DATA Nugget

Why so blue? The determinants of color pattern in killifish
Featured scientist: Becky Fuller from The University of Illinois

Research Background:

In nature, animals can be found in a dazzling display of different colors and patterns. Color patterns serve as signals to members of the animal's own species, or to other species. They can be used to attract mates, camouflage with the environment, or warn predators to stay away. When looking at the diversity of colors found in nature, you may wonder, why do animals have the color patterns they do? One way to study this question is to look at a single species that has individuals of different colors. This variation can be used to uncover the mechanisms that determine color.

The color polymorphism in bluefin killifish – males display anal fins in blue, red, or yellow. Photos by Tony Terceira.

The bluefin killifish is a freshwater species that is found mostly in Florida. They are found in two main habitats, springs and swamps. An intriguing aspect of this species is that male bluefin killifish are brightly colored with many different color patterns. The brightest part of the fish is the anal fin, which is found on the bottom of the fish by the tail. Some males have red anal fins, some have yellow anal fins, and others have blue anal fins. This variation in color is called a **polymorphism**, meaning that in a species there are multiple forms of a single trait. In a single spring or swamp you may see all three colors!


Becky in the field, with her colleague Katy, collecting fish in the Wakulla Spring.

Becky is a biologist studying bluefin killifish. One day, while out snorkeling for her research, she noticed an interesting pattern. She observed that there were differences in the polymorphism depending on whether she was in a spring or a swamp. Springs have crystal clear water that can appear blue-tinted. Becky noticed that most of the males in springs had either red or yellow anal fins. Swamps have brown water, the color of iced

Becky's family helping her out in the field!

tea, due to the dissolved organic materials in the water. Becky noticed that most of the males in swamps had blue anal fins. After noticing this pattern she wanted to find out why this variation in color existed. Becky came up with two possible explanations. She thought males in swamps might be more likely to be blue (1) because of the **genes** they inherit from their parents, or (2) because individual color is responding to environmental conditions. This second case, where the expression of a trait is directly influenced by the environment that an individual experiences, is known as **phenotypic plasticity**.

Becky had to design an experiment that could tease apart whether genes, plasticity, or both were responsible for male anal fin color. She did this by collecting male and female fish from the two habitat types, breeding them, and raising their offspring in clear or brown water. If a father's genes are responsible for anal fin color in their sons, then fathers from swamps would be more likely to leave behind blue sons. If environmental conditions determine the color of sons, then sons raised in brown water will be blue, regardless of the population origin of their father.

Becky and her colleagues collected fish from two populations in the wild - Wakulla Spring, and 26 Mile Bend Swamp - and brought them into the lab. These two populations represent the genetic stocks for the experiment. Fish from Wakulla are more closely related to each other than they are to fish from 26 Mile Bend. In the lab, they spawned female fish with male fish from the same population: females from Wakulla spawned with males from Wakulla, and females from 26 Mile Bend spawned with males from 26 Mile Bend. After the offspring hatched from their eggs, half were put into tanks with clear water (which mimics spring conditions) and half in tanks with brown water (which mimics swamp conditions). For the brown water treatment, Becky colored the water using 'Instant, De-caffeinated, No-Sugar, No-Lemon' tea. They raised the fish to adulthood (3-6 months) so they could determine their sex and the color of the son's anal fins. Becky then counted the total number of male offspring, and the number of male offspring that had blue anal fins. She used these numbers to calculate the proportion of sons that had blue anal fins in each treatment.

