotine and/or caffeine exposure during early development causes gastrulation defects in zebrafish*. Students discussed four scientific articles (two research articles and two review articles). Students selected their experiment from the list of instructor designed caffeine and/or nicotine experiments, made solutions, and treated embryos. They returned to add fixative to the embryos, and the next day, to wash embryos in PBS. During the next class meeting, students divided the fixed embryos into two sets. One set was used to measure epiboly progression using classroom dissecting microscope, and the other set was used to perform phalloidin staining.

Students made phalloidin solution and incubated the embryos in stain at room temperature overnight. The instructor acquired images using a confocal microscope in the faculty research lab. Images were then uploaded to the Google drive. Students re-examined epiboly progression in phalloidin-stained images.

Students data showed that nicotine exposure (1.5–1.7 mM) affects epiboly and gastrulation progression. Early exposure (2–8 hpf) reduced epiboly progression, indicating that nicotine affects early gastrulation stage embryos. Student generated data examining effect of 1.6 mM nicotine exposure on epiboly progression is shown in Figure 2A–E. Phalloidin staining confirmed the epiboly delay ($p < 0.01$ for all treatment conditions, Fig. 2F–G′). Students also analyzed the effect of caffeine exposure (1.3–1.5 mM) on epiboly progression. Caffeine exposure, like nicotine exposure, reduced epiboly progression ($p < 0.01$, for all treatment conditions).

Example student data is shown in Figure 2H. Combination treatment using lower concentrations of nicotine (1.1–1.3 mM) and caffeine (1.1–1.3 mM) produced effects on epiboly progression similar to higher concentrations of individual compounds ($p < 0.01$, for all treatment conditions). Student-generated data (Fig. 2I) show epiboly delay caused by 1 mM nicotine plus 1.2 mM caffeine treatment. These are novel findings showing effects of these environmental toxicants on gastrulation.

Student-designed CURE investigations

For the Introductory Biology CURE, the final part of the semester was dedicated to student-designed experiments and to developing scientific communication skills. To enhance student's critical thinking, students were given the freedom to ask a research question and design research experiments to address that question. First, student teams did a literature

