INQUIRY & INVESTIGATION

Multidisciplinary Inquiry-Based Learning Using an Ex Ovo Chicken Culture Platform: Role of Vitamin A on Embryonic Morphogenesis

PHILIP R. BUSKOHL, RUSSELL A. GOULD, SUSAN CURRAN, SHIVAUN D. ARCHER, JONATHAN T. BUTCHER

Abstract

Embryonic development offers a unique perspective on the function of many biological processes because of embryos' heightened sensitivity to environmental factors. This hands-on lesson investigates the effects of elevated vitamin A on the morphogenesis of chicken embryos. The active form of vitamin A (retinoic acid) is applied to shell-less (ex ovo) cultured chick embryos, which are highly accessible and intrinsically spawn inquiry. The student activities mirror the scientific research process, including review of scientific literature, hypothesis formation, experimental design, interpretation of data, and re-evaluation of the initial hypothesis. This exercise supports instruction on developmental biology, biophysics, animal research, and experimental design and is motivated by a clinically relevant health issue.

Key Words: *Retinoic acid; inquiry; embryo; developmental biology; biophysics; vitamin A.*

Congenital defects occur in 2.5% of all near- or full-term babies (Dolk et al., 2010). These malformations can affect multiple body systems and are caused by both genetic and environmental factors. Chromosomal rearrangements or local mutations within the genome can disrupt normal development, such as with Down and Patau syndromes. Environmental factors are chemical, molecular, and mechanical stimuli that can guide or misdirect embryonic development. Common negative environmental factors include maternal substance abuse, pollutants, nutritional deficiencies, and/or medications. In this exercise, we investigate the role of a nutritional environmental factor, vitamin A metabolite, in directing embryonic chicken development.

The chick embryo is an excellent animal model in which to study human morphogenesis.

Retinoic acid (RA) is the active form of vitamin A and has been correlated with severe congenital defects at both surplus and deficient levels of maternal consumption (Zile, 2010; Ackermans et al., 2011). Retinoic acid regulates cell differentiation, proliferation, and developmentally related gene expression through interactions with RA receptors located on the DNA (Lanska, 2010). The specific RA signaling mechanisms for tissue morphogenesis are still an active area of research. Excessive consumption of vitamin A supplements has been correlated with increased risk of congenital defects of newborns (Rothman et al., 1995). Furthermore, the increased use of RA for medicinal purposes, such as treatment of acne (Tretinoin), underscores the importance of clarifying the role of RA in formation of birth defects.

The chick embryo is an excellent animal model in which to study human morphogenesis of the heart, limbs, and eyes (Davey & Tickle, 2007). Advantages of using the chick embryo for research include short development times, no maternal sacrifice, and ease of access for observation and treatment administration. Shell-less (ex ovo) chick culture furthers this access by allowing real-time observation of the whole chick and its complement of extra-embryonic vasculature. Ex ovo culture is an intrinsically inquiry-based classroom activity, in that the natural response of students is to ask "How can a chick embryo develop without a shell?" This activity engages that enthusiasm by exploring the role of vitamin A in development and the negative consequences of vitamin A excess. The teaching activities include an interactive background of RA scientific literature, hypothesis-driven experiments, hands-on ex ovo culture with RA-treated embryos, systematic measurement of growth, and interpretation of data in the context of the reviewed literature. This exercise supports instruction in developmental biology, molecular signaling, animal research, and experimental design and is motivated by a clinically relevant health issue using current scientific research.

○ Preparing for the Study

Materials & Methods

- Fertilized white Leghorn chicken eggs (local poultry farm)
- Egg incubator and rocker (HovaBator, GQF Mfg., $60–100)
- Cling wrap
- 70% ethanol
- Rubber bands (no. 16, 2.5 inches)
- Rubber gloves and safety glasses
- 9-oz. plastic cups (diameter 3 inches)

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- 100-mm-diameter Petri dish
- Whatman filter paper, size 3
- 1.5-mL centrifuge tubes (optional)
- Forceps
- All-trans retinoic acid (Sigma-Aldrich R2625, $30)
- Dimethyl Sulfoxide (DMSO, <10 mL)
- USB Microscope Veho VMS-100 (~$70) or alternative large working distance scope

Safety: Retinoic acid and DMSO should be handled with gloves and safety glasses.

