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Publication Date

2024-07-16

DOI

10.1002/bmb.21851

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Received: 24 October 2023 | Revised: 5 June 2024 | Accepted: 24 June 2024

DOI: 10.1002/bmb.21851

ARTICLE

From primers to pipettes: An immersive course introducing high school students to qPCR for quantifying chemical defense gene expression

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Funding information
National Institute of Food and Agriculture, Grant/Award Number: CA-D-ETX-2526-H; UC Davis Academic Senate Large Grant; The PAm Costco Scholarship

Abstract

We created a 2-week, dual-module summer course introducing high school students to environmental toxicology by teaching them quantitative polymerase chain reaction (qPCR) as a way to quantify gene expression of chemical defense proteins in response to exposure to environmental pollutants. During the course, students are guided through the various stages of a successful qPCR experiment: in silico primer design and quality control, total RNA extraction and isolation, cDNA conversion, primer test PCR, and evaluation of results via agarose gel electrophoresis or UV/Vis spectra. The course combines lectures, discussions, and demonstrations with dry and wet laboratory sections to give students a thorough understanding of the scope, utility, and chemical principles of qPCR. At the end of the course, the students are taught how to analyze qPCR data and are encouraged to discuss their findings with other classmates to evaluate their hypotheses and assess possible sources of error. This course was designed to be easily adaptable to multiple test species, chemical exposures, and genes of interest. To explore both terrestrial and aquatic toxicology, the students use honey bees (*Apis mellifera*) and mosquitofish (*Gambusia affinis*) as test organisms, as well as ABC-type efflux transporters, antioxidant enzymes, and cytochrome P450 enzymes as endpoints for assessing gene expression. We share this course setup and applied protocols to encourage others to design and offer similar courses that give high school students a hands-on introduction to a broad swath of environmental toxicology research and an opportunity to develop scientific skills necessary for university-level research.

KEYWORDS

active learning, gene expression, high school, involvement of high school students in research, molecular biology, transport through membranes

Zeke T. Spooner and Angela M. Encerrado-Manriquez are co-first authors.

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1 | INTRODUCTION

Environmental toxicology is a multidisciplinary field of study at the interface of chemistry, biology, and pharmacology that is rarely given attention during secondary education in the United States, instead typically being reserved for higher education settings.[1] Perhaps one of the main challenges educators dread when attempting to introduce high school students to toxicology is the lack of previous laboratory experience the students may have, putting them at risk of toxic chemical exposures.[1] Nevertheless, its multidisciplinary nature provides a unique opportunity for students to connect the concepts of analytical chemistry and molecular biology, developing their critical thinking skills in the study of toxic chemicals and their effects on people and the environment. Furthermore, environmental toxicology allows for chemistry and biology concepts learned in class to be applied into real world scenarios, further developing the student's curiosity for science. Given that some students' strengths lean toward biology, while others are more interested in chemistry, and still others are keen about both fields, and considering the safety of the students, we selected a field of study within environmental toxicology for the course—chemical stress-induced gene expression—that we believed would interest all types of students through a focus on chemical detoxification and the biochemical principles underlying gene expression, while providing a safe space for students to take their first steps into applied laboratory sciences.

Chemical stress-induced gene expression not only brings these broad fields together in the context of environmental toxicology but allows for the use of quantitative molecular biology techniques suitable for a high school level of instruction. The quantitative polymerase chain reaction (qPCR) is a popular, simple, and rapid method of quantifying nucleic acids.[2] Because it is easy to learn, minimally hazardous, technically simple, and broadly applicable, it is an ideal method for giving hands-on experience to secondary students new to the field. Historically, qPCR has been used in the field of toxicology to examine toxicant-induced changes in gene expression.[3,4] Many studies have shown that environmental exposure to a multitude of xenobiotic chemicals, including pesticides,[5] household chemicals,[6] natural biotoxins,[7] and even transportation pollutants (such as emissions from cars[8] and oil from maritime activity[9]) can cause changes in gene expression of exposed organisms.

Although a wide variety of organisms are affected by unwanted chemical exposures in their environment, this course will focus on two model species: the Western Mosquitofish (*Gambusia affinis*) and the Western honey bee (*Apis mellifera*). As its name suggests, the mosquitofish is widely used to control mosquitoes in small bodies of water such as agricultural and urban ponds.[10] Living in these environments can expose these fish to industrial, agricultural, and household chemicals that could affect gene expression. For this course, we use mosquitofish caught from the UC Davis Wastewater Treatment Plant (WWTP), where they are used for mosquito control in semi-treated sedimentation tanks. This wastewater may contain urban runoff, household chemicals, and other contaminants related to human waste. This potentially harmful chemical environment could elicit a change in gene expression in these fish to better fend off chemical accumulation and toxicity. Unexposed control fish will be obtained from a UC Davis IACUC-approved biological specimen supply company (Carolina Biological Supply Co., Burlington, NC). Our second model species is widely familiar to high school students. Honey bees are essential pollinators worldwide and the primary commercially used pollinator in the United States, adding billions of dollars in revenue to crop production yearly.[11] Although mitigation strategies are in place during commercial pollination, honey bees are often exposed to multiple pesticides simultaneously. These pesticides (spanning insecticides, herbicides, fungicides, and miticides) are potentially toxic to individual bees and the general hive. They also could affect the detoxification mechanisms of the hive, resulting in a change in gene expression to lessen toxicity.[12] We thought the billion-dollar almond industry in the United States would offer students a real-life example, given that they are one of the main users of pollination services. We identified three pesticides currently used to control pest populations in almond orchards: Diflubenzuron, Methoxyfenozide, and Pyriproxyfen (Figure 1). The bees used in this class had been previously exposed to these pesticides in a controlled laboratory setting to study their effects on detoxification gene expression.

The goal of this course is to introduce university-level research in environmental toxicology to high school students. The use of environmentally relevant and easily accessible model species is key to achieving this aim. At its core, this course is an environmental toxicology research project that uses qPCR as its main tool to understand chemical exposure effects on detoxification gene expression. Throughout the course, students are taught the chemical principles behind qPCR and chemical detoxification and how to design a qPCR experiment and perform the steps to quantify the gene expression of ABC-type transporter proteins, cytochrome P450 enzymes, and antioxidant enzymes in mosquitofish and honey bees. However, the flexibility of qPCR allows for the easy adjustment of which genes and organisms are studied. In this article, we present the methods we used for administering this course and an organizational map that can be adapted to the design of other courses

FIGURE 1 Chemical structures of three different pesticides used to expose honey bees studied in this course. All three pesticides belong to the group of insect growth regulators (IGR) that are used to selectively inhibit the life cycle of pest insects.

targeting specific genes, chemical detoxification pathways, and organisms of interest.

