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Practical Applications of the HHMI Stickleback Evolution Virtual Lab

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The Howard Hughes Medical Institute Stickleback Evolution Virtual Lab introduces students to concepts and techniques used to analyze the morphology of organisms. The goal of the lab is to understand aspects of evolutionary adaptation by exploration and measurement of pelvic structures of the threespine stickleback fish (*Gasterosteus aculeatus*) from living and fossil populations. Students also investigate the association between patterns of evolutionary change and known genetic mechanisms underlying pelvic reduction in living and fossil sticklebacks. The lab exercises are based on genuine field specimens, which students measure to graph, statistically analyze, and compare with other students. (Virtual lab url: **http://media.hhmi.org/biointeractive/vlabs/stickleback/index.html**)

Keywords: threespine stickleback, fish, pelvic reduction, evolution, ecology, fossil record, *Pitx1*, natural selection

Link to Supplemental Materials

http://www.ableweb.org/volumes/vol-35/park/supplement.htm

Introduction

The Howard Hughes Medical Institute (HHMI, 2013 a,c) Stickleback Evolution Virtual Lab (**http://media.hhmi.org/biointeractive/vlabs/stickleback/index.html**) explores patterns of evolutionary change in present and past natural populations of threespine stickleback fish (*Gasterosteus aculeatus*) (Fig. 1). The lab design allows students to directly measure traits important to the ecology and evolution of this species by making comparisons of population traits living under different selective pressures. Through a series of online tutorials and activities, students practice science and explore the roles of natural selection and genetic mechanisms for evolution, using measurements obtained by stickleback biologists working in the field and the lab. The specific trait studied in the lab is the fish pelvic skeleton, a structure similar to the hip and hindlimbs of humans and other tetrapods.

Instructors may find the lab useful for introducing and reinforcing the integration of genetics, developmental biology, ecology, and evolution. For example, the lab can be used to illustrate predator-prey relationships and environmental selection pressures in an ecology unit. As many students will be familiar with tetrapods, the lab could also supplement comparative anatomy and other vertebrate zoology topics. The lab activities can be performed in any sequence the instructor chooses. A major focus of the lab is the evaluation of real data using statistics. Tools built into the virtual lab allow students to graph their results and apply statistical tests (i.e., chi-square and simple regression analyses).

The lab also includes examples of the process of research such as collecting and preparing specimens for data collection (i.e., staining fish and preparing fossils), which can be used to teach students necessary (but at times, tedious) elements of real research. The activities, videos, and concise text with bolded key terms engage students and guide their inquiry into stickleback evolution. Interspersed quizzes assess students' mastery of the ecology and evolution of stickleback pelvic reduction. This lab also complements the HHMI short film **The Making of the Fittest: Evolving Switches, Evolving Bodies** (HHMI 2013d).