Preparation of Incubator & Chick Embryos

Fertilized white Leghorn chicken eggs should be obtained from a local poultry farm. These fertilized embryos can be stored in a cooler maintained at 13°C (55°F) for up to 3–4 days until ready for use. Prior to the start of your activity, clean out the incubator with soap and water to remove contaminants. Dilute bleach or 70% ethanol can be used to sterilize the incubator. Line the base of the incubator with aluminum foil for easy clean-up and place the thermometer inside. Preheat the incubator to 37°C (99°F) and let stand 1 hour to confirm constancy of temperature. Place eggs blunt-side-up in the incubator and turn on the rocker to start incubation (day 0). Include a three-quarters-full 9-oz. cup of water inside the incubator to maintain humidity.

Ex Ovo Culture of Chick Embryos

After 72 hours (day 3), prepare hammocks for ex ovo culture (Figure 1). Fill three-quarters of each 9-oz. plastic cup with warm water. Cut about a 20 × 20 cm (8 × 8 inch) square of cling wrap and place loosely inside each cup. Cling wrap should be in contact with water and form a loose hammock. Secure cling wrap to the cup with a rubber band (Figure 1A). Spray the plastic wrap with 70% ethanol to prevent contamination. Remove eggs from incubator after 72 hours of incubation. Sterilize eggs by spraying the surface with 70% ethanol. Lay the eggs horizontally on an egg carton for 1–2 minutes to allow the embryo to rotate (Figure 1B). Using a sharp edge (like the edge of a metal bucket or a glass beaker), tap the egg gently until there is a small dent on the underside of the egg (Figure 1C). Put thumbs in opposite sides of the dent and open the shell directly above the hammock (Figure 1D). The entire yolk, egg white, and embryo should fall into the hammock softly (Figure 1E). If the embryo is not on the top side of the yolk, gently rotate the egg yolk with fingers (using gloves). Place a 100-mm-diameter Petri dish on top of the cup to seal the embryo. Transfer the embryo back into the incubator, set at 37°C, with a water cup to maintain humidity (Figure 1F). Observe embryos as long as you would like or as directed by the activity. We recommend that the students perform the ex ovo culture under close supervision of an instructor or teaching aid.

Motivate Study & Discuss Background

Before investigating the effect of vitamin A on the embryonic chick, it is important to motivate this study. Using the information outlined in the introduction, attempt to connect the overuse of vitamins and/or congenital birth defects with the students' experience with or perceptions of the issue. We found that this was best done through a brief but interactive large-group discussion. In addition, we recommend discussing normal embryonic development, particularly