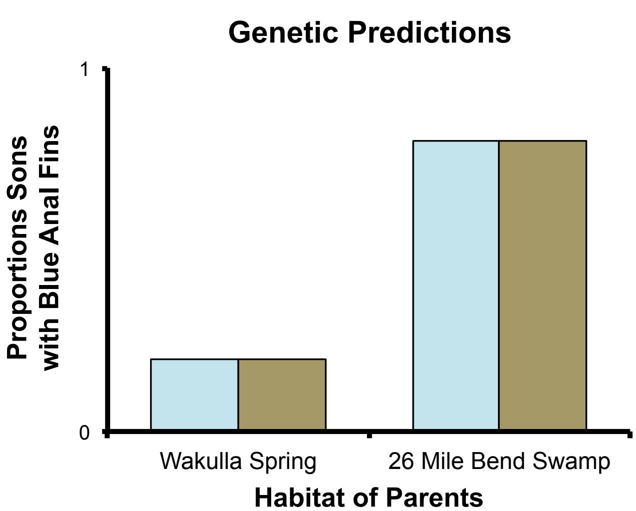
Scientific Question: Is male anal fin color determined by genetics, environment, or both?

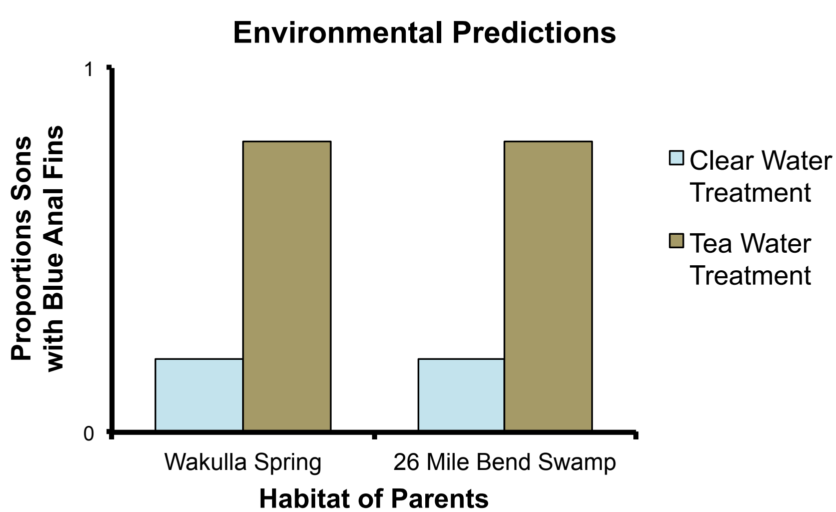
What is the hypothesis? Find the two hypotheses in the Research Background and underline them. A hypothesis is a proposed explanation for an observation, which can then be tested with experimentation or other types of studies. Having two alternative hypotheses means that more than one mechanism may explain a given observation. Experimentation can determine if one, both, or neither hypotheses are supported.

Check for Understanding: After reading the introduction, students should be able to:

- Discuss reasons why animals come in so many different colors.
- Understand that male bluefin killifish have a color polymorphism, meaning individuals can have different colors. For example, the male bluefin killifish in these populations can have blue, yellow, or red anal fins.
- What observations did Becky make about the frequency of each color in the two habitats? Were certain colors found more often in the swamp versus spring habitats? *Males with blue fins are found mostly in swamps with brown water, while males with red or yellow fins are found mostly in springs where the water is clear.*
- Understand that the color pattern in male fish is potentially both a genetic and plastic trait. The trait in the son may be influenced by the genes of the dad, and also the habitat where the sons are raised.
- Consider why the color polymorphism may exist in the bluefin killifish. Would a male with a blue anal fin be more or less visible in a swamp or spring? What about males with red or yellow anal fins? Would it be advantageous for a male killifish to blend in or stand out in his environment?
- Describe the experimental design, and discuss the importance of the type of tea that Becky used to color the water brown. Why did she make sure the tea had no caffeine, sugar, or lemon? *Becky was trying to replicate the swamp habitat by using instant tea to color the water for her brown water treatment. Tea releases chemicals from the leaf, including tannins. Tannins are the same chemicals that turn the swamp water brown. To make sure she didn't introduce any additional chemicals, she had to choose a tea that was unflavored and had no caffeine or sugar as these chemicals may have affected the fish in unknown ways.*

Draw your predictions: Becky hypothesized that the color of the bluefin killifish's anal fin is determined by potentially two factors: (1) the genes they inherit from their parents, and (2) the environment that they are raised in. Below, draw your predictions that arise from each of these hypotheses. In your first graph, draw your predictions if male offspring anal fin color is genetic and determined only by the genes they inherit from their parents. In your second graph, draw your predictions if male offspring anal fin color is plastic and determined only by the environment they are in.