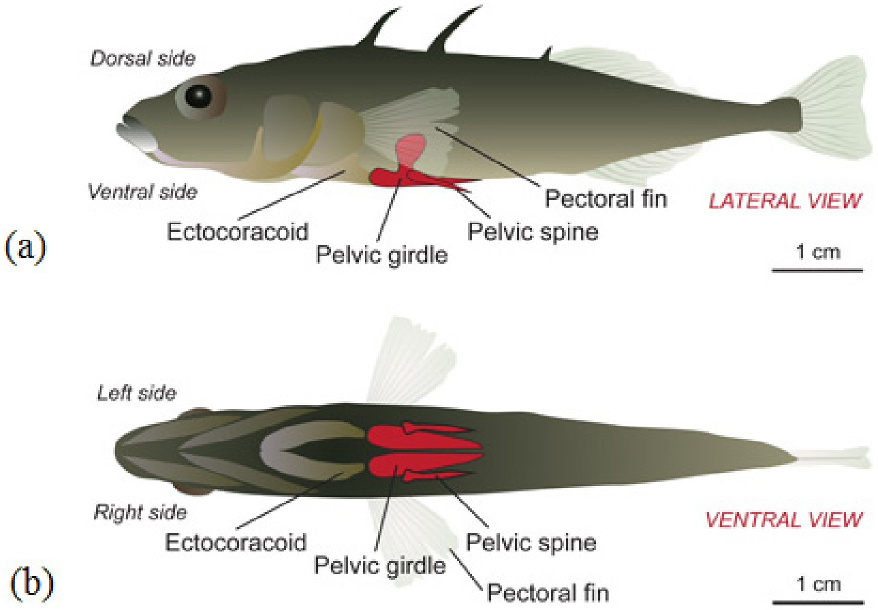

Figure 1. Diagram of threespine stickleback fish. (a) Lateral view, anterior to left. (b) Ventral view, anterior to left. Pelvic structure colored red in both images. Original artwork by Heather McDonald.

The virtual lab highlights the following major concepts: (1) natural selection acts at the level of populations and can drive the evolution of traits such as complex skeletal pelvic components; (2) different environments can impose selective pressures that result in divergence of traits; (3) mechanisms of evolutionary change can be revealed by studying ecologically contrasting populations; (4) patterns of evolution can be analyzed using fossils from different stratigraphic layers; (5) statistics are an essential tool for evaluating outcomes of research studies. After having completed the lab, students are challenged to synthesize a variety of complicated biological concepts. By focusing on research concerning a single species, the threespine stickleback, students are guided in discovering the conceptual links among Mendelian genetics, molecular genetics, development of phenotypes, ecology, evolution, and paleontology.

Scientific Background

Threespine Stickleback as a Model Organism

The threespine stickleback (*Gasterosteus aculeatus*) is a small anadromous fish, widely distributed across the Northern Hemisphere. The species is found in a wide range of freshwater habitats and exhibits diverse morphological, behavioral, physiological, developmental, and reproductive traits that vary in relation to habitat type. Live stickleback are easy to maintain in aquaria, and *in vitro* fertilization can be used to produce hundreds of progeny for laboratory studies. Freshwater populations have evolved from marine populations repeatedly over millions of years as well as very recently, allowing their use as independent evolutionary replicates derived from a common marine ancestor (Bell, 1995). These useful biological properties have led to extensive research on *G. aculeatus* (Wootton, 1976; Bell and Foster, 1994; Östlund-Nilsson et al., 2007), including the production of powerful genomic tools (Kingsley and Peichel, 2007) and more than 170 separate genome sequences using individuals from phenotypically diverse populations (Jones et al., 2012; Broad Institute, 2013). Thus, it is possible to trace major phenotypic differences to DNA sequence changes, and to place those differences into an ecological context.

Pelvic Reduction in Living Stickleback Populations.

One of these phenotypic differences, pelvic reduction, occurs in threespine, ninespine, and brook sticklebacks (Klepaker et al., 2013). It has been reported in threespine stickleback from lakes in Scotland, Norway, Iceland, Canada (Quebec and British Columbia), and USA (Alaska). Extensive pelvic reduction has been reported from 40 lake populations of *G. aculeatus* from Cook Inlet, Alaska (Bell and Ortí, 1994), an area that was deglaciated within the last 22,000 years (Reger and Penny, 1996). The geographical distribution of populations with pelvic reduction both within Cook Inlet and around the world indicates that the trait has evolved independently several times (Bell and Ortí, 1994): the presence of different alleles of *Pitx1* that are associated with pelvic reduction in some populations supports this interpretation (Chan et al., 2010). Extensive pelvic reduction occurs in Cook Inlet populations only from lakes that both lack predatory fishes and have water with low ionic concentration (Bell et al., 1993). A predation experiment using a Cook Inlet population with a wide range of pelvic phenotypes indicated that stickleback with pelvic reduction are eaten by trout more frequently than specimens with full pelvic structures (Lescak and von Hippel, 2011). Other experiments do not support hypotheses that insect predation (Lescak et al., 2012) or low ionic strength water (J. L. Rollins pers. comm., 2013) select for extensive pelvic reduction.