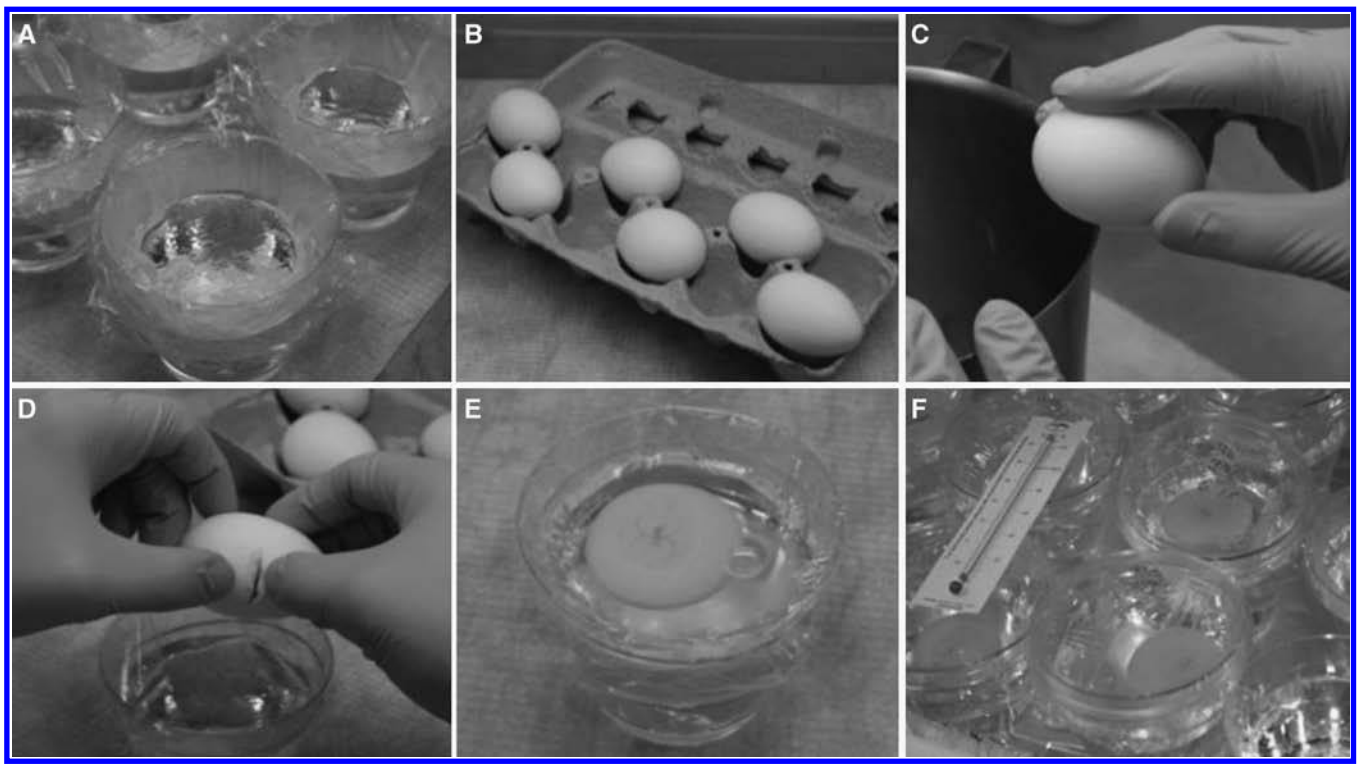

Figure 1. Step-by-step ex ovo culture technique. (A) Prepare hammocks in plastic cups. (B) Lay eggs on blunt side to prep for cracking. (C) Use sharp edge to create narrow crack on center of egg. (D) Carefully open eggshell directly above hammock and cup. (E) Rotate embryo to top side of egg yolk. (F) Place in incubator at 37°C (99°F).

pertaining to chick development (Hamburger and Hamilton embryo atlas images provided in the supplement and/or online at http://www.butcherlab.com).

Sufficient background information is needed to further place excess vitamin A exposure in context and facilitate the students' hypothesis formation. The students were given excerpts from introduction sections of scientific and medical journal articles to analyze. Field-specific literature was paraphrased to increase the readability of the excerpts. With each excerpt, we included a series of questions that assessed reading comprehension and the acquisition of scientific concepts. Although reading technical literature can be difficult for students, completion of the exercise empowered the students and broke down the false notion that scientific research can be understood only by scientists. The exercise is similar to scientific reading-comprehension questions on many standardized tests. It can also be used as a graded activity, which provides early feedback for both student and teacher on the progress of the project. We recommend small groups of two or three students, preferably with a range of ability, for this exercise. An initial read-through as a large group can remove any class-wide gaps in understanding. An example excerpt and questions are shown below, and additional excerpts can be found online at http://climb.bme.cornell.edu/embryo.php.

Background Information Example

Read the following excerpt from Ritchie et al. (2003) and answer the following questions.

> Vitamin A is an essential nutrient required for normal embryonic development. Both an excess and deficiency during gestation will result in abnormalities. Vitamin A (in its more common metabolic form, retinoic acid [RA]) is teratogenic when orally administered to pregnant rats, mice, hamsters, guinea pigs, rabbits and dogs. The induced malformations occur in all body systems including the central nervous system (CNS), the face, the cardiovascular system (ventricular septal defect, aortic arch anomalies), the limbs, the urogenital system, the respiratory system, and the gastrointestinal system. […] The question then arises, would similar malformations be induced in humans exposed to RA? Teratogenic outcomes have reportedly been associated with exposures >40,000 IU [IU – international unit of biological activity] or >10,000 IU. Equally reports have also suggested that supplemental RA intakes of <10,000 IU have no increased risk of major malformation. This contradictory data in humans highlights the importance of laboratory animal data in risk estimation.

Background Questions to Help with Hypothesis Generation

1. List the stages of chick embryonic development and illustrate normal morphology.
2. List the reported body systems that are affected by vitamin A excess during development.
3. What does this excerpt indicate about the role of vitamin A in development?
4. The authors indicate that there is a "contradiction" in the human data. What are the contrary/conflicting data?
5. Why is the contradiction important to settle?
6. What are the animal models for vitamin A research mentioned in this excerpt?
7. Why are animal models important for this research?
8. Why are different animal species important for this research?