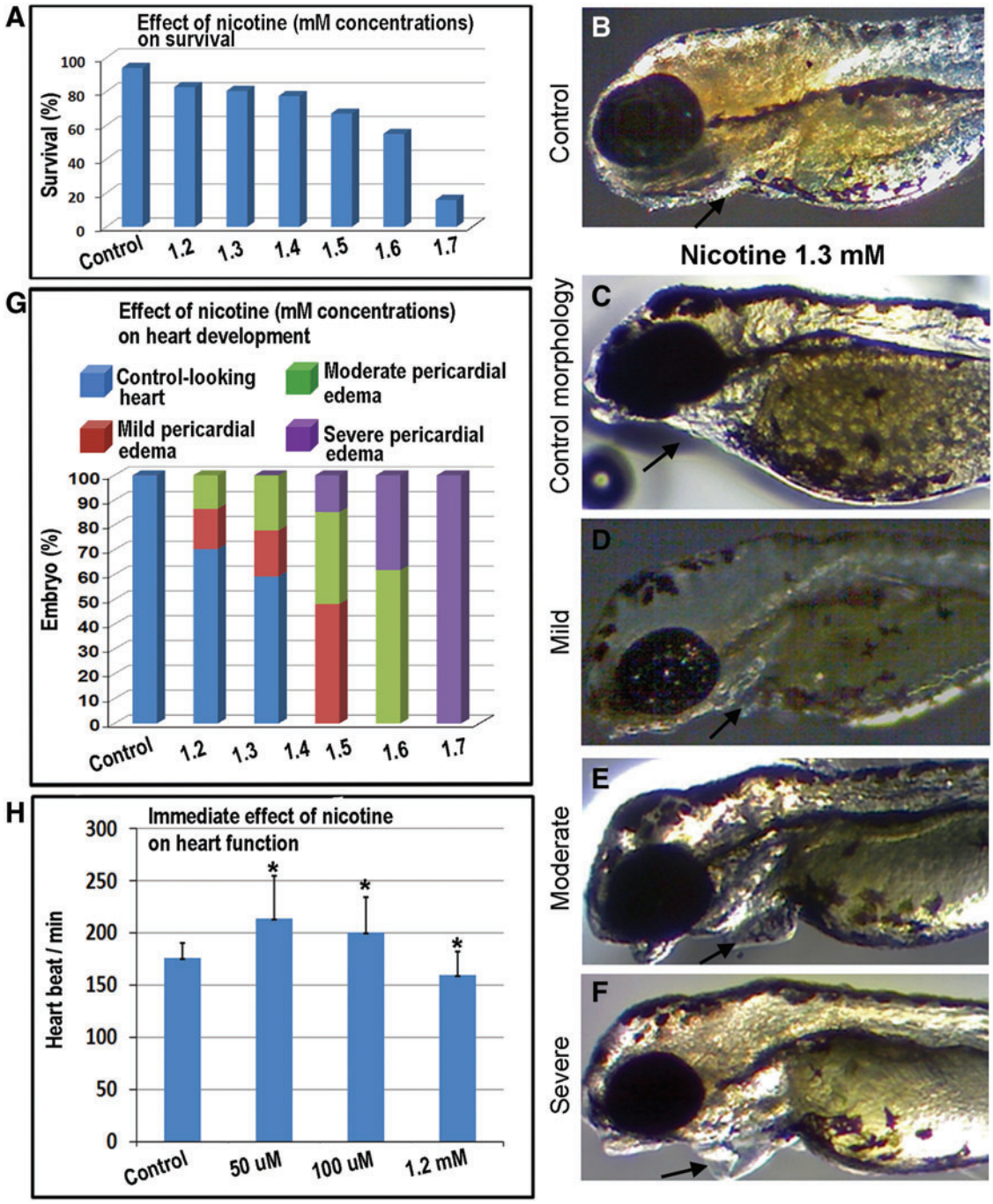

FIG. 1. Example of introductory biology student generated images and quantification of defects after nicotine exposure. **(A)** Nicotine exposure from ~4 to 48 hpf affected survival rate in a dose-dependent manner. **(B)** Control embryo showed normal heart at 3 dpf. **(C–F)** 1.3 mM nicotine-treated (4–48 hpf) embryos showed either control heart morphology **(C)**, mild **(D)**, moderate **(E)**, or severe **(F)** pericardial edema at 3 dpf. *Arrow*: heart. **(G)** Quantification of pericardial edema after nicotine exposure (1.2–1.7 mM). **(H)** Quantification of heart rate in control embryos after transient nicotine exposure. Fifty and 100 μm nicotine exposure increased heart rate, but 1.2 mM decreased heart rate, $^*p < 0.01$. dpf, days post fertilization; hpf, hours post fertilization. Color images available online at www.liebertpub.com/zeb

survey to find three possible research ideas related to environmental toxins, potential teratogens, or compounds that may rescue the effects of teratogens. Instructors evaluated the scientific questions and feasibility of the experiments and chose one for further investigation. Students then submitted a detailed research proposal using research proposal guidelines that included (1) An explanatory paragraph about what is being tested and why; (2) A brief literature review to support the hypothesis; (3) Description of the substance to be tested; (4) Concentrations to be tested; (5) Rationale for concentrations; (6) Description of exposure protocol; and (7) a Reference list. Instructors gave feedback on proposals. Modified