Genetic Mechanisms Underlying Pelvic Reduction

In most threespine stickleback populations with pelvic reduction, a major gene responsible for the trait is *Pitx1*, which encodes for a homeodomain transcription factor (Chan et al., 2010). It is expressed during development of the normal pelvis but not when the pelvis is severely reduced (Cole et al., 2003). Pelvic girdle reduction maps to the *Pitx1* gene in populations across the species' range, but at least five other genes can contribute to pelvic reduction (Shapiro et al., 2004; Cresko et al., 2004; Coyle et al., 2007). Reduced *Pitx1* alleles have overlapping deletions of a short DNA sequence upstream of the *Pitx1* protein-coding region; insertion of a functioning artificial *Pitx1* gene (i.e., transgenesis) into individuals from populations with pelvic reduction restores pelvic girdle expression (Chan et al., 2010). Remarkably, vestigial pelvic structures are usually larger on the left side than the right (Bell et al., 2006). This asymmetry appears to be caused by silencing of the *Pitx1* gene and normal expression of a duplicate gene, *Pitx2*, on the left side of the body.

Pelvic Reduction in Fossil Stickleback

A threespine stickleback known as *Gasterosteus doryssus* occurs in 10 million year-old lake sediments of the Truckee Formation in western Nevada, USA. Fossils of *G. doryssus* exhibit dramatic pelvic girdle evolution (Bell, 2009). During the first 93,000 years of its occurrence in a well-studied geological section of the Nevada site, the fossil lineage initially exhibited extreme pelvic reduction and a weak, long-term trend for complete loss of the pelvis. That lineage was apparently replaced by a second *G. doryssus* lineage that initially had full pelvic structures, and during the next 5000 years, it re-evolved pelvic reduction. At the same time that pelvic reduction was evolving in the second lineage, microscopic wear patterns on the teeth indicate that it shifted from feeding on benthic prey to plankton (Purnell et al., 2007). Re-evolution of pelvic reduction in the second lineage was probably due to directional natural selection (Hunt et al., 2008). Predatory fish were absent from the lake in which *G. doryssus* occurred, which is consistent with the absence predatory fishes from Alaskan lakes in which pelvic reduction occurs (see above). Like modern stickleback with pelvic reduction, pelvic vestiges in fossil stickleback tend to be larger on the left side, implicating *Pitx1* in pelvic reduction in the fossil form. Thus, pelvic reduction first occurred at least 10 million years ago in threespine stickleback, and the genetic and ecological factors for its evolution have remained basically unchanged.

Lab Overview

As an educational resource, the Stickleback Evolution Virtual Lab has been integrated into biology, ecology, and environmental science courses. It is entirely virtualized (it is not a hybrid lab). Students are introduced to the lab with background information in the form of text and professionally produced illustrations, animations, and short videos intended to engage different learning styles. The videos are optional, but in addition to reinforcing key concepts, they show students what it is like to actually do field research. The lab also consists of a student notebook and tools for making measurements and analyzing data. Formative assessments are provided via several quizzes that test student understanding of key concepts taught in this lab; the quizzes are multiple choice and students receive immediate feedback after answering each question. An overall final quiz provides summative assessment. The lab provides extensive support for instructors on the Introduction page and also contains links to additional supporting materials, including a compelling short documentary on stickleback evolution titled **The Making of the Fittest: Evolving Switches, Evolving Bodies** (HHMI 2013d)

Tutorials

The lab has two tutorials, one for living fish and one for fossil fish, to train students in characterizing pelvic phenotypes. Students practice identifying and "scoring" three character states of the stickleback pelvis (see below). They receive immediate feedback after scoring each specimen to enhance accuracy, efficiency, and confidence in scoring the fish.