Experimental Design

After reading the background material and discussing it as a class, divide the students into groups of three or four to prepare for the experiment. Ask each group to formulate and record a hypothesis on what will happen when RA is applied to the vasculature network of the ex ovo cultured embryo as compared to a normal embryo. Remind the students to develop a testable hypothesis, considering ahead of time which metrics they will use to evaluate the hypothesis. In this module, embryo tip-to-tail length and eye diameter were the two morphological parameters used to compare between treatments. "Tip-to-tail length" is defined as the longest line drawn between any two points in the embryo (Figure 2). Other measurement ideas include wing bud length, limb bud length, embryo area, heart rate, and vasculature branching.

Example Hypothesis & Proposed Metrics

We hypothesize that excess vitamin A administered to the embryo vasculature will affect the entire embryo and will result in stunted

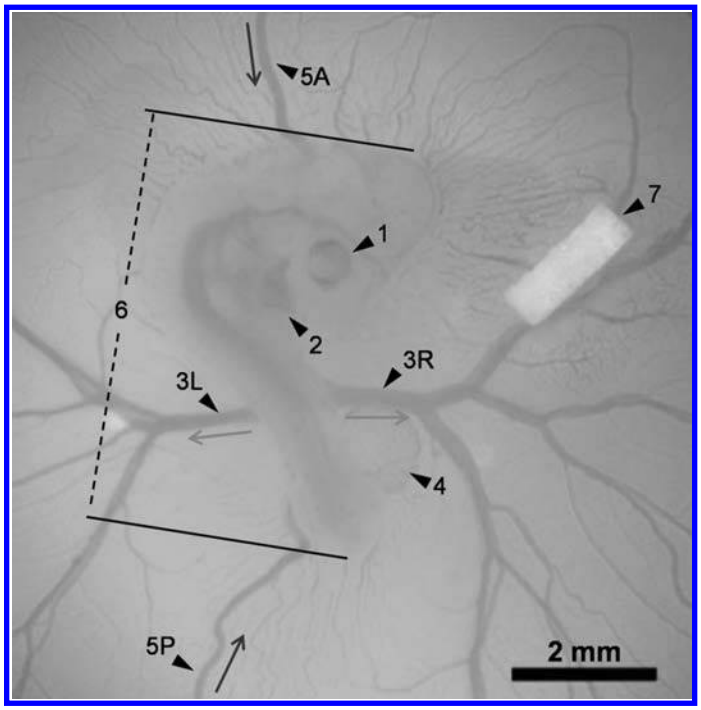

Figure 2. Image of chick embryo in ex ovo culture at 4.5 days of incubation. Annotations: 1 = eye, 2 = heart, 3L = left vitelline arterty, 3R = right vitelline artery, 4 = chorioallantoic membrane, 5A = anterior vitellene vein, 5P = posterior vitellene vein, 6 = tip-to-tail length, and 7 = RA filter paper.