Teacher Note: Teacher Note: You will notice that the prediction graphs included in the teacher guide reflect that there was some variation found in nature. If anal fin color were completely heritable, there would still be some genetic variation in this trait within the populations (Becky observed some, but not all, males were blue in both the spring and swamp). If your students draw their prediction graphs without this variation present (they have bars at 100% and 0%), this still reflects that they understand the role of the treatments and can separate out the genetic and plastic components of anal find color, but they have not incorporated the variation found in nature into their predictions.

Scientific Data:

Use the data below to answer the scientific question:

Father #	Parent Population	Water Treatment	Total # of Sons	Total # of Sons with Blue Anal Fins	Proportion Sons w/ Blue Anal Fins
1	Wakulla	clear	10	0	0
2	Wakulla	clear	33	1	0.03
3	Wakulla	clear	32	0	0
4	Wakulla	clear	26	0	0
5	Wakulla	clear	15	0	0
6	Wakulla	clear	25	0	0
7	Wakulla	clear	28	0	0
8	Wakulla	clear	17	0	0
9	Wakulla	clear	11	0	0
1	Wakulla	brown	16	0	0
2	Wakulla	brown	23	0	0
3	Wakulla	brown	26	0	0
4	Wakulla	brown	31	2	0.06
5	Wakulla	brown	31	1	0.03
6	Wakulla	brown	23	1	0.04
7	Wakulla	brown	42	0	0
8	Wakulla	brown	50	1	0.02
9	Wakulla	brown	14	0	0
10	26 Mile Bend	clear	29	1	0.03
11	26 Mile Bend	clear	21	0	0
12	26 Mile Bend	clear	17	1	0.06
13	26 Mile Bend	clear	16	0	0
14	26 Mile Bend	clear	31	1	0.03
15	26 Mile Bend	clear	34	6	0.18
16	26 Mile Bend	clear	14	0	0
17	26 Mile Bend	clear	31	0	0
10	26 Mile Bend	brown	38	11	0.29
11	26 Mile Bend	brown	6	3	0.50
12	26 Mile Bend	brown	24	2	0.08
13	26 Mile Bend	brown	17	3	0.18
14	26 Mile Bend	brown	20	5	0.25
15	26 Mile Bend	brown	28	5	0.18
16	26 Mile Bend	brown	25	0	0
17	26 Mile Bend	brown	42	3	0.07

Parent Population	Water Treatment	Average Proportion Sons w/ Blue Anal Fins	Standard Deviation (SD)	Sample Size (N)	Standard Error (SE)
Wakulla	clear	0.003	0.010	9	0.001
Wakulla	brown	0.018	0.018	9	0.006
26 Mile Bend	clear	0.038	0.038	8	0.013
26 Mile Bend	brown	0.194	0.194	8	0.068

**Standard deviation (SD) tells us about the amount of variation in the data. A large SD means there is a lot of variation around the mean, while a small SD means the data points all fall very close to the mean. Standard error (SE) is SD divided by the square root of the sample size (N), and tells us how confident we are in our estimate of the mean. A large SE means we are not very confident, while a small SE means we are more confident.*

Teacher Note - Error Bars: You can have students add error bars to their graphs to deepen this discussion or remove SE or SD from the table for younger students. Standard deviation (SD) is the most common measure of variation for normally distributed data. It is a measure of the average distance of all values from their mean. The smaller the bars, the less variation around the mean. Standard error (SE) is the SD divided by the square root of the study's sample size ($SE=SD/\sqrt{n}$). Unlike SD, SE reflects uncertainty in our estimate of the mean. The larger our sample size and the less variation in the data, the more confident we can be in our estimate of the mean. Upper error bars are calculated by adding one SE or SD to the mean, and lower bars are calculated by subtracting one SE or SD from the mean.