Experiment 1: Analyze Fish from Contemporary Lake Populations

Students first prepare samples for study by performing a bone stain on fish specimens. Samples from two lake populations, one with and one without pelvic reduction, are then studied. Pelvic phenotype data is collected; each specimen is scored based on whether it has a complete pelvis, reduced pelvis, or absent pelvis (Fig. 2). The data that students acquire is summarized in a histogram. The instructor can implement one of two graphing options: (1) students can download their raw data and generate their own graph using external spreadsheet application software (e.g., Microsoft® Excel), or (2) at the push of a button, the students can access built-in software that auto-generates the graph. Comprehension of concepts and the ability to apply them after having read the text, watched the videos, and analyzed data from Experiment 1 is assessed by an interactive quiz. Instructors can also have students carry out chi-square tests to statistically evaluate their results, the interpretation of which is assessed via a separate quiz. Data and quiz questions are based on real research findings by stickleback biologists. Through these quizzes, students are guided in making scientifically relevant inferences about the real selective pressures that best explain trait differences among ecologically contrasting stickleback populations.

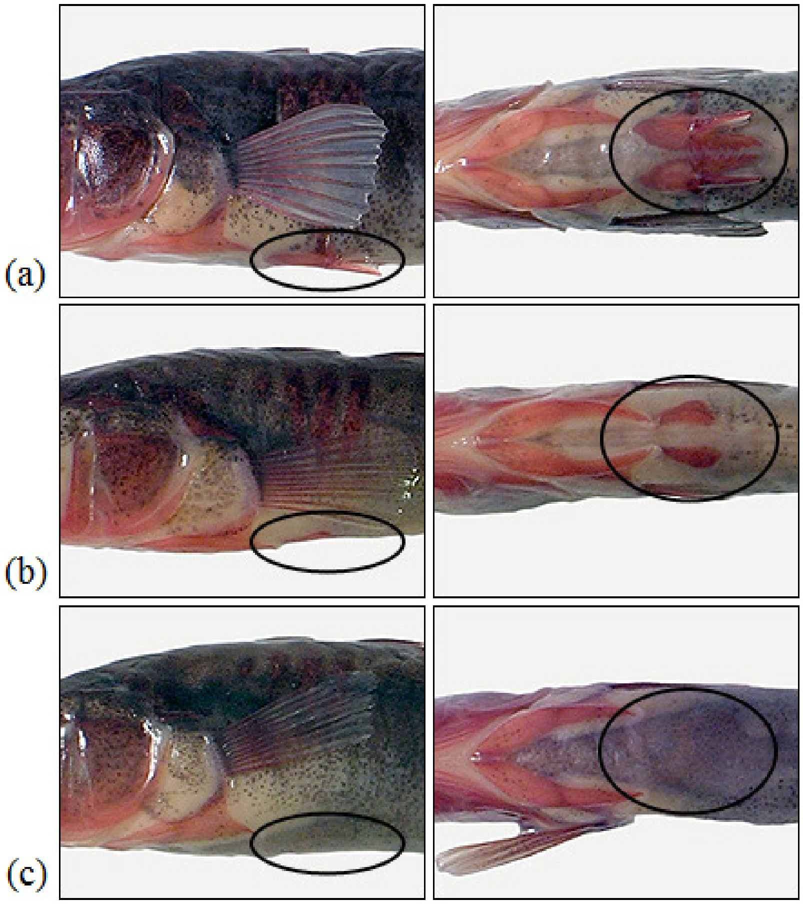

Figure 2. Pelvic phenotypes in living stickleback. Specimens with (a) complete, (b) reduced, or (c) absent pelvic morphology are shown. Pelvic area is marked with a solid circle. Anterior is left.

Experiment 2: Analyze Fossil Fish

Students set up for this study by preparing a fossil fish and then watching a short video of Bell preparing an actual fossil. Students collect data on fossil fish from two stratigraphic layers that derive from the same Nevada fossil deposit, which was once a lake bed. Fish from these two layers are separated by 9,000 years of evolution and represent the same population sampled at different times. These two layers (i.e., layers 2, 5) are a subset of a larger set of six successive stratigraphic layers that span a total of 15,000 years (Fig. 3).