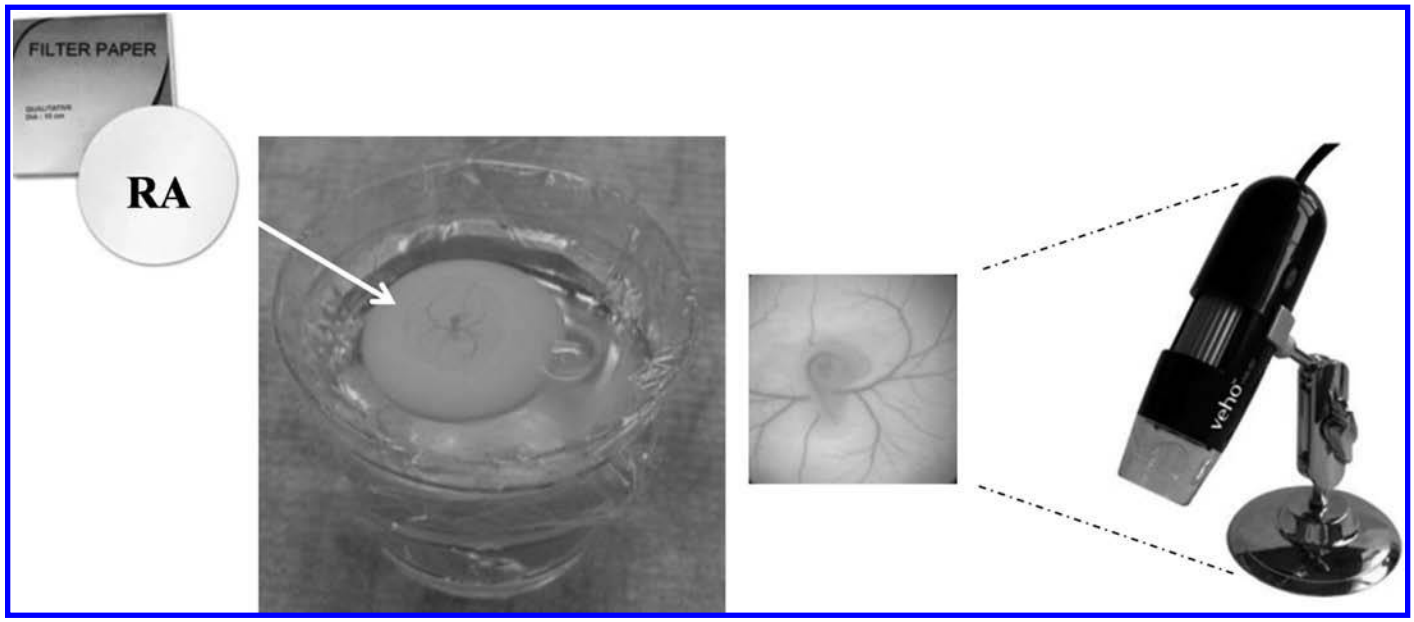

Figure 3. Experimental design consists of soaking filter paper in different concentrations of retinoic acid (RA) and placing it on the embryonic vasculature. Following several days of incubation, a USB microscope can capture the downstream effects on development.

growth and reduced eye size. We will use tip-to-tail length as a metric of embryo growth and directly measure eye diameter.

Control experiments are an essential component of any well-designed scientific study. Spend a brief but intentional time explaining the types and purpose of controls with the students. Solicit their assistance in identifying the most appropriate controls for this study. We recommend two controls, an untreated group (standard control) and a filter paper with DMSO only (sham control). The standard control will provide the benchmark of normal development to compare with RA treated, and the sham control to verify that the effects observed are not due to the filter paper or the RA solvent, DMSO.

○ Conducting the Investigation

Environment Perturbation Study – Addition of Vitamin A (Retinoic Acid)

From previous dose-dependent and temporal responses, we have determined that an RA concentration of 1 mg/mL applied at day 4 of incubation induces morphological changes by day 7 of incubation. We recommend that the teacher mix the RA treatment and administer it to the embryo. The RA and DMSO should be handled with gloves and safety glasses. Cut the Whatman filter paper into punches or strips 2–5 mm in diameter. Dilute 1 mg of RA in 1 mL of DMSO in a 1.5-mL tube. Place the filter-paper sections in the 1.5-mL tube and let soak for 10–15 minutes. Using forceps, remove the filter paper from the RA solution and place it on the ex ovo culture. We suggest placement of the filter paper on the right side of the embryo, within the vasculature (Figure 2). Capture images of the embryo and vasculature sequentially at day 4, day 5, day 6, and day 7 using the USB-microscope (Veho VMS-100; Figures 3 and 4). Measure the predetermined metrics of tip-to-tail length and eye diameter from these images using the open-source software ImageJ (National Institutes of Health; http://rsbweb.nih.gov/ij/). Simple instructions for ImageJ are available at http://climb.bme.cornell.edu/embryo.php. Hand measurement using a caliper or ruler is a viable alternative. Finally, record the measurements in tables for each metric (Figure 5).

Analysis & Interpretation of RA Treatment Results

An essential part of the scientific process, and often the most challenging, is to quantitatively analyze and correctly interpret the