The simplest measure of spread or variation in a data set is the range, which is the difference between the largest and smallest values in the data set. For students unfamiliar with SD or SE, a discussion of range can help bring their attention to not only central tendency in the data, but also variation around the mean. Be sure to note with students that though range is easy to calculate, it can be misleading; one outlier can make it appear the data set is much more spread than it is.

What data will you graph to answer the question?

Independent variables: Water treatment (clear, brown) & Parent population (Wakulla, 26 Mile Bend)

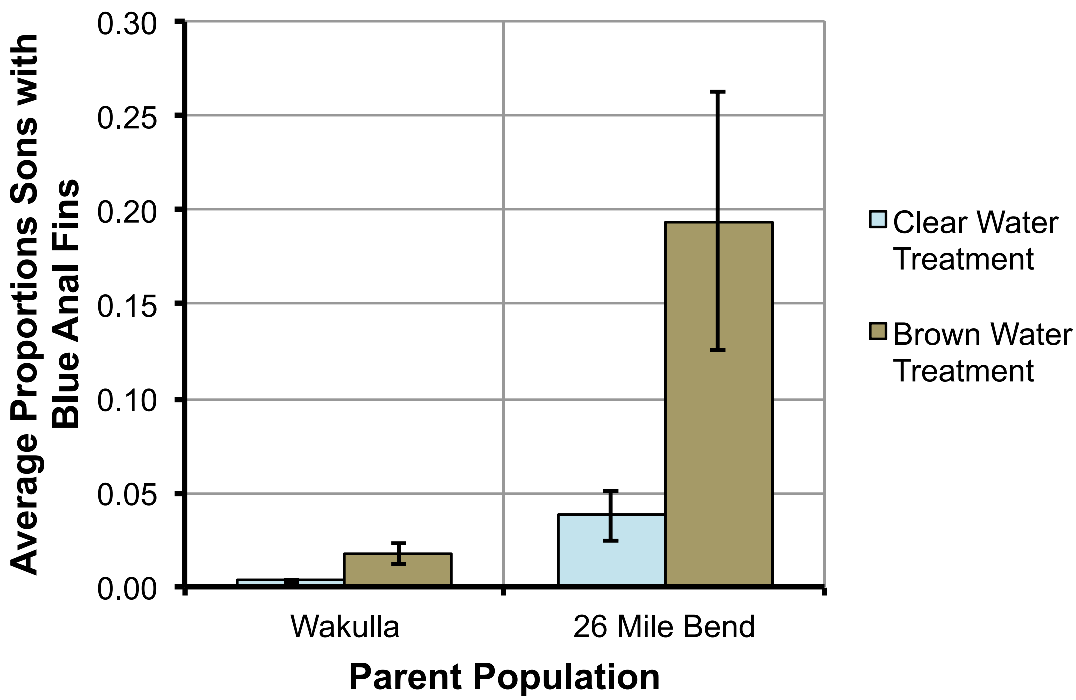
Dependent variable: Average proportion of sons with blue anal fins

<u>Draw your graph below</u>: Identify any changes, trends, or differences you see in your graph. Draw arrows pointing out what you see, and write one sentence describing what you see next to each arrow.

Teacher Note: Students can become overwhelmed when they try to interpret graphs, figures, or data tables. The Identify and Interpret (I^2) strategy, developed by the Biological Science Curriculum Study (BSCS), is a way to help students make sense of the information by breaking it down into smaller parts. In the I^2 strategy, students first *identify* changes, trends, or differences. They draw an arrow to each observation and then write a "What I see" comment. These comments should simply be what the student observes, such as a positive slope on a graph or increasing numbers in a data table. After students have made all their observations, they should *interpret* the meaning of their observations by writing a "What it means" comment for each.