1.1 | Learning objectives

We created a set of learning objectives that reflected our expectations for students after completing the two-module course. These objectives reflect our goal of creating a low-stakes classroom environment where students can explore their budding interest in environmental toxicology. These learning goals emphasize not only detoxification, but a deep understanding of the methods used in the course to study it. Learning goals were designed around key chemical principles, with students reinforcing their comprehension through lectures, a laboratory manual (see Supporting Information A in Data S1), and collaborative discussions, culminating in their application in hands-on laboratory work.

1. Students will demonstrate proficiency in the use of bioinformatic software and modern databases through a series of guided assignments (see Supporting Information A in Data S1).
2. Students will demonstrate a grasp of the scientific method and its application to toxicological research through oral discussion and hypothesis development and evaluation.
3. Students will demonstrate knowledge of the basic chemical and biochemical principles of detoxification through in-class oral discussions of important detoxification proteins that correspond to the genes analyzed in the class laboratory project.
4. Students will demonstrate an understanding of the theory, applications, and practice of qPCR through laboratory exercises, guided assignments, and oral discussion. Examples of emphasized topics include nucleic acid chemistry and the chemical and biochemical differences between DNA and RNA, the thermodynamics of nucleic acid binding and extension as it relates to primer binding and the phases of PCR, and the physical chemistry behind fluorescence and why this is important for qPCR.
5. Students will demonstrate key laboratory skills (including safety procedures and pipette work) with an emphasis on developing and improving technique and safe handling of chemicals through laboratory exercises.
6. Students will further demonstrate the ability to ensure high-quality, quantifiable data through the implementation of standards (such as nucleic acid ladders in gels and reference genes in qPCR) and quality control at each step of the qPCR process.

2 | METHODS

2.1 | Experimental overview

Figure 2 shows the modular setup and individual tasks of the experimental workflow for the course. This two-

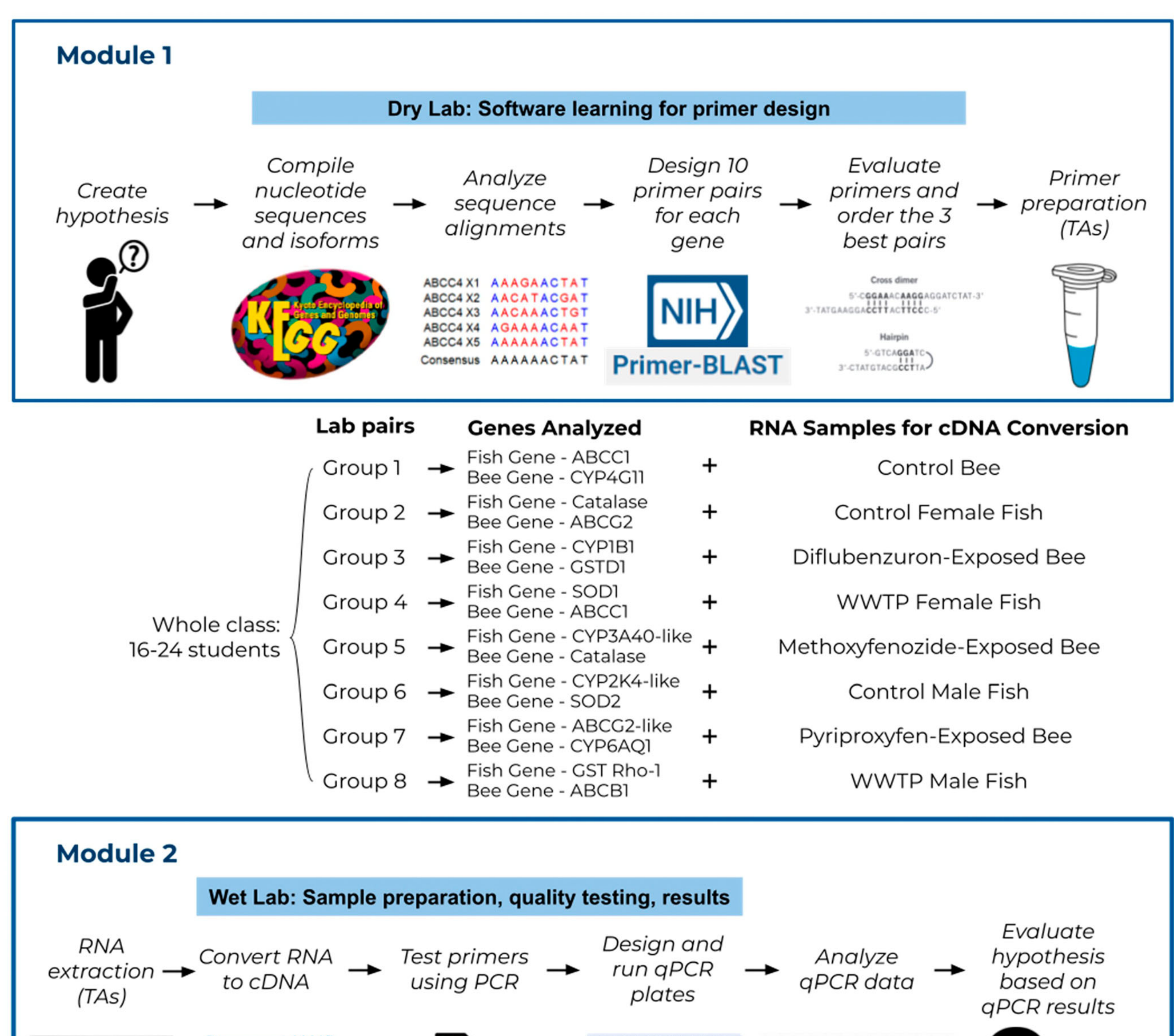

FIGURE 2 Organizational map showing course workflow and class assignment distribution. Days began with lectures and discussions based on the course content of that day, followed by TA walkthrough demonstrations of software use and laboratory techniques, and ended with students applying their knowledge in various activities and assignments. During Module 1, students become familiar with open-access modern databases and software commonly used in academic fields to study genes of interest and create qPCR primers. During Module 2, students move to a wet lab environment where they practice their laboratory skills by executing a qPCR protocol from start to finish. The middle diagram shows how we divided students into groups to distribute work, including how we allocated genes for them to research and RNA samples to perform cDNA conversion.

module course can be adapted for 16–24 high school students, divided into groups of two or three, that meet for 6 h of class time per day. These 6 h are generally divided into 1-h lectures, discussions, and walkthroughs in the mornings, with 3-h hands-on activities and guided assignments in the afternoons. The first module takes place in a dry lab setting, where students receive lectures on the chemical and biochemical principles behind cellular detoxification and PCR and familiarize themselves with modern bioinformatics databases and software used

for sequence analysis and primer design. The second module moves to the wet lab, where students are trained to carry out the steps of a qPCR experiment, ensure the quality of their samples at each step, and analyze their qPCR data. Before starting any type of wet lab activities, a class is specially designated to give students instruction on laboratory safety and etiquette, as well as an introduction to micro-pipetting.