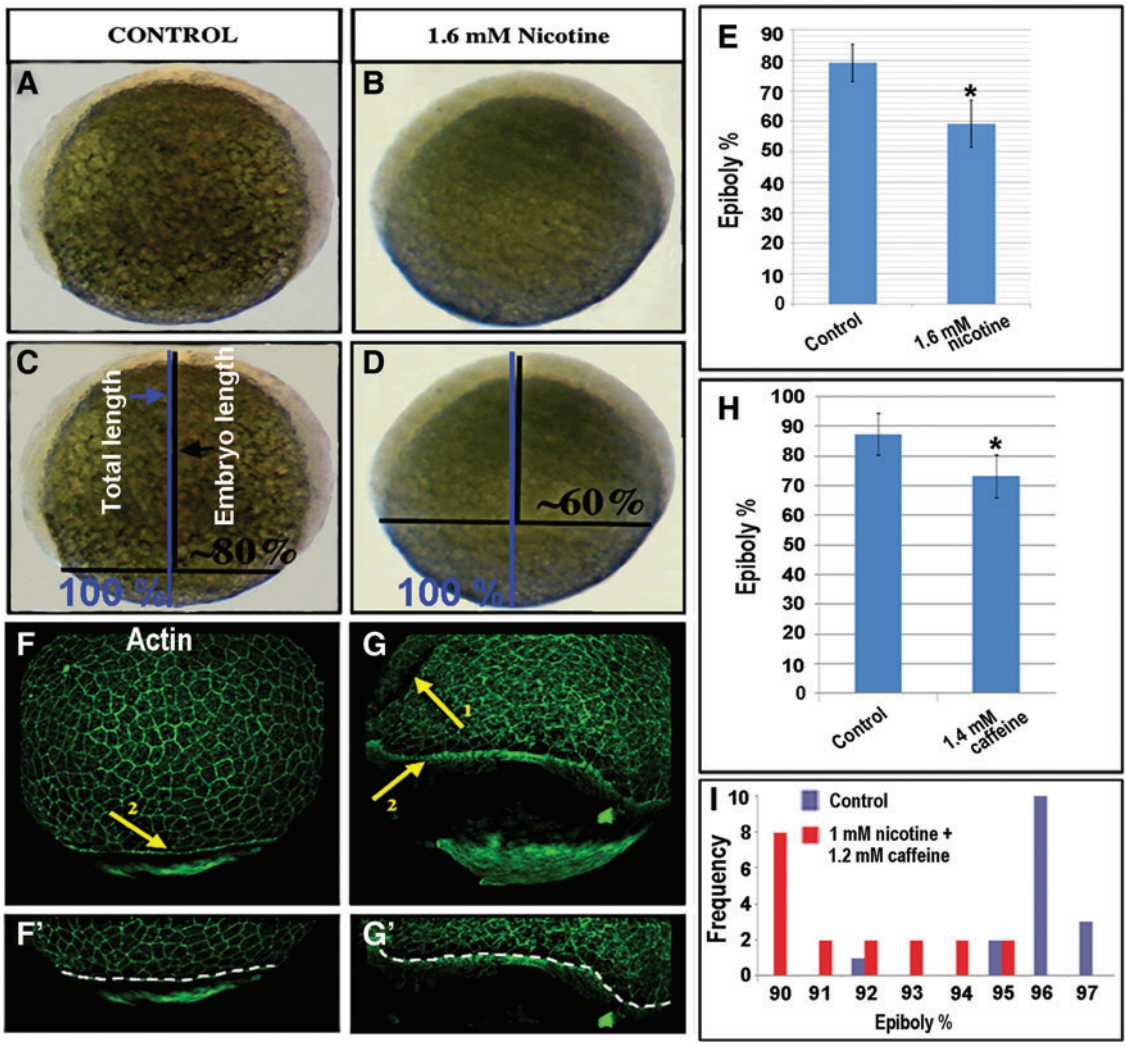

FIG. 2. Example of cell biology student generated images and quantification of defects after nicotine and caffeine exposure. **(A–E)** Nicotine-exposed (1.6 mM) embryos showed epiboly delay, **(A, C)** control, **(B, D)** nicotine-exposed embryo. *Blue* and *black lines* showed the measurements for epiboly used in ImageJ analysis. Graph showed quantification of epiboly progression after nicotine treatment **(E)**; $*p < 0.01$. **(F, G)** Phalloidin stained (F-actin staining) embryos showed delayed epiboly progression. *Arrows*, 1: actin disintegration; 2: delayed epiboly. **(F′, G′)** Phalloidin-stained embryos highlighted with *white dotted line* showed uneven epiboly progression in the nicotine-exposed embryo. **(H)** Graph showed quantification of epiboly progression after 1.4 mM caffeine treatment; $*p < 0.01$. **(I)** Graph shows percent epiboly versus frequency of control or 1 mM nicotine plus 1.2 mM caffeine-treated embryos. Color images available online at www.liebertpub.com/zeb

proposals were submitted after incorporating instructor's comments. About two dozen compounds (Supplementary Table S2) were selected for study: (1) Environmental toxins (nitrate, lead, and others); (2) Over-the-counter medications (dextromethorphan, acetaminophen, and others); (3) Potential teratogenic agents (ethanol, nicotine, coumarin, red wine, and others); (4) potential rescue agents for teratogenic compounds (EGCG, folic acid, vitamin B12, turmeric, and others); and (5) chemicals with immediate effects on heart function after exposure (caffeine).