Students score fossil fish for possession of a complete pelvis, reduced pelvis, or absent pelvis (Fig. 4). The data that students acquire is then incorporated into the larger dataset that summarizes fossil fish data from layers 1, 3, 4, and 6 (see above). Data for all six layers is summarized using a line graph. The instructor has two graphing options for students: (1) students can download their raw data and generate a line graph using external spreadsheet application software (e.g., Microsoft® Excel), or (2) at the push of a button, the students can access built-in software that auto-generates the graph. Comprehension of concepts and the ability to apply them after having read the text, watched the videos, and understood the data from Experiment 2 is assessed by an interactive quiz. Instructors also can have students carry out simple rate-of-change analyses, the interpretation of which is assessed using a separate quiz. Data and quiz questions are based on real research findings by stickleback biologists. Through these quizzes, students are guided in making scientifically relevant inferences about the patterns of evolutionary change recorded in the stickleback fossil record. This experiment illustrates how fossil analysis provides insights into evolution over time, including intermediate forms.

Experiment 3: Pelvic Asymmetry

Students begin by preparing samples for study by performing a bone stain on fish specimens. Samples are from two lake populations, both with pelvic reduction. Students score pelvic asymmetry by determining if a fish has pelvic vestiges that are larger on the left, larger on the right, or roughly equal in size (no asymmetry) (Fig. 5). The data that students acquire is summarized in a histogram. The instructor has two graphing options for students: (1) students can download their raw data and generate a graph using external spreadsheet application software (e.g., Microsoft® Excel),

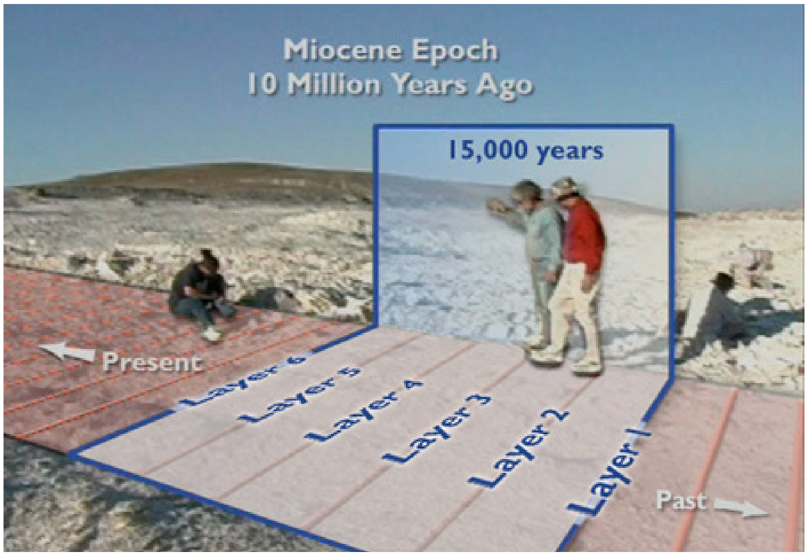

Figure 3. Diagram of Nevada site with stickleback fossils. Strata used for the virtual lab span 15,000 years of evolution.