Below are examples of student observations and interpretations for the data:

- I see that across both water treatments there are more blue sons in the 26 Mile Bend population.
    - This means that a son's anal fin color is partially due to their genetics, or the population there parents came from.
- I see that in both populations there are more blue sons in the brown water treatment.
    - This means that a son's anal fin color is plastic and responds to the clarity or color of the water they are in.
- I see that the effect of the water treatment was greater for the 26 Mile Bend population.
    - This means that there is an interaction between a son's genetics and the environment that they are in. Sons from 26 Mile Bend are able to respond more to the environment.



Interpret the data:

Make a claim that answers the scientific question.

For males, genetics (i.e. population of origin), environment, and an interaction between the two affect the expression of blue anal fin coloration.

What evidence was used to write your claim? Reference specific parts of the table or graph.

The genetic effect can be seen when we compare Wakulla and 26 Mile Bend populations within a single treatment. When offspring are raised in clear water conditions, there are very few sons with solid blue anal fins in Wakulla crosses (0.3% of males). There are more sons with solid blue anal fins in clear water conditions for 26 Mile Bend parents (3.8% of males). The same effect can be seen when we compare the two populations in the brown water treatment. Again, we see there are fewer sons with blue anal fins for Wakulla parents than for 26 Mile Bend parents (1.8% in Wakulla, 19.4% in 26 Mile Bend).

The environmental effect can be seen when we compare the two water treatments in parents from each population. Even though spring fish (Wakulla) have very few sons with solid anal fins, they have more of them in the brown water treatment (0.3% vs. 1.8%). The effect is even more pronounced for swamp fish (26 Mile Bend). Again, we see that there are many more sons with blue anal fins in brown water than there are in the clear water treatment (3.8% vs. 19.4%).

Teacher Note: We see that both hypotheses are supported - the expression of the phenotype depends on both the genetics of the parents (genetic), and on the environment experienced by the sons (plastic). To help students identify this point, you can have them compare their graphs back to the graphs that they drew in their Prediction sections. They will note that the results do not match either prediction graph exactly, and have aspects of both.

However, there is more going on than just the genetics and environment affecting the phenotype of the sons. There is also an interaction between the genetics and environment. This point will likely be the trickiest feature of the data for students to identify. To prompt students to consider this point, ask whether the data show the effects of the water treatment differ depending on the population that the father came from. Students can then identify that there is a greater effect of the water treatment for fathers from 26 Mile Bend than there is for fathers from Wakulla. Stated in another way, we see that the effect size of the treatment depended on the genetics (or population) of the father.

When the effect of one independent variable (water treatment) depends on the level of another independent variable (population of parents) this is known as an interaction between variables. In this case there is an interaction between genetics and the environment, known as a “GxE interaction”. We can use statistical tests such as analysis of variance to verify the interaction has a significant effect.

Advanced: The data show that there is a greater effect of the water treatments for the swamp population than there is for the spring population. Stated in another way, we see that the swamp population responds more strongly to the different environments (change of 15.6%) than does the spring population (change of 1.5%). The magnitude of the effect of the environment on the expression of the phenotype differs between the two populations.

Explain your reasoning and why the evidence supports your claim. Connect the data back to what you learned about how a bluefin killfish's genes and environment may affect their color.