In collaboration with the UC Davis Pre-College Program, this course has been successfully run in person and online for three consecutive summers (2021–2023). In addition to the instructor, three TAs provide instructional and laboratory support during the course. In the interest of time and conservation of materials, students are divided into groups of two or three to perform laboratory procedures and computational analysis so that the work is equally divided. The class was designed to be informative, interactive, and minimally stressful, so assignments are not graded, and coursework can be completed entirely during class time (i.e., no homework). However, students are expected to engage in a class discussion at the end of the course, sharing their research project results and their interpretation of them.

2.2 | Specimen acquisition

Prior to the course, honey bees (nurse bees) are collected with the help of our collaborators at the laboratory of Dr. Julia Fine from the USDA-ARS facility (Davis, CA), while mosquitofish are collected from two sources—unexposed fish from the Carolina Biological Supply Company (Burlington, NC) and exposed fish from the UC Davis Wastewater Treatment Plant (Davis, CA). Fish are euthanized in accordance with an approved IACUC-approved protocol (#23388) by use of 1 g/L Buffered Tricaine Methanesulfonate (Spectrum, New Brunswick, NJ, USA). Honey bees are euthanized by flash freezing in liquid nitrogen. Upon euthanasia all frozen tissue is stored at −80°C until homogenization.

2.3 | MODULE 1: Bioinformatic databases and software for sequence analysis and primer design

2.3.1 | Generating research hypotheses

One of the first lessons in the first module is designed to give students the necessary background information on both species and exposures. As a result, each group of students is encouraged to develop a hypothesis about the relative expression of detoxification genes in each of their experimental systems. Here are some examples of hypotheses that students created for their research project:

- "There will be increased expression of defense proteins in honey bees exposed to almond-crop pesticides than those not exposed."
- "There will be increased expression of some defense proteins, like xenobiotic efflux transporters, and decreased expression in others, like antioxidant enzymes, in honey bees exposed to almond-crop pesticides relative to unexposed bees."
- "There will be increased expression of defense proteins in mosquitofish from wastewater sedimentation tanks relative to store-bought mosquitofish."
- "There will be a difference in expression of defense proteins in male and female mosquitofish from wastewater sedimentation tanks."
- "There will be a difference in expression of defense proteins in male and female mosquitofish, regardless of chemical environment."

2.3.2 | Bioinformatics and primer design

The first module involves hands-on assignments that teach students how to begin a qPCR project by compiling information from databases and analyzing it using modern software. To properly analyze and create primers for their assigned ABC-type transporter, students must first learn how to search for and extract relevant information from genetic databases. For each software application and database, students are given an in-class assignment (see Supporting Information A in Data S1). All assignments serve as an intermediate tool to assess what students have learned on a particular day and allow students to practice their skills and apply their knowledge to their research project.

1. *Kyoto Encyclopedia of Genes and Genomes (KEGG) and the National Center for Biotechnology Information (NCBI)*: Databases on genes and proteins used to extract names, sequences, accession numbers, gene IDs and nucleotide and protein lengths.[13] The goal is for students to use these databases to compile a list of genes and isoforms for their assigned defense genes in the organisms of interest. In Assignment 1A (see Supporting Information A in Data S1), students fill out a table listing relevant metadata for all isoforms of their genes of interest.
2. *NCBI Basic Local Alignment Search Tool (BLAST)*: An online tool that searches the NCBI database for similar nucleotide or amino acid sequences to the search

query.[14] The goal is for students to ensure that all assigned genes and isoforms were found by running the genes they retrieved from KEGG and NCBI through this tool. Students then tabulate this information for use in isoform analysis and primer design. In Assignment 1B (see Supporting Information A in Data S1), students upload a printout of the top 100 BLAST results for their genes of interest.

3. *CLC Main Workbench 23.0.4 (Qiagen, Aarhus, Denmark)*: Software for nucleic acid and protein sequence comparison and analysis. The goal with CLC is for students to create alignments of all isoforms for a given gene and see how different the isoforms are. If the isoforms are very different, then different primers need to be designed for them; if they are largely the same, then one primer pair can be used to capture all the isoforms. In Assignment 2 (see Supporting Information A in Data S1), students upload printouts of their isoform alignments and log any gaps or areas of non-consensus in a spreadsheet.
4. *NCBI Primer BLAST and Beacon Designer Free Edition (Premier Biosoft International, Palo Alto, CA)*: Two different types of software used to design PCR primers[15] and analyze the quality of those primers, respectively. The goal is for students to design three primer pairs for their given genes. Using Primer BLAST, 10 qPCR primer pairs are generated for each gene. These primers were between 18 and 25 nucleotides in length. The targeted amplicon sizes (products of qPCR amplification) were between 80 and 200 nucleotides. The students are then tasked with picking the optimal three primer pairs from the 10 contenders by evaluating their ability to dimerize and form hairpins (as estimated by Beacon Designer). The open-source software we use for the class is recommended for qPCR primer design for SYBR® Green assays.[16] In Assignment 3A (see Supporting Information A in Data S1), students complete a spreadsheet that documents the sequences, properties, and Beacon Designer analysis of their 10 contenders and highlight their top three picks.
5. *SnapGene® Viewer (from Domatics; available at snapgene.com)*: Software used for the visualization and annotation of genes. The goal for students is to ensure that their primers will bind to the gene sequence of interest and produce the predicted amplicon by annotating their genes with their primers. In Assignment 3B (see Supporting Information A in Data S1), students use SnapGene® Viewer to perform restriction enzyme digestions on their genes of interest and a plasmid and virtually predict how their resulting fragments would visualize on an agarose gel after the digestion.

After deciding on the top three primers for each gene, the results are compiled, and the instructor and TAs order these primers from an online sequencing service, such as Eurofins Genomics (https://eurofinsgenomics.com).

2.4 | MODULE 2: Sample preparation, quality checks, qPCR run, and data analysis

2.4.1 | Lab and chemical safety, etiquette, and micro-pipetting techniques

For high school students with little to no experience in the laboratory setting, learning good safety habits is of utmost importance. A 2-h safety lecture was developed in which we instructed students on proper techniques of donning and doffing PPE with an emphasis on glove usage, principles of safe laboratory practices and chemical handling, and proper micropipette operation. Students are required to wear lab coats, gloves, and safety glasses at all times in the lab. The reagents used are nonhazardous and there are only a few risks associated with their use (these include the use of the flammable liquid ethanol and handling very hot agarose solution); nonetheless, students are instructed on proper chemical handling in order to minimize risk of exposure and spills. Students are required to use heat-resistant gloves while handling hot glassware during the gel electrophoresis lessons.