Students made the necessary compound solutions and performed treatments outlined in Supplementary Table S2 to examine the effect of those compounds on (1) development, (2) immediate effect on heart function. Embryos were imaged and analyzed by students using techniques developed earlier in the CURE. In the next four class periods, students repeated the experiments 3–4 times and analyzed results. A summary of these experiments is shown in Supplementary Table S2.

Enhancing science communication skills through the zebrafish CURE

Throughout the semester, students were assigned to write different sections of a scientific article. Introductory Biology students wrote an abstract on their initial body length and eye measurement data, methods and results sections to describe experiments completed in the second portion of the instructor-designed experiments. For the student designed portion, students used their research proposal guidelines to carry out the investigations they designed and planned. Rigorous writing exercises provided a tremendous opportunity to develop critical thinking and communication skills during this research process.

Biology departmental poster sessions were organized for both Introductory Biology and Cell Biology CUREs. A draft poster was sent to instructors for review and critique before printing. Faculty, graduate students, undergraduate students, and various guests were invited. Students presented their posters with great confidence, and they discussed their work and potential next steps for the research projects. Results from the pre- and post-CURE survey indicated gains in presentation skills as a course element (Fig. 3A) and as a learning outcome (Fig. 3B), as described below.

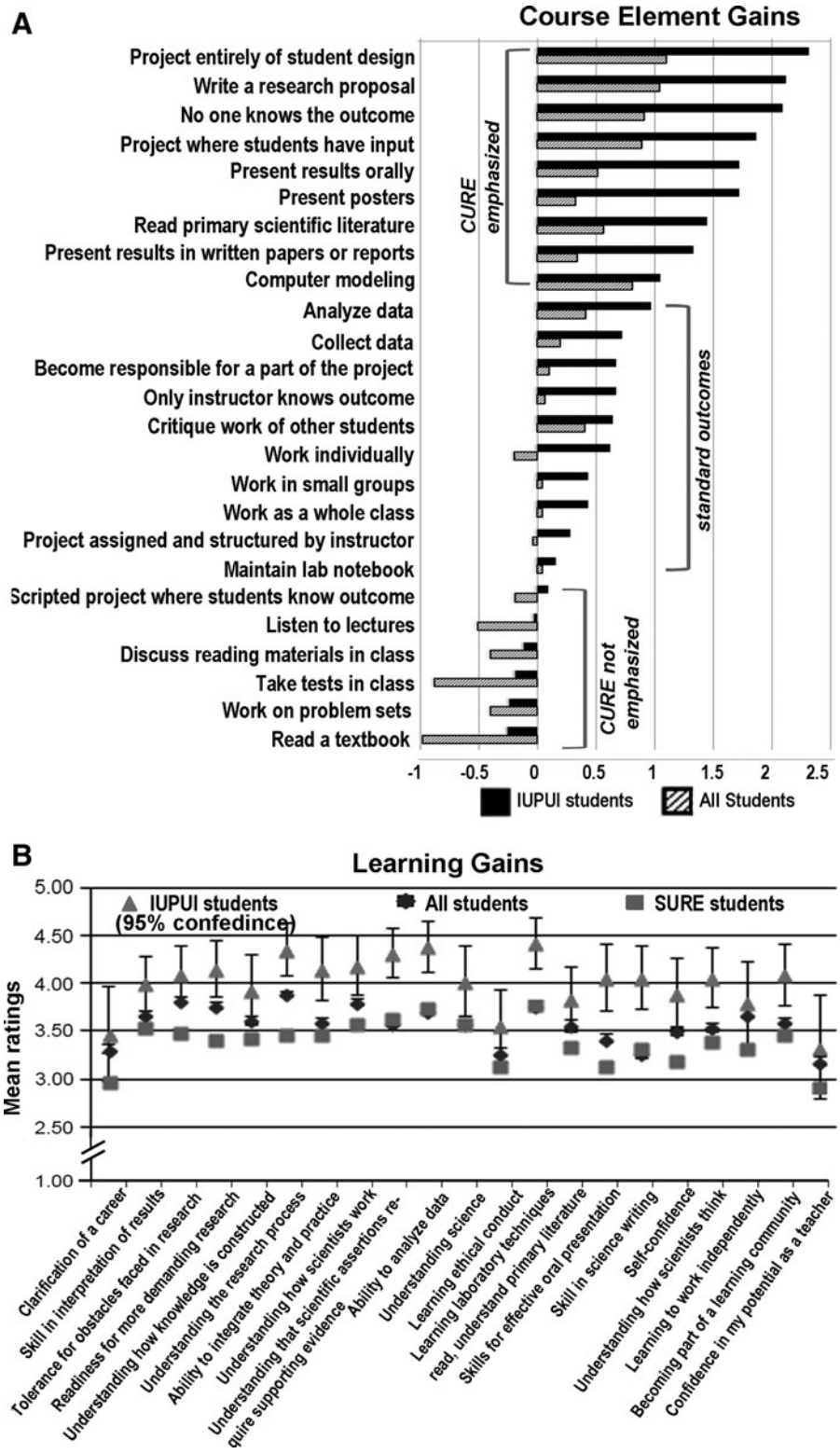

FIG. 3. Results of course-based undergraduate research experience (CURE) survey showed course elements gains and learning benefits after the course. **(A)** Course element gains comparing Indiana University-Purdue University Indianapolis (IUPUI) students data with ''all student data.'' Students were asked the same questions before and after the course. The 5-point scale, where 1 = no or very small gain to 5 = very large gain was used to rate the gain. The differences of means of postcourse and precourse were used to represent the data. *Bracketed* ''CURE emphasized'' elements were targeted in the CURE, ''standard outcomes'' were general laboratory course elements and ''CURE not emphasized'' elements were de-emphasized in the CURE. **(B)** Comparing learning gains between IUPUI students, all students taking CURE survey, and students taking a Summer Undergraduate Research Experience (SURE) survey. The scale was 1–5, with 5 being the largest gain. Mean were used to represent the data. Survey showed higher learning gains obtained by IUPUI students taking this course. Error bars represent lower and upper bounds of the confidence intervals around the mean.