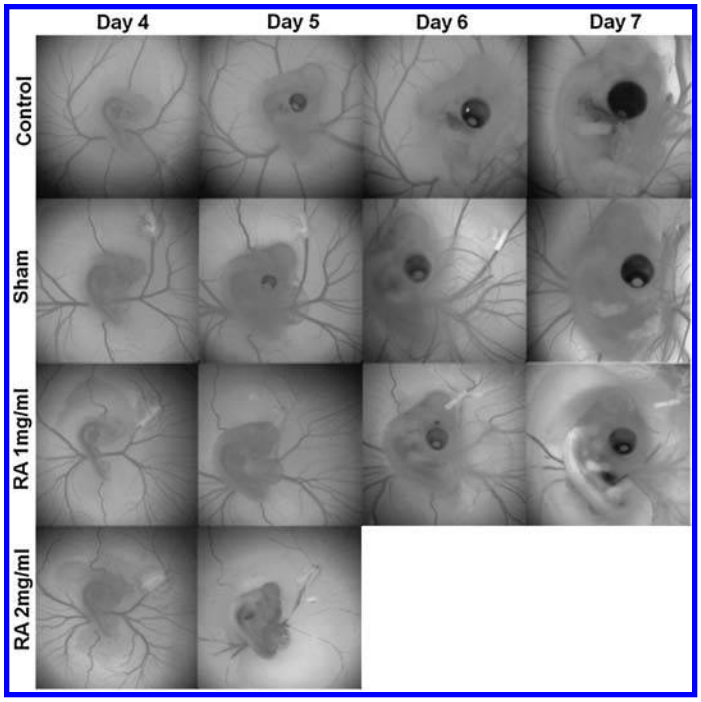

Figure 4. Retinoic acid perturbation study data set. Array of ex ovo chick images across treatment and over time. The RA treatment stunted embryo development and even resulted in embryo lethality at higher dosages.

Tip-to-Tail Length (mm)	Day 4	Day 5	Day 6	Day 7
Control	0.76	1.22	1.62	1.92
Sham	0.85	1.14	1.52	1.85
RA (1mg/ml)	0.79	0.96	1.19	1.56
RA (2mg/ml)	0.72	0.92	N/A	N/A

Eye Diameter (μm)	Day 4	Day 5	Day 6	Day 7
Control	96	221	334	443
Sham	120	195	315	419
RA (1mg/ml)	78	150	253	328
RA (2mg/ml)	95	116	N/A	N/A

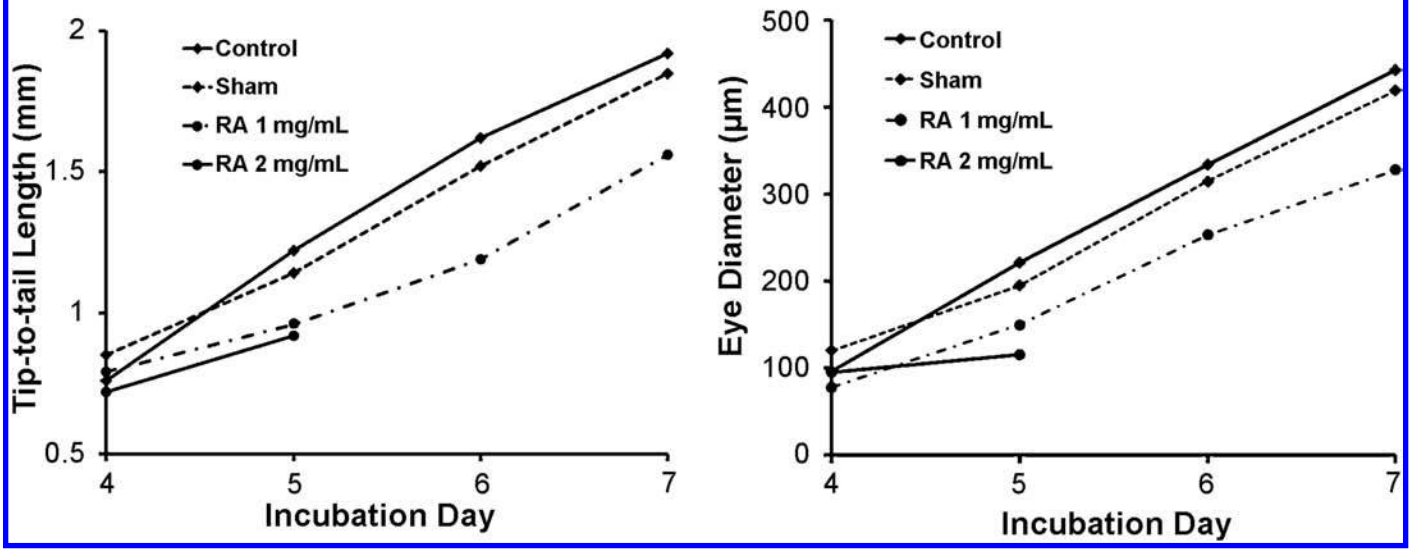

Figure 5. Quantification of eye diameter and tip-to-tail length for retinoic acid perturbation study using open-source software (ImageJ) provided by the National Institutes of Health.

data. To assist with this task, we recommend that the students plot the data, either digitally or by hand, for each output measured (Figure 4). Instruct the students to use the data to confirm or deny their initial hypothesis. If the hypothesis is confirmed, the students must prepare a series of arguments from the data to support their claim. If the hypothesis fails, the students must generate a new hypothesis based on the results of the study. It is also important to consider the implications of these results in a broader context. For instance, ask the students how these results might be related to human development, or how the other unmeasured features of the embryo, such as the internal organs, might be affected by the treatment.