2.4.2 | Agarose gel electrophoresis

Throughout the course, students are trained in using agarose gel electrophoresis to evaluate the quality of their obtained tRNA and generated cDNA and primer pairs. Students are taught about agarose polymerization and fluorescent compounds (i.e., stain and loading dye) used for visualizing bands, then create two gels: a "real" gel containing stain and a "mock" gel that did not contain stain. This is so every group has a chance to cast and load an RNA or cDNA gel (despite there being more groups than necessary gels). The students in this course create a total of 4 mock gels, 2 tRNA gels, 2 cDNA gels, and 4 test PCR gels (Figure 3).

Gels are 2% agarose (Bio-Rad, Hercules, CA, USA) in 1× TAE buffer (Bio-Rad, Hercules, CA, USA) stained with SYBR® Safe stain (Bio-Rad, Hercules, CA, USA) by spiking 5 μL per 50 mL of gel into the hot solution before pouring. Before the students test real samples, they are trained in creating mock gels (without stain) and loading store-bought food coloring dye to practice the gel

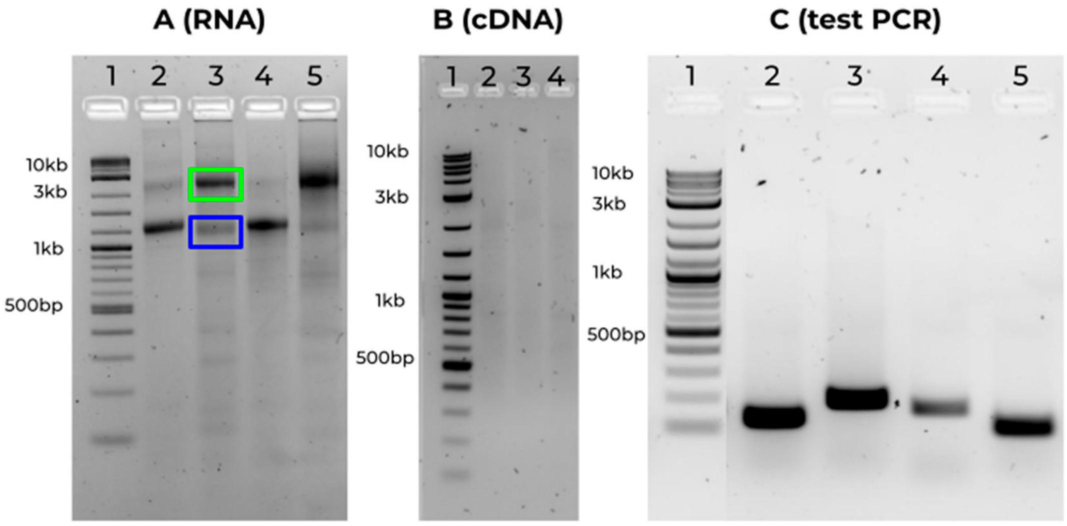

FIGURE 3 Agarose gel electrophoresis to evaluate quality of RNA and DNA products. The agarose gel is 2% agarose in TAE buffer. The first lane (1) in each gel contains a 1 kb Plus DNA ladder #N3200S from NEB. Individual ladder bands below 1 kb are in steps of 100 bp. (a) Honey bee and mosquitofish RNA gel with honey bee RNA samples in lanes 2 and 4 and mosquitofish RNA samples in lanes 3 and 5. The green box indicates the ubiquitous 28S RNA, and the blue box indicates 18S RNA to better evaluate total RNA extraction efficiency and quality. (b) Honey bee cDNA gel with cDNA samples in lanes 2–4. (c) Primer test PCR for honey bee qPCR primers, demonstrating successful and clean amplification of ABCB1 (lanes 2–3) and ABCG4 gene fragments (lanes 4–5).

loading procedure. When loading the dyes with sample, students mix 3 μL of sample with 0.6 μL of Monarch 6× Purple Loading Dye (New England Biolabs, Ipswich, MA, USA) on parafilm and load the resulting mixture into the gel lanes. Marker lanes are used as internal standards for all gels, so students are instructed to load 3 μL of Monarch 1 kb DNA ladder (New England Biolabs, Ipswich, MA, USA). A Mini-Sub Cell GT System with 75 W PowerPac power supply (Bio-Rad, Hercules, CA, USA) is used for running agarose gels for 60 min at 100 V before viewing them on a ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA, USA). A preliminary written gel loading design of each gel is encouraged to help the students keep track of their samples during the loading process of their research project.

2.4.3 | Pre-lab preparation: Total RNA (tRNA) extraction

Due to time constraints in this 2-week course and the advanced difficulty of the protocol, a total RNA extraction is performed by the TAs beforehand and stored at −80°C until use. Tissues are homogenized using the BeadBug™ 6 Six-Position Homogenizer (Benchmark Scientific, Sayreville, NJ, USA) and 2.8 mm steel beads (Omni International, Kennesaw, Georgia, USA). The Monarch® Total RNA Miniprep Kit (New England Biolabs, Ipswich, MA, USA) is used to obtain high-quality RNA from the honey bee and mosquitofish samples. Whole bees weighing approximately 100 mg and whole fish weighing approximately 200 mg are used in this protocol, and samples are homogenized in buffer provided with the kit for three cycles of 30 s at 6 m/s with 45-min dwell periods. Four different RNA samples are extracted for each model species: for bees, one unexposed control sample and three samples exposed to different pesticides (pyriproxyfen, diflubenzuron, and methoxyfenozide); and for fish, two control samples and two wastewater-exposed samples. Only nurse bees are used, while one male and one female mosquitofish are used for each treatment.

RNA quality is important because it can affect the validity of all downstream processes, and poor extractions of RNA could prevent students from obtaining viable results. Therefore, RNA yield and possible protein contamination are assessed before the class using a DS-11+ Nanovolume and Cuvette Mode Spectrophotometer (DeNovix, Wilmington, DE, USA), while RNA quality and integrity is assessed using agarose gel electrophoresis (see Figure 3a) by the TAs prior to any student wet lab activities.

2.4.4 | Total RNA (tRNA) to complementary DNA (cDNA) conversion

After obtaining total RNA, including messenger or mRNA, which describes the transcriptional variance in the genes of interest, students must convert the RNA into a form suitable for PCR amplification through cDNA

TABLE 1 Tabulated volumes of reagents necessary to carry out cDNA conversion using the SuperScript IV First-Strand Synthesis System from Invitrogen.