Evaluating the effectiveness of the CUREs and student's feedback

Introductory Biology course. In fall 2014, we administered the CURE survey developed by David Lopatto[40] to evaluate the effectiveness of the course. Out of 65 students, 38 students took the precourse surveys and 31 students took postcourse surveys. The CURE survey results demonstrated substantial benefit of this course (Fig. 3 and Supplementary CURE survey document). Students gained tremendously in areas that were the primary focus of the CURE, including developing student designed projects, reading primary literature, writing a research proposal, analysis of results from novel experiments, poster presentation, and presenting results in written papers (Fig. 3A). The CURE survey showed students' gains in general laboratory outcomes, including collecting data, analyzing data, taking responsibility for the project, critiquing the work of other students, working individually or as a team, and maintaining lab notebook.

Students showed no change or negative change in the course elements excluded from our CURE by design (Fig. 3A). Student learning outcomes that benefited considerably include understanding of how a scientist works on real problem, increased interest in science, high ability to analyze information, understanding laboratory techniques, increased ability to read and understand primary literature, communication skills giving oral presentation and, importantly, becoming part of learning community (Fig. 3B).

The CURE survey comparison between IUPUI students data with ''all students data'' showed that IUPUI students gained substantially in each of the items of course elements (Fig. 3A). The ''all students data'' was generated from CURE surveys performed between June 1, 2014 and January 6, 2015 by the group of David Lopatto where 5057 and 4962 students from colleges across the nation participated in pre- and post-surveys, respectively. Comparing learning gains between IUPUI students, all students taking the CURE survey, and students taking a SURE survey showed much higher learning gains obtained by IUPUI students taking the Introductory Biology course (Fig. 3B). Overall assessment of the Introductory Biology course was extremely satisfactory. Assessment questions asked in the CURE survey are shown in Table 3.

Cell Biology course. To evaluate Cell Biology laboratory course, an in house survey was conducted at the end of the course in spring, 2014. The survey suggested that students learned substantially (Table 3). All students who responded to the survey (17 out of 21) reported that they enjoyed the course. Students agreed that authentic research laboratories should be included in other courses as well. Students reported improvement in scientific confidence, performing analysis, interpreting results, and presenting research data. Surveys indicated that the workload was appropriate, and tutorials given by the instructors provided an adequate introduction to the topics (Table 3).