In this study, a "genetic effect" is shown by the two populations having different proportions of a phenotype for a given environmental condition. In other words, if the offspring of Wakulla parents differ from the offspring of the 26 Mile Bend parents in one of the treatments (e.g. clear or brown water), then this must be due to genetic factors. The reasoning here is that all the other environmental conditions are the same. The fish were reared in the same water conditions, fed the same food, experienced the same temperatures and light regimes, etc. Hence, if the offspring from Wakulla parents differ from the offspring of 26 Mile Bend parents, then this must reflect something that they inherited. Also note that simply seeing that one offspring has a different phenotype than another one is not enough. Rather, we must see consistent differences between genetic strains. In this case, the two populations are the different genetic strains.

In this study, an "environmental effect" is indicated when the two water treatments differ within a population. Stated another way, if there are differences in the phenotypes of offspring raised in clear water compared to those raised in brown water within a single cross type (Wakulla or 26 Mile Bend), then this must reflect effects that are induced by the environment. In the data above, we see that there are more male offspring with blue anal fins in brown water in both the spring and swamp populations. This indicates that something about the environment is different in the two water treatments and causes phenotypes to develop in different ways.

One thing to note about the 'environmental effect' is that one has to be careful to be sure that you are manipulating the desired thing. In this case, Becky wanted to create a treatment that mimicked swamp water. The interpretation is that the difference in the water clarity caused the difference in the expression of the phenotype. However, there could conceivably be other things that vary as an effect of the treatments. For example, there was less algae growth in brown water tanks compared to clear water tanks. While Becky tried to remove the excess algae, one could argue that this excess algae could cause the differences in male color pattern. The treatment clearly caused a large effect, but the treatment itself might affect multiple aspects of the environment.

Did the data support one, both, or either of Becky's two alternative hypotheses? Use evidence to explain why or why not. If you feel the data was inconclusive, explain why.

The data tell us that all three types of variation (genetic, environmental, and an interaction between genetics and environment) affect the presence of blue anal fin color in nature. In the brown water treatment with tannin-stained water, we find sons that are more likely to express blue coloration.

Your next steps as a scientist: Science is an ongoing process. What new question do you think should be investigated? What future data should be collected to answer this question?

See following Teacher Notes.

Teacher Note: Student responses may vary, and they will probably generate a wide diversity of questions for in this system. Remember, if your class wants to send their questions about the study system to Becky, the scientist studying Bluefin killifish, they can email them to datanuggetsk16@gmail.com!

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Becky's future research is addressing the following themes:

- One noticeable aspect of our study was that there were very low levels of blue expression - even for the swamp offspring raised in brown water. In swamps in nature, we can find up to 70-80% of the population with males with solid blue anal fins. Yet, in our experiment, the treatment with the highest level of solid blue sons only had 20% blue males. One possibility is that for both males and females, we got the eggs from the spawning substrate and then raised them in plastic containers in clear water for about 2-3 weeks. This means that all of the offspring experienced clear water conditions for 2-3 weeks. If water color determines fin color as early as the egg stage, this may have contributed to low levels of blue males. To test this, we are conducting an experiment where we performed population crosses (spring, swamp, and variable) and raised the offspring in one of the following treatments (1- parents spawn eggs in tea and offspring are raised in tea; 2 - parents spawn eggs in tea and offspring are raised in clear; 3 - parents spawn eggs in clear and offspring are raised in tea; 4 - parents spawn eggs in clear and offspring are raised in clear). From this experiment we hope to determine whether there is a critical time period when animals must receive the environmental cue.
- The pattern also raises the question of 'Why be blue in a swamp?' We are performing mate choice studies, male/male competition studies, and predation studies to determine whether these color morphs differ in either mating success or susceptibility to predation and whether this varies as a function of lighting environment.
    - Students can collect data on this question themselves. What do you think the colors would look like under the different lighting environments? Do an experiment. Print out the color checker included below. Then, get an aquarium and fill it with water. Look at the color checker under both clear water and tea-stained water. You can use any type of tea. Compare the colors. Or better yet, take a picture of the color checker in clear and brown water and then use the picture to get the RGB values from some of the squares. Be careful to keep your illumination (i.e. the light from above) constant when you do this.