Reagent	Volume
DEPC-treated water	V_{H2O} (as calculated above)
5× SSIV buffer	4 μL
10 mM dNTP mix	1 μL
100 mM DTT	1 μL
Rnase inhibitor	1 μL
Prepared total RNA	V_{RNA} (as calculated above) up to 11 μL
SuperScript IV reverse transcriptase	1 μL
Oligo d(T)$_{20}$ primer	0.5 μL
Random hexamers primer	0.5 μL
Total	20 μL

conversion (Table 1). The cDNA for the qPCR reaction is made using previously prepared tRNA and SuperScript IV First-Strand Synthesis System (Invitrogen, Thermo Fisher Scientific, Waltham, MA). Students are guided through the process of calculating the amount of RNA to use in order to yield 1000 ng of cDNA for each reaction according to the following equation:

$$[\text{RNA}]\ (\text{ng}/\mu\text{L}) = \frac{1000\,\text{ng}}{V_{\text{RNA}}(\mu\text{L})} \rightarrow V_{\text{RNA}}\ (\mu\text{L}) = \frac{1000\,\text{ng}}{[\text{RNA}]\ (\text{ng}/\mu\text{L})}$$

$$V_{\text{H}_2\text{O}} = 11\,\mu\text{L} - V_{\text{RNA}}$$

The above equation details the calculations needed for determining the volumes of RNA and water necessary to synthesize 1000 ng of cDNA during cDNA conversion. Students were guided through these calculations prior to the cDNA conversion protocol. The calculations assume 100% conversion from RNA to cDNA, and the company protocol for 20 μL reactions is used. The only modification made concerned the primers used for the reverse transcriptase. Rather than 1 μL of Oligo d(T)$_{20}$ or 1 μL of random hexamers, our protocol uses 0.5 μL of each (additionally, the optional RNA removal step is not performed).

The students then get additional practice with agarose gel electrophoresis by analyzing the quality of their converted cDNA in another set of gels (Figure 3b).

2.4.5 | Primer test PCR

In order to maximize the quality and consistency of our primers and the generated cDNA, all three primer pairs ordered for each gene are tested using a test PCR reaction. Test PCR is carried out using GoTaq® Green Master Mix (Promega, Madison, WI, USA). The company protocol outlines a 25 μL reaction, but the protocol used scales this down to 20 μL but keeps the proportions of reagents consistent. In a PCR tube, the students combine 10 μL of GoTaq master mix with 7 μL of water and 1 μL each of cDNA, forward primer, and reverse primer. The steps for the PCR incubation conditions are as follows:

- 2 min at 95°C ("hot start")
- 30 cycles of: 30 s at 95°C; 30 s at 64°C; 1 min at 72°C, and
- 5 min at 72°C for the final elongation of products.

After incubation, 4 μL of the same 6× Purple Loading Dye is combined with the completed PCR reaction, and 20 μL of the resulting mix is loaded into a 2% agarose gel (created using the same method described above) and run for 30 min at 100 V. The students are instructed to evaluate the quality of their primers by examining the gel and determining whether one clear, strong band appears at the correct amplicon length. Students look for one strong band at the predicted amplicon size on the resulting gel (Figure 3c). A lack of bands, a very faint band, the presence of multiple bands, or dense primer clouds could indicate a poor primer pair.

3 | RESULTS

3.1 | Product and data quality assessments

3.1.1 | RNA quality assessment

As previously mentioned, the quality of reagents and products is tested through various means for each step of the qPCR protocol. Our main method of validation is through using agarose gel electrophoresis, a widely-accepted way of evaluating RNA quality.[17,18] Most of the extracted total RNA is typically ribosomal RNA, which is primarily visible as two clear bands representing the two ribosomal subunits 28S (green box, Figure 3a) and 18S (blue box, Figure 3a). Students are taught to first look at the ladder spread to evaluate the quality of the run (M, Figure 3a), checking for good separation between bands and no misshapen bands. Then, students look for prominent bands in their sample wells corresponding to these two indicator bands in their gels as a quick and easy way to evaluate the quality of their provided total RNA samples. A secondary method to determine RNA quality is using the DeNovix Nanodrop. This instrument allows us to determine the RNA's yield and contamination levels by the absorbance ratio at 260 nm (which corresponds to

nucleotides in DNA and RNA) with contaminant absorbing at either 280 nm (e.g., proteins, detergents) and 230 nm (e.g., solvents, salts). The RNA samples are deemed high-quality if the 260/280 and 260/230 ratios are above an accepted threshold of 1.7 and ideally close to 2.0 for pure RNA.

3.1.2 | cDNA quality assessment

As seen in Figure 3b, an agarose gel is also used to evaluate the quality of the cDNA that the students create. As seen by the figure, the cDNA gels should always show a consistent smear from high-molecular weight to low-molecular weight, indicative of full conversion of small to large mRNA templates into cDNA.

3.1.3 | Primer quality assessment

As shown in Figure 3c, an agarose gel is used to assess the results of the test PCR as well. The qPCR amplicons vary in length but are generally between 80 and 200 bp. A strong band is expected to be seen at the predicted amplicon length for each primer pair.

3.2 | qPCR: Experimental setup

Once optimal primers and high-quality cDNA are obtained, the students assess the relative expression of their genes of interest using qPCR. Prior to running qPCR plates, 96-well diagrams are made during class time. Reaction sizes are set to 20 μL to keep volumes at a reasonable amount. Samples are loaded into each well following the company protocol recommendations: 10 μL of SsoAdvanced Universal SYBR® Green Supermix (Bio-Rad), 7 μL of PCR-grade water, 1 μL of the 10 μM solutions of forward and reverse primer, and 1 μL of the 10 ng/μL prepared cDNA. This is incubated in a QuantStudio 3 qPCR system (Thermo Fisher Scientific, Waltham, MA, USA) in three cycles:

- A hold stage for 30 s at 98°C,
- A 40-cycle PCR stage of 15 s at 98°C followed by 30 s at 60°C,
- A melt curve stage of 15 s at 95°C followed by 1 min at 60°C,
- And a final 15 s at 95°C.

Multiple reference genes (stable, highly expressed genes used to account for differences in expression unrelated to the treatment) are used for both honey bees and mosquitofish. The pre-selected honey bee reference genes are glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ribosomal protein S5 (RS5). Mosquitofish reference genes are Beta Actin 1 (ACTB1) and ribosomal protein L8 (RPL5).

3.3 | qPCR: Data quality assessment

A qualitative way to assess whether amplification has occurred in any given well in a qPCR plate is by examining the amplification plots (Figure 4a). Generally, successful qPCR reactions display a sigmoidal amplification curve.[19] Seeing an array of sigmoidal curves indicates that the qPCR was successful in most (if not all) of the wells. Two examples of good-quality and poor-quality amplification plots from the students' qPCR experiments are depicted in Figure 4b,d, respectively. During the analysis, students are shown how to assess wells for potential contamination and/or nonspecific amplification using the melt curve data (Figure 4c,e).[20] If a given well has more than one melting point, then there is a possibility that the well was contaminated. Students are encouraged to observe how their results change when including or excluding the data that is deemed untrustworthy by the melt curve analysis. Oftentimes, the wells with multiple melting temperatures have a vastly different cycle threshold (C_T) value than those with a single melting temperature. Including this in the averaged cycle threshold across their three primer sets can alter the results significantly, especially for contaminated wells in control gene plates, as those plates are used for comparison with all of the other plates.