Students comments on the course model were very positive and included the following: ''I enjoyed actually doing lab work with a real research project rather than do labs from a lab manual''; ''I enjoyed getting a wet lab experience and completing meaningful research. I also appreciated the experience of creating a poster and presenting data''; ''Real life applications behind the research'' and ''I like the level of independence but still appreciate the structure that came with it.'' The students also gave constructive criticism, for example, ''I enjoyed the work done in the lab as well as the instruction I received. I do feel it would be nice to have the major projects (bioinformatics and effect of environmental toxicants on development) spread throughout the semester, as it is difficult to perform both big projects one after another.'' Student feedback on how to improve the course included the following comments: ''I'd suggest that you don't break up the dates, and do it all as one big section, so it all is streamlined and together'' and ''I would like a better Photoshop tutorial (or Adobe Illustrator). Although the pdf provided helped, I had to relearn Photoshop on my own, which I imagine is harder for people who didn't already have experience with it.''

Table 3. Assessment of Research Laboratories

Statement	*Average rating*
Students' attitude assessment after cell biology laboratory course	
After the lab, I have a better understanding of how to approach a research question.	4.59
This course helped increase my skills in performing experiments, analyzing data, interpreting results, and presenting research data.	4.41
I felt the workload was appropriate for the lab.	4.47
The tutorials given by the instructors provided adequate introduction.	4.41
The authentic research lab met my expectations.	4.18
Authentic research labs should be included in other courses as well.	4.59
I am planning to continue in any biomedical field after graduation.	4.12
Did you enjoy the lab? Yes or No	100% (Yes)
Overall assessment of introductory biology laboratory course (CURE result)	
This course was a good way of learning about the subject.	4.48
This course was a good way of learning about the process of scientific research.	4.68
This course had a positive effect on my interest in science.	4.45
I was able to ask questions in this class and get helpful responses.	4.52

Likert scale was used: 1 = Strongly disagree, 2 = Disagree, 3 = Neither agree nor disagree, 4 = Agree, 5 = Strongly agree, except for one yes or no question.

Discussion

Developing a research framework through a zebrafish CURE

CUREs are increasingly becoming an effective and scalable way of broadening participation of undergraduates in research experiences.[14,47] The results reported here support our primary hypothesis that participation in a zebrafish CURE develops undergraduate students' skills in research, communication, and collaboration. Participation in a zebrafish CURE was also effective in developing skills related to a deeper understanding of the research process, experience in analyzing data, tolerance for

overcoming obstacles faced in research, and enhanced science identity, as reported by other investigators implementing CUREs.[6,7,44,45] The complete set of course element and learning gain effects from the zebrafish CURE shown in Figure 3.

Integrating research and teaching through a team approach

Our overall goal was to develop an authentic research project that integrated of the five dimensions of a CURE as described by Auchincloss *et al.*,[14] including broadly relevant or important work, use of scientific practices, collaboration, iteration in discovery research, and communication of scientific findings (Supplementary Fig. S1).

This zebrafish CURE was developed by a faculty team whose members share common interests in developmental biology research and science pedagogy. Our projects were built on the expertise from department members who study zebrafish developmental biology, particularly the effects of ethanol on development[18–21] (J.A.M., S.S., and P.M.). The instructional team included three faculty experienced in teaching the course and laboratory, with no previous expertise in zebrafish (K.A.M., M.A.V., and G.W.C.). The research sponsor (J.A.M.) consulted on experimental design and interpretation. The research assistant professor (S.S.) provided the key role in developing the specific content for each lab and becoming part of the laboratory teaching team, and a graduate student (P.M.) in the research sponsor's lab was a teaching assistant for lab sections, helping those research teams and supporting the required animal husbandry tasks. Development of teams with shared interests undergraduate science education has been reported as an effective and practical way to support the large-scale pedagogical change needed to fully integrate CUREs into a departmental curriculum.[14,47,48]

Student research projects were centered on the hypothesis that nicotine and caffeine exposure during early development affects gastrulation and heart development in zebrafish. The experiments were designed to be sufficiently simple and self-explanatory but able to collect accurate data on observable features, such as body length, eye size, pericardial swelling, epiboly progression, and survival/mortality. The students had no experience with using zebrafish, but within two lab periods, they learned the basics of the zebrafish model system, image analysis to measure morphological traits, and statistical analysis of results. Students performed different experiments as part of a large range of concentrations being tested, using common protocols that could be used to test a wide variety of teratogens. This design allowed students to compare similar experiments, facilitated by using a common Google drive shared by all sections and classes.