Example Data Analysis & Interpretation Worksheet

Using the generated tables, line graphs, and previous understanding of embryonic development and RA, work through the following questions as you analyze the data (Figure 5).

1. Compare the tip-to-tail length and eye diameter between the control and RA treatments. Did addition of RA stimulate or stunt growth?
   - Although the control and treated embryos had roughly the same starting tip-to-tail length, by day 7 the control tip-to-tail length was 25% greater than the 1 mg/mL RA treatment. The 2 mg/mL RA treatment embryo died before day 7. These results suggest that RA stunted and/or terminated the growth of the embryo.
2. Are the sham results different from the control? What do the sham results indicate about our method of treatment?
   - The sham results do not appear to be significantly different from the control. This indicates that the defects were not induced by the filter paper or DMSO, but instead can be wholly attributed to the addition of RA.
3. Approximate the growth rate of each treatment by determining the slope of the tip-to-tail curves. Which condition had the fastest growth rate? Which was the slowest?
   - Control – 0.39 mm/day, Sham – 0.34 mm/day, RA 1 mg/mL – 0.25 mm/day, RA 2 mg/mL – 0.2 mm/day. The control embryo had the fastest growth rate and the 2 mg/mL RA treatment had the slowest. The control growth rate was roughly twice as fast as the 2 mg/ml RA treatment. This result agrees with the conclusion in question 1, that RA stunted the embryo's growth.
4. From our data, we see that RA treatment affected the *global* development of the embryo, yet the treatment was applied *locally*. Why do you think this is?
   - Even though the RA is applied over a small area away from the embryo, it was placed on a blood vessel of the vasculature network. This particular placement on a vessel facilitated the spread, and uptake, of RA in the embryo body. This emphasizes the substantial role the circulatory system plays in nutrient consumption and trafficking, demonstrating how high local concentrations of harmful agents can have global consequences due to rapid transport throughout the developing embryo.

5. Explain how an optimum (not too high and not too low) human exposure to vitamin A measurement could be determined from these data.
    - The upper-bound dosages for human embryos could be approximated from this study's data by scaling according to body weight or blood volume.

○ Discussion

The embryo provides a unique lens through which to study general (cell activity, organ systems) and development-specific (cell differentiation, morphogenesis) processes in biology and physics. Watching the embryo induces fundamental questioning of why some structures develop first versus later on, how the embryo is feeding itself, and how processes change with growth. Even if it's just in a biology classroom, the effects of physical properties (diffusion, transport, and mechanical forces) can be incorporated into our lesson framework. Instructional versatility enables the breadth of content to be appropriately scaled to student ability or knowledge base. For instance, observation, measurement, and prediction of normal development may be sufficient for middle school biology students, whereas the RA perturbation model may be more appropriate for upper-level high school students. In the following, we present further background information and ethical issues with animal research to extend the applicability of this lesson (Figure 6).

Figure 6. Engagement of students during the ex ovo chick culture and analysis of experimental results.

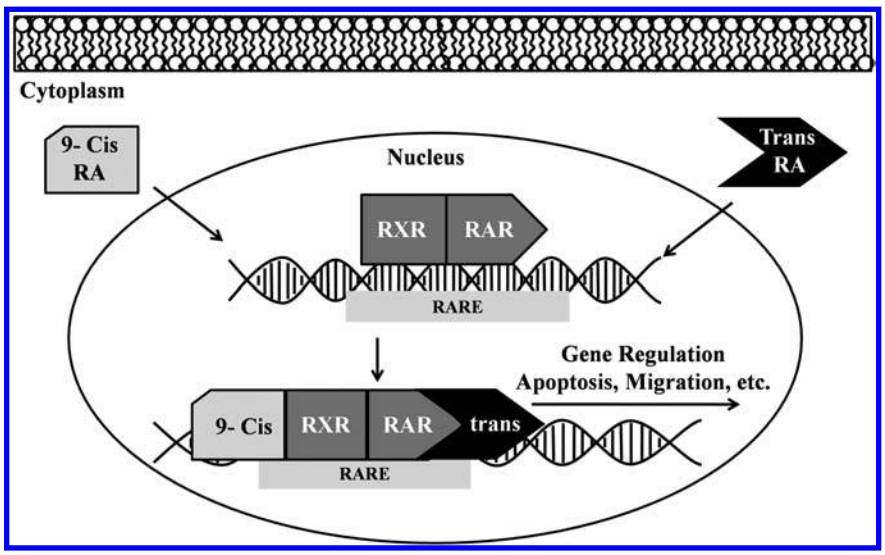

Figure 7. Metabolic and molecular pathway of retinoic acid (RA) describing the mechanism through which RA regulates gene regulation (for advanced classrooms).