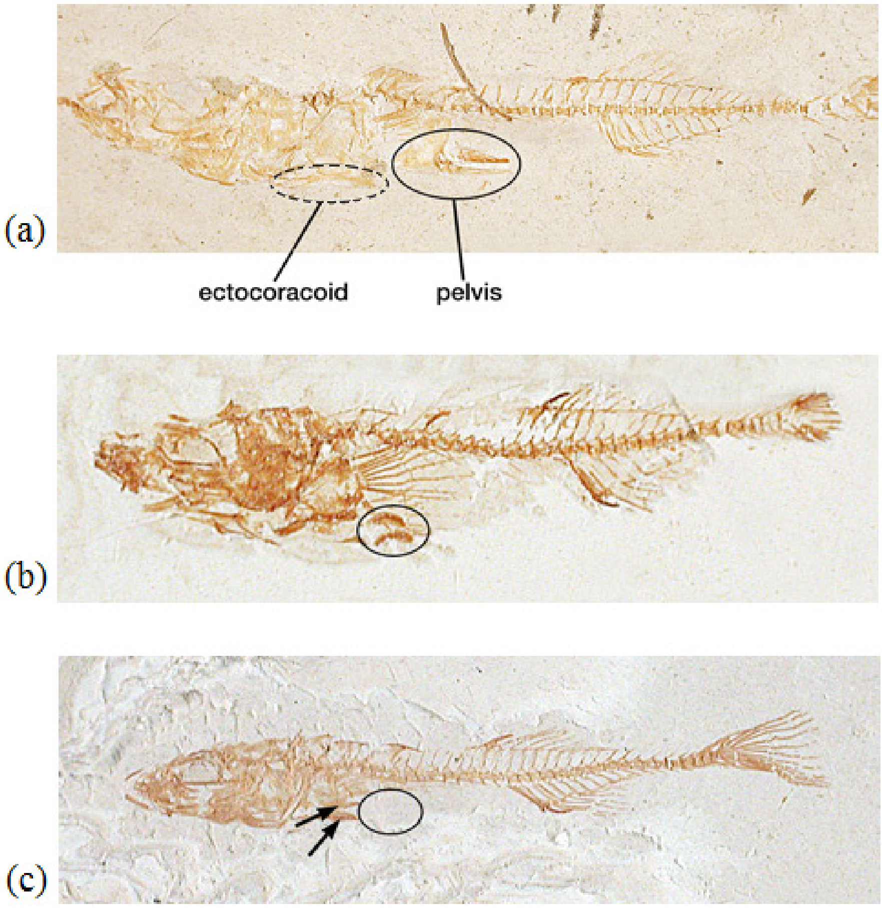

Figure 4. Pelvic phenotypes in fossil stickleback. Specimens with (a) complete, (b) reduced, or (c) absent pelvic morphology are shown. Pelvic area is marked with a solid circle. Arrows or dotted circle indicate location of ectocoracoid bone. Suitable ventral images are not available for fossil stickleback. Anterior is left.

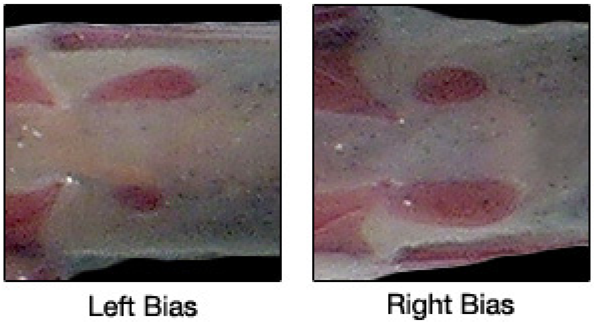

Figure 5. Pelvic asymmetry in living stickleback with pelvic reduction. (a) Ventral view of specimen with pelvic vestige larger on the left (Left Bias). (b) Ventral view of specimen with pelvic vestige larger on the right (Right Bias). Anterior is left.

or (2) at the push of a button, the students can access built-in software that auto-generates the graph. Comprehension of concepts and the ability to apply them after having read the text, watched the videos, and analyzed data from Experiment 3 is assessed by an interactive quiz. Instructors can also have students carry out chi-square tests to statistically evaluate their results, interpretation of which is assessed using a separate quiz. Data and quiz questions are based on real research findings by stickleback biologists. Through these quizzes, students are guided in making scientifically relevant inferences about the directionality of pelvic asymmetry being a signature of specific underlying genetic mechanisms.

Time Requirements

Instructors and students may choose to use the lab in different ways. Students can perform just one, two, or all three experiments. They can do portions of the lab at home and others in class; they can also skip some sections, such as the quizzes or the more advanced statistical analysis sections.

The lab tracks individual student progress, allowing for completion of the lab over multiple sessions, provided the same computer is used throughout. If students do portions of the lab at home, they can print out a progress report for their instructors. Instructors can use the lab multiple times in different classes and to cover different topics. A recently added feature allows a single computer to track the progress of up to 8 students. The lab has been updated recently since its launch in October 2012, by adding new videos and features, based on student and reviewer feedback.

The following recommendations for classroom implementation of the lab are based on time requirements for 90 minute sessions. They are meant to be suggestive, and adjustments certainly should be made according to the specific needs of the instructor.