The research project explored the following: (1) effects of the same teratogenic compound at different concentrations, (2) effects of same teratogenic compound for different exposure periods in development, and (3) effects of various student identified compounds. This iterative design of flexible protocols, established checks and balances for reproducibility, and openly shared student's research data are key features reported by others to successfully incorporate faculty research into the curriculum.[44,49]

Collaboration is a critical skill in scientific research, bringing diverse perspectives and viewpoints to complex problems.[50,51] To accurately reflect authentic laboratory research, CUREs must incorporate multiple opportunities for collaboration, allowing students to work interactively to address different parts of the same question. Students in the zebrafish worked collaboratively in the lab section and course, but each student was also responsible for the completion of their portion of the project outside class lab work and research without conflict. Thus, students experienced the advantages of working in a collaborative research team, as recommended by many recent national calls.[4,47,48,50,51]

Novel findings and new knowledge generated by the CURE

In our CURE, nicotine exposure during zebrafish embryogenesis produced defects during early development, and defects were persistent to later stages of development. Numerous studies have implicated nicotine exposure *in utero* as factors in neural,[52] behavioral, and cognitive defects[22–24] in studies of animal models and human patients. Nicotine exposure also produces lung defects that may lead to asthma and other diseases.[25–27] Cardiac defects in nicotine-exposed offspring could be induced by stress, but cardiac malformations have not been reported.[28,29,53] Previous zebrafish studies showed defects in motor neuron pathfinding were induced by nicotine exposure during embryogenesis.[52] Our studies used higher concentrations of nicotine than in previous studies, perhaps producing a more extreme phenotype. However, this allowed students to observe and measure changes and generate new knowledge.

Caffeine effects on development were previously described in other experimental models.[54] Effects of caffeine exposure during zebrafish embryogenesis were also detected in this CURE. The combined treatment of nicotine plus caffeine caused more severe defects. This is significant because mothers often use nicotine and caffeine during pregnancy. Future studies could examine developmental effects of nicotine and caffeine when combined with alcohol.

Acute effects of nicotine exposure on heart rate in zebrafish embryos were also examined. This part of the project allowed us to begin discussions with the class, introducing pharmacology concepts on receptor occupation, activation, and attenuation.

Students also observed the actin cytoskeleton and cell shapes in different layers in early embryos. This showed that the students could perform delicate techniques and produce very high quality data. The instructors imaged these embryos using a confocal microscope, but the students were introduced to advanced microscopy concepts and image analysis methods.

In the final portion of the CURE, students proposed their own experiments. Many ideas were pursued, including experiments on various toxicants and experiments testing substances that could protect embryos from toxicants. The enthusiasm for this portion of the CURE was overwhelming. The student researchers were extremely creative and worked hard to explore new ideas. Some compounds that were proposed were not feasible, for example, testing controlled substances like drugs of abuse. However, the instructors were able to discuss possibilities with the students and arrive at feasible alternative experiments. Extra work was required from the instructors to set up the proposed student experiments. The experiments were very successful. Testing their own ideas was very rewarding and engaging, which produced an outstanding feeling of accomplishment among the students.

Conclusion

Development of research experiences in the early undergraduate STEM curriculum is a key recommendation by the 2011 *Vision and Change*[3] and the 2012 PCAST[4] reports. Our CURE was successfully provided to over 130 undergraduate students. Students generated reproducible data. Students' data was compared with data obtained from previous semesters, extending continuity of the project. These courses fostered high-level discussions and generated novel findings that will be extended in successive semesters. We conclude that the CURE approach provides an efficient, scalable means for extending research opportunities to a larger number of students than is afforded by independent study and summer programs. This approach produced substantial excitement and enthusiasm in all students and substantial learning gains.

The CURE introduced students to research, which they might even consider in their future careers. Beyond generating novel findings, learning about the effects of different drugs on development provided students opportunities to learn real life lessons that would have impact beyond the classroom. The level of engagement, enthusiasm, and excitement among the students led to increased learning and greater course satisfaction, which, in our opinion, was the most significant outcome of this CURE approach.

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Disclosure Statement

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