Metabolic & Molecular Aspects of Study – Mechanism of Action

Vitamin A is broken down into all-*trans*-RA and 9-*cis*-RA, which is then transported to the nucleus of the cell bound to cytoplasmic RA-binding proteins. Within the nucleus, all-*trans*-RA binds to RA receptors (RAR) and 9-*cis*-RA binds to retinoid receptors (RXR). RAR and RXR form RAR/RXR heterodimers, which bind to regulatory regions of the chromosome called RA response elements (RARE). Binding of all-*trans*-RA and 9-*cis*-RA to RAR and RXR respectively allows the complex to regulate the rate of gene transcription. In the case of all-*trans*-RA applied systemically to a developing chick embryo, complexity arises because cells and tissues will respond to the RA differently. For example, in the developing limb bud, tissue regression has been largely associated to the molecular control of self-induced cell death (apoptosis). Likewise, brain defects have been largely associated to the molecular control of cell migration. Therefore, we suggest that a generalized mechanism of action be used for explanation. Upon excess RA administration, stunted growth may be the result of abnormal cell migration or apoptosis, which is regulated directly by the transcriptional complex (RA and 9-*cis*-RA to RAR and RXR) (Figure 7, adapted from http://lpi.oregonstate.edu/infocenter/vitamins).

Bioethics of Animal Research

Using live embryos within the classroom will inevitably spark a conversation about humane treatment and ethics. We feel that this is an extremely important aspect and should not be overlooked. From a purely regulatory standpoint, all use of vertebrate animals in research, teaching, and testing is regulated by the Institutional Animal Care and Use Committee (IACUC, http://www.iacuc.org/). Chick embryos younger than embryonic day 15 (E15) are assumed to be unable to experience pain, because of limited neural development. It is recommended that E14 or younger embryos be euthanized by hypothermia, typically by placing the eggs in a –20°C freezer. Chick embryos from E15 to pre-hatching should be euthanized by decapitation, anesthetic agents, or another rapid and humane method. Studies using embryos within 3 days of hatching or hatchlings must be reviewed by the normal IACUC procedure for vertebrate animals. In our experiments, embryos will be euthanized at day 7, which is only one-third of the way through their 3-week hatching cycle. Therefore, the embryos cannot experience pain and are exempt from any IACUC protocols. This, however, will not limit conversation about the ethics of animal research within the

classroom. We encourage teachers to lead a classroom discussion on the role of animals in scientific research and use this time effectively to discuss humane treatment and ethics. From our experience, the level of concern and opinions will vary from class to class. It is imperative to gauge student sensitivity in a considerate and objective manner before conducting the experiments. Resources for animal research ethics can be found on the Web at http://www.ethics.org.

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Appendix. Addressing the *National Science Education Standards*.

Standard	Value of This Activity
Teachers should successfully convey unifying concepts of science.	Students will successfully design, conduct, report and evaluate the effects of RA on embryonic development. Using mathematics, students will process and report data to understand the origin of congenital defects through environmental factors.
Teachers should engage students in various methods and active learning through scientific inquiry.	During our investigation, we implement multiple methods of inquiry leading to scientific knowledge. Students determine the effects of RA on embryonic development that require them to develop concepts and relationships from their observations, data, and inferences in a scientific manner.
Teachers should organize safe and effective learning environments that promote the success of students and the welfare of all living things.	Students will treat all living organisms used in the classroom or found in the field in a safe, humane, and ethical manner and respect legal restrictions on their collection, keeping, and use. Classroom discussions on using animals for experimental systems have been highlighted.
Teachers should create a community of diverse learners who construct meaning from their science experiences for further exploration and learning.	The experiments have been organized to engage students in a collaborative learning environment. In addition, technological tools such as ImageJ allow students to collect and process data and facilitate the learning of science.