Option 1

Students complete the two tutorials, read the text, and watch the videos in the Introduction and Overview tabs as homework before coming to class. One 90-minute session can be used to complete the first two laboratory experiments (data collection and quizzes) and statistical analyses. The instructor can begin by presenting slides 1-14 of the prepared Microsoft® Powerpoint presentation "**Park et al 2013 stickleback pelv_reduction.ppt**" (available on the article supplements web page), followed by running the class in lock-step. Individual students or pairs of students work on the first experiment for a set amount of time, after which the instructor reviews answers to the Experiment 1 quizzes with the class. This can be followed by going over slides 16-23 in the Powerpoint presentation. The instructor can have students continue on to Experiment 2 in lock-step and then show slides 25-41 in the Powerpoint presentation. Class can conclude with the HHMI short film **The Making of the Fittest: Evolving Switches, Evolving Bodies** (HHMI, 2013d). Experiment 3 can be administered as a separate future lesson or as homework. The instructor can present the remainder of the Powerpoint presentation after reviewing student results of Experiment 3 with the class.

Option 2

Students complete the two tutorials, read text, and watch the videos in the Introduction and Overview tabs as homework before coming to class. One 90-minute session can be used to complete the first two laboratory experiments (data collection and quizzes) and statistical analyses. The instructor can begin by presenting slides 1-14 of the prepared Microsoft® Powerpoint presentation "**Park et al 2013 stickleback pelv_reduction.ppt**." Individual students or pairs of students can work at their own pace. The remainder of the Powerpoint presentation can be returned to as needed. At the end of class, the instructor can assess student understanding of major concepts using the quizzes from experiments 1 and 2. The class can conclude by watching the HHMI short film **The Making of the Fittest: Evolving Switches, Evolving Bodies** (HHMI, 2013d). Experiment 3 can be administered as a separate future lesson or offered as homework.

Option 3

Students complete the two tutorials, read the text, and watch the videos in the Introduction and Overview tabs as homework before coming to class. One 90-minute session can be used to complete all three laboratory experiments (data collection and quizzes), but the statistical analyses are skipped. The instructor can begin by presenting slides 1-14 of the prepared Microsoft® Powerpoint presentation "**Park et al 2013 stickleback pelv_reduction.ppt,**" followed by having individual students or pairs of students work at their own pace or in lock-step. The remainder of the Powerpoint presentation can be returned to as needed. Class can conclude by watching the HHMI short film **The Making of the Fittest: Evolving Switches, Evolving Bodies** (HHMI, 2013d). If the instructor is able to print out the statistical analysis quiz from each experiment before class, students can save their raw data, print or e-mail the data to themselves, and for homework, carry out the statistical tests by hand and complete hard copies of the statistical analysis quizzes.

Option 4

Students complete the two tutorials, read the text, watch the videos in the Introduction and Overview tabs, and perform experiments 1 and 2 as homework before coming to class. One 90-minute session can be used to review graphs and quizzes from experiments 1 and 2. The instructor can begin by presenting slides 1-41 of the prepared Microsoft® Powerpoint presentation "**Park et al 2013 stickleback pelv_reduction.ppt**." This can be followed by having individual students or pairs of students carry out Experiment 3 in class. The instructor can have students work at their own pace or in lock-step. Class can conclude with the Final Quiz and then watching the HHMI short film **The Making of the Fittest: Evolving Switches, Evolving Bodies** (HHMI, 2013d).

Comments Regarding Implementation of Experiments

The options above separate experiments 1 and 2 from Experiment 3 for several reasons. Experiments 1 and 2 are conceptually similar; Experiment 1 investigates ecological and evolutionary mechanisms of pelvic reduction, while Experiment 2 explores patterns of pelvic reduction over evolutionary time. Thus, the first two experiments can be seen as complementary to each other in terms of prior scientific background needed, concepts conveyed, and comprehension of research findings. Thus, it is recommended that experiments 1 and 2 be administered together. They can serve as an introduction or review of ecology and evolution concepts.