3.4 | qPCR: Data analysis and reporting

The final qPCR data is given to students for in-class discussion and analysis. Students are guided through the use of the Livak Method for analyzing the data they obtain.[19] They are taught to identify C_T values, eliminate erroneous data points based on melt curve analysis, and calculate ΔC_T and $\Delta\Delta C_T$ values. The ΔC_T and $\Delta\Delta C_T$ calculations are as follows:

$$\Delta C_T = C_T(\text{chemical defense gene}) - \text{geometric mean of all } C_T(\text{reference gene})$$

$$\Delta\Delta C_T = \Delta C_T(\text{treated sample}) - \Delta C_T(\text{control sample})$$

Students are then instructed to find C_T values in their data sheets and to calculate ΔC_T (for comparing the

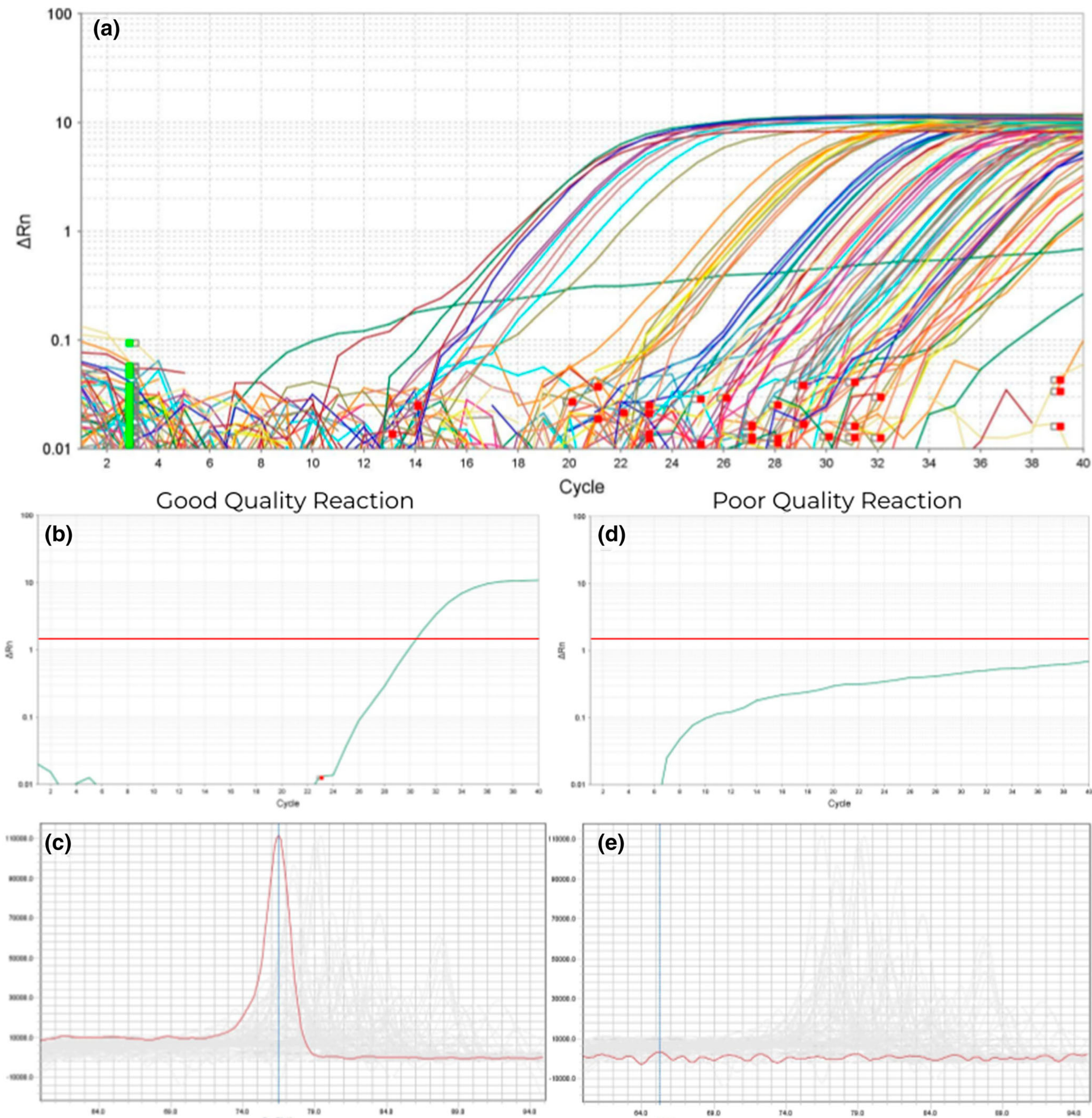

FIGURE 4 Amplification plot of honey bee control samples and examples of good and poor-quality qPCR reactions based on gene amplification and melt curve profiles. These plots were generated by the qPCR machine analyzing the qPCR plate that students loaded using primers they designed and tested with their converted cDNA. (a) Full amplification plots for all genes analyzed in this course shows the variance in expression of different genes. (b) A successful qPCR reaction for gene SOD2, with the signal crossing the threshold value of 1.418 as indicated by the red line; this is validated by (c) the melt curve plot for the same well, which has a single, strong peak. (d) An unsuccessful qPCR reaction for gene SOD2, with the signal failing to cross the threshold value; this is further demonstrated by (e) the melt curve plot for the same well, which has several weak peaks and melting temperatures.

expression of their gene of interest relative to a reference gene), $\Delta\Delta C_T$ (for comparing relative expression of treatment and control organisms), and $2^{-\Delta\Delta CT}$ (to understand the fold change in expression across treatments).[21] An example of some of the students' $\Delta\Delta C_T$ values is displayed in Figure 5.

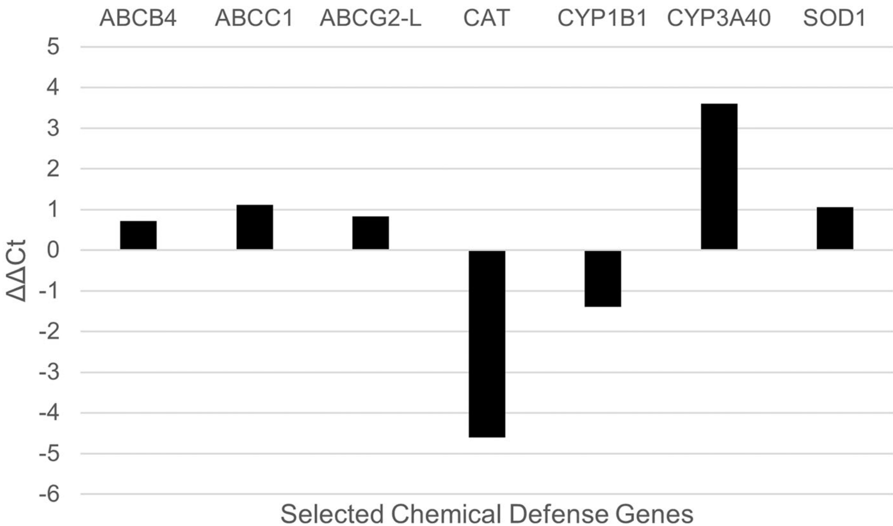

FIGURE 5 An example bar graph displaying the $\Delta\Delta C_T$ values for relative expression of chemical defense genes in exposed versus unexposed mosquitofish. Results are displayed for selected genes of the female wastewater-exposed fish versus the female control fish. Negative $\Delta\Delta C_T$ values indicate relative upregulation of a gene (in the exposed vs. unexposed treatments) while positive $\Delta\Delta C_T$ values indicate relative downregulation of a gene. These values are on a logarithmic scale (every time the $\Delta\Delta C_T$ increases by 1, the gene expression doubles). Here, the $\Delta\Delta C_T$ values show a slight downregulation of the ABCB4, ABCC1, ABCG2-L, and SOD1 genes, a slight upregulation of the CYP1B1 gene, a strong downregulation of the CYP3A40 gene, and a strong upregulation of the Catalase gene.

4 | STUDENT LEARNING ASSESSMENT

All results from this 2-week course are reviewed and discussed in an open group discussion session at the end of the second week. The analyzed data from each group is displayed in class for better relative comparison of gene expression profiles, stratified by species, sex, and gene of interest. Students were encouraged to discuss their research project data and relate it to their initial hypothesis. In addition, students can peer-evaluate data from other groups (and previous year data) that could inform their results and help reject or confirm their hypothesis.

4.1 | Intermediate learning assessments via discussions and in-class assignments

Our discussions and in-class assignments help us understand to what extent students are achieving the learning goals presented above. In-class discussions are focused on the first three learning goals. These discussions go in-depth on topics such as detoxification proteins, the PCR process, and qPCR data analysis. They give students an opportunity to share what they have learned and ask questions and gives us an idea of what concepts need further reinforcement. The primary focus of in-class assignments is on the fourth learning goal, bioinformatics, as well as the fifth learning goal, hypothesis testing. The assignments were designed to give students an opportunity to demonstrate their ability to use modern databases and their command of state-of-the-art bioinformatics software in the context of answering their hypotheses. Additionally, these assignments emphasize chemical principles underlying the first three learning goals. For example, the primer design assignment (Assignment 2 in Supporting Information A in Data S1) has students practice determining which primers are the highest quality for a qPCR reaction by using software to analyze the thermodynamics of primer byproduct formation reactions and using the resulting free energy values to evaluate a given primer pair. Both discussions and assignments are reviewed by the instructor and TAs when planning lectures and improving the course for future years.

An additional intermediate learning assessment is conducted during the laboratory introduction to determine if students demonstrate proficiency in basic laboratory safety principles and skills (see Supporting Information A in Data S1). Throughout the course, the instructors examine data from students' successful completion of assignments and their demonstration of conceptual understanding during oral discussions to measure progress toward the learning objectives. To illustrate the effectiveness of the course, some of the student assessment data is provided demonstrating achievement of the specific learning objectives:

- During the final discussion, all (100%) of the students successfully evaluated their hypotheses based on their results, showing the achievement of Learning Objective #2 (*evaluating hypotheses*) and Learning Objective #3 (*applying chemical detoxification knowledge and critical thinking*).
- In the primer design activities, all (100%) students in the 2022 and 81% in 2023 designed successful primers for their assigned genes, indicating progress on Learning Objective #1 (*bioinformatics proficiency*) and Learning Objective #4 (*PCR laboratory skills*).
- During laboratory days, zero safety incidents were registered, and all (100%) students demonstrated their ability to follow laboratory safety guidelines and wear appropriate PPE. Furthermore, a notable improvement in pipetting ability of all (100%) students throughout the course demonstrates achievement of Learning Objective #5 (*lab safety and pipetting skills*).
- During the agarose gel and qPCR activities, all (100%) students included appropriate DNA ladders, controls, and reference genes, satisfying Learning Objective #6 (*understanding the importance of standards and controls*).

4.2 | Summary, interpretation, and discussion of individual results

Students' mosquitofish qPCR was quite successful each of the three times the course has been offered. When comparing female exposed and control mosquitofish, many genes had $\Delta\Delta C_T$ values of roughly ± 1 or ± 2, indicating minimal differences in exposure across treatments. This was fairly consistent across all mosquitofish comparisons. However, the difference in chemical environment was correlated with changes in two genes—Cytochrome P450 3A40-like (CYP3A40-L) and Catalase. CYP3A40-L had a $\Delta\Delta C_T$ equal to 3.6, indicating that there is a fold change of 0.082, or 12.1 times decrease in expression of this gene relative to the control. On the other hand, Catalase experienced the opposite effect, with a $\Delta\Delta C_T$ value of -4.6, indicating that there is a fold change of 24.3. This would indicate that CYP3A40-L is not related to mosquitofish's defense against wastewater contaminants, while Catalase may play a role.

The C_T values for a given gene from students' qPCR in this class could vary dramatically, leading to inconsistent results. Honey bee results, in particular, experienced high deviation in C_T values and ΔC_T values, especially due to extreme variance in reference gene values. Some genes had differences of 10–15 in C_T values across treatments, with a value for one plate being $\sim$20 and others being >30. This is especially problematic for reference genes, which are supposed to be stable across treatments. Additionally, some results were inconclusive due to a lack of amplification or excessive contamination for all technical replicates of a gene on a given plate. This is an important learning experience for students, since it illustrates how technical errors associated with lab inexperience can lead to poor-quality data that is difficult to salvage for later analysis and interpretation.

4.3 | Hypothesis testing and error analysis

With these varied results, students are tasked with a multifaceted analysis of the complex results of their experiments on the final day of this course. Students are introduced to different sources of error in science, including systematic, random, and personal errors, and are instructed to consider these while evaluating their hypothesis.[22] Additionally, students are asked to come up with scientific reasons that could have led to their inconsistent results or results that disagree with their initial hypothesis. For example, some of the more inconsistent results may have been caused by operator error (a thorough discussion of the prominence of error in this kind of teaching laboratory is discussed below), but a variety of reasons may be responsible for causing downregulation of a certain gene instead of upregulation. Students came up with thoughtful explanations for some of their research project results, such as:

- "Lower expression of a certain gene in exposed bees may be due to that gene not being involved in the defense against that particular chemical. It is then downregulated so other genes can be upregulated."
- "Higher expression of a certain gene in female fish over male fish may be due to the female fish being pregnant and needing more of that gene to help protect her young."
- "Variance in gene expression could be due to sample differences, such as non-homogenous exposure to a certain chemical."