Experiment 3 could be offered as an immediate follow-up to the first two experiments, or it could be offered at a later time. Unlike the first two experiments, Experiment 3 requires students to explore the link between genes and phenotype. Thus, students may need a basic understanding of Mendelian genetics (e.g., laws of inheritance of discrete traits) and molecular genetics (e.g., gene regulation, function of enhancers) before performing Experiment 3. The website BioInteractive.org (HHMI, 2013a) offers supplemental active learning activities concerning Mendelian genetics and molecular genetics that may help students bridge knowledge gaps in order to link the major concepts of the first two experiments with the third experiment. Additional information about these supplemental activities is provided below:

Using Genetic Crosses to Analyze a Stickleback Trait.

This active learning activity was created by Ann Brokaw, Laura Bonetta, and Dennis Liu, and it supports the short film **The Making of the Fittest: Evolving Switches, Evolving Bodies** and the **Stickleback Evolution Virtual Lab**. Students apply Mendelian laws of inheritance to analyze outcomes of monohybrid crosses. Genetic crosses between stickleback fish with a full pelvis from one population and stickleback with pelvic reduction from another population are simulated. Using photograph cards of actual research specimens, students collect data and analyze outcomes of subsequent F1 and F2 crosses. Students can compare their results with actual research data from crosses performed by stickleback biologists (c.f., Cresko et al., 2004), using fish from a variety of different populations. All resources for this activity can be accessed on the BioInteractive.org website (HHMI 2013e), which also includes extension activities such as a chi-square analysis.

Modeling the Regulatory Switches of the Pitx1 *Gene in Stickleback Fish.*

This active learning activity was created by Eriko Clements, Ann Brokaw, Laura Bonetta, Satoshi Amagai, and Dennis Liu, and it supports the short film **The Making of the Fittest: Evolving Switches, Evolving Bodies** and the **Stickleback Evolution Virtual Lab**. It teaches students basic concepts about eukaryotic gene regulation by focusing on the function of the *Pitx1* gene in the development of pelvic structures in the threespine stickleback fish. Students learn about eukaryotic gene transcription and regulation by handling manipulatives that model molecular components involved in these processes. Students work individually or in groups to describe and predict the phenotypic consequences of a variety of developmental scenarios, including a functioning *Pitx1* gene versus a non-functioning *Pitx1* gene. Students can compare their results with what is known about the genetic mechanisms underlying pelvic phenotypes in natural populations with or without pelvic reduction. All resources for this activity can be accessed on the BioInteractive.org website (HHMI 2013b).

Student Outline

See Appendix A (Student Handout – Basic Worksheet) and Appendix B (Student Handout – Advanced Worksheet). Both worksheets cover the same concepts, but the Advanced Worksheet includes chi-square analysis. These documents were written by Ann Brokaw and Laura Bonetta for BioInteractive.org and are used with permission. These documents are current as of date of the present work. To check for possible updated versions of these documents, see the BioInteractive.org website (HHMI 2013f). Appendices A-C are in Online Supplements.

Materials

The Stickleback Evolution Lab has been available online at no cost since October 2012; it can also be ordered at no cost on CD-ROM so that it can be used without Internet access. The lab was developed using HTML5. As of October 2013, the following browsers are supported: Microsoft Internet Explorer (version 8 or greater), Mozilla Firefox (version 9 or greater), Google Chrome (version 9 or greater), Apple Safari (version 5 or greater). For the virtual lab to work appropriately, JavaScript and cookies must be enabled and any pop-up blockers must be disabled. Browser plug-ins are not required to use this virtual lab. The **Stickleback Evolution Virtual Lab** is also accessible on tablet-sized mobile devices (e.g., iPad), best viewed horizontally. Please note that screen zooming is not fully supported on smaller devices (e.g., iPhone).

Notes for the Instructor

Teacher Materials

See Appendix C (Teacher Materials – Advanced Worksheet) in Online Supplements. Appendix C was written by Ann Brokaw and Laura Bonetta for BioInteractive.org and is used with permission. It is current as of the date of this publication. Visit BioInteractive.org (HHMI 2013f) for possible updated versions of this document. Appendices A-C are in Online Supplements.

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