4.4 | Self-reflection and troubleshooting

As an additional exercise incorporated into the final discussion, students were asked what steps could be taken to improve results were the experiment to be repeated. This was an opportunity for students to demonstrate their knowledge of the experimental process and what factors impact the quality of the results. Students were encouraged to apply the concepts they had learned in lecture to

this reflection. Students brought up various potential improvements, such as refreshing ice baths more often to maintain temperature-sensitive reagents during long lab days and improving the organization of qPCR workflow to minimize chances of user error. Students also explained how they would avoid errors by applying their knowledge from lectures and discussions to their project. Some of the students' main reflections and troubleshooting ideas were:

- "Do a test run of all installed software for this course on my computer/tablet to make sure that it will start."
- "Practice pipetting of small volumes to get more familiar with it before doing the real samples."
- "Attend an Excel software crash course to practice basic spreadsheet functions and formulas."

4.5 | Exit questionnaire

Students were provided with the opportunity to give critical feedback for improvement of the course upon completion. In addition to gaining an understanding of how much students enjoyed the course, this exit survey allowed us to better understand what big-picture takeaways the students took from the course, and how the course influenced them as budding scientists. Details on the questions asked in the survey are provided in the Supporting Information B in Data S1.

5 | EDUCATIONAL IMPACTS

5.1 | College and career preparation

One of the great strengths of this course is that it gives students a broad introduction to environmental toxicology as a field and illuminates the different career paths that stem from a degree in environmental toxicology. In addition to completing the main project of the class, which focuses on chemical detoxification and qPCR applications, we invited several other faculty members involved in toxicology research as guest lecturers, took the students on field trips to other environmental toxicology laboratories and testing facilities, and included an hour-long lecture dedicated to toxicology career paths at the end of the course. The goal of these activities is to help students think about their newfound knowledge and interests, give them an idea of other types of toxicological research on and off campus, and gain new perspectives on the different careers a toxicologist can pursue. Additionally, students are encouraged to ask questions about their specific interests so we can provide personalized advice for finding the right field for them.

5.2 | Software and bioinformatics proficiency

Computer proficiency and the ability to use and troubleshoot software is vital even for students not planning on going into toxicology research. Many students entered the class not having a working knowledge of basic computer functions, such as navigating through files and using Microsoft Office. Through this class, students can practice their basic computer skills while applying them to a field they are interested in and learning advanced skills in software usage and database proficiency. Students were much more comfortable using programs like MS Excel and PowerPoint by the end of the course and left having software skills transferable to other scientific fields.

5.3 | Interdisciplinary critical scientific thinking

Students gained an advanced understanding of the scientific methods and techniques applied in environmental toxicology through this course. We emphasized forming hypotheses, testing them in the lab, and coming up with conclusions based on their obtained results. By the end of the research project, students were not only able to arrive at conclusions but also think of alternative explanations for their results and create new scientific questions based on them.

6 | COMMON PITFALLS AND PRACTICAL CHALLENGES

6.1 | User and technical errors

The most prominent sources of error in the experiments described above revolve around students lacking laboratory experience. Even in college, undergraduate students often struggle with pipetting as a skill and keeping track of which reagents go in which tubes.[23] This is one of the biggest challenges of teaching the wet lab part of this course. As such, at the beginning of the second module, before any experiments begin, students are introduced to laboratory safety and proper micropipette operation, including an opportunity to practice pipetting and weighing water samples. During this time, students receive one-on-one help with their pipetting by the TAs so that they can understand basic pipet operation, such as first and second stops, as well as proper form and hand positioning for maximum accuracy. Nevertheless, developing mastery in pipetting technique comes from practice over time.

Additionally, PCR and qPCR are organizationally challenging protocols to run. Because of this, instructors pipette especially sensitive and/or expensive reagents. Additionally, all reagents and buffers used in the lab course are previously tested for contamination and expiration by the TAs. With the students doing most of the pipetting for cDNA conversion, primer test PCR, and qPCR, many errors still occur, including adding incorrect volumes and forgetting to add primers or template cDNA. Some of these errors are weeded out through the primer test PCR and melting curve analysis, but even the need to remove some of these data points decreases the overall data quality. Despite the course's focus on quality assessment, we recognize that qPCR is difficult to master, and errors will occur while learning it. With this in mind, we emphasize student improvement in technical laboratory skills over the course's duration in addition to the quality of their data. For young adults enthusiastic about a potential career in science, this is arguably more important than having consistent results. That said, at least one of the samples of every student's qPCR plate ran successfully in our courses and students were able to perform a complete data analysis.

6.2 | Limited knowledge in fundamental science

Another significant challenge comes from the lack of background coursework in molecular biology and biochemistry. Environmental toxicology is conceptually challenging, and most students have limited background in this field. Surveying students showed varying levels of experience in biology and chemistry, the two most important courses in secondary education for understanding this course's content. Some students had yet to take a chemistry course in high school, and others had not taken any biology course. This proved to be an immense challenge since PCR and qPCR requires significant background knowledge to understand. It is also where TAs come into play; while the instructor can focus on teaching about environmental toxicology and PCR, TAs can help students grasp new and confusing terms so they understand fundamental biological and chemical concepts. The emphasis on teamwork also allows students to help fill in each other's gaps in knowledge.

7 | CONCLUSION

This two-module course provides motivated and advanced high school students with an introduction to environmental toxicology through the lens of gene expression and molecular biology. The core of the course is the execution of a complete qPCR experiment from start to finish, taking students through two modules: a dry lab focusing on understanding gene expression and designing primers through modern databases and software, and a wet lab focusing on carrying out the steps of a qPCR reaction, including cDNA conversion, primer test PCR, qPCR, and qPCR data analysis. Through the course, students gain a thorough understanding of molecular biology techniques for application in environmental toxicology and develop important laboratory skills. The course is designed to encourage students to work hard, challenge them with difficult content, and be an enjoyable experience that motivates them to pursue their scientific interests. As a pre-college class, it gives students a taste of university-level science research and prepares them for the rigors of college-level scientific coursework.

ACKNOWLEDGMENTS

The authors would like to thank UC Davis Continuing and Professional Education (CPE) program for providing funding for laboratory materials and class teaching assistants, as well as administrative support and out-of-class supervision and activities of students. Additionally, we thank Lily Hinh and Amara K. Pouv, previous teaching assistants for this class, for their work helping develop the course. Finally, we thank the UC Davis Waste Water Treatment Plant (WWTP) management and the USDA-ARS Honey Bee Research Lab at UC Davis to provide specimen for this course. Marvin was used for drawing, displaying and characterizing chemical structures, substructures and reactions, Marvin 17.21.0, Chemaxon (https://www.chemaxon.com).

FUNDING INFORMATION

The research reported in this publication was supported by the NIFA-USDA (CA-D-ETX-2526-H) and a UC Davis Academic Senate Large Grant to S.C.T.N. and the 2022–2025 PAm-Costco USA Scholarship for A.M.E.-M.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Spooner ZT, Encerrado-Manriquez AM, Truong TT, Nicklisch SCT. From primers to pipettes: An immersive course introducing high school students to qPCR for quantifying chemical defense gene expression. Biochem Mol Biol Educ. 2024. https://doi.org/10.1002/bmb.21851