Agonistic Behavior in *Betta splendens*
Developing an Experimental Protocol

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You and your team, equipped with an ethogram template and your experience observing *Betta splendens* behavior, are now ready to design a research project. You will not be manipulating the environment of your fish. You will be observing its natural reactions to visual stimuli of your team's choice.

I. Experimental Design: Resources

Each team will be supplied with male and female *Bettas* in individual containers.
You will also have available:

- paper cutout **models** ("puppets") of
  - male and female Bettas
  - different colors, sizes, positions
- **mirrors** of various sizes (and convex/concave)

Paper fish models will usually elicit a response, though not as strong as the response to a reflection. However, the subject will also not habituate as quickly to a static model as to a mirror. Why might this be the case?

If using a paper model, move it slowly up to the subject and then wave it slightly to attract the subject's attention. Try to use similar technique and movement in each trial, to avoid introducing human error into your experiment.

Male *Bettas* respond strongly to the sight of another *Betta*, whether male or female. Consider the responses of your fish to same sex or different sex stimulus.

Do not feel obligated to use or limited by the resources we provide. Your research project should reflect your team's interest and creativity. Use any devices your team feels are necessary for a good research project that still stays within our guidelines.

You might consider using **videos** or **other stimuli** on an electronic device.
This could require some creative engineering of a set-up that will allow you to
(1) display the stimuli to your fish in a repeatable, consistent manner and
(2) prevent damage to your electronic device
But the results could make the effort worthwhile.

II. Experimental Design: Hypotheses

When you have finished observing fish and creating an ethogram, meet with your team to discuss ideas based on your literature search and lab observations. Each individual team member may wish to write a list of ideas, and then the team can confer to discuss all ideas and decide which ones to use (and modify).

Consider your **observations** of *Betta splendens* behavior.
What **questions** related to the fish's behavior do your observations elicit?

Confer with your team members and ask questions about

- aggression
- agonistic display
- mate choice/sexual selection

The focus of your research is for you and your team to decide.

In designing your experiment, consider some of the following questions, and also try to conjure original questions of your own.

- What are the possible functions of agonistic display in male *Betta splendens*?
- Is there a "trade off" between conflicting evolutionary needs (e.g., the need to attract a mate vs. the need to avoid attracting predators)?
- Does agonistic display between two males change if there are other fish observing the contest? Does their sex have an effect?
- How might certain aspects of the display behavior have been adaptive in the wild ancestors of these fish?
- Why do males bother with display? Why not just launch into battle?
- Wild *Betta splendens* are not as brightly colored, and do not have fins as long and showy as the domestic variety you are observing. How might artificial selection have affected the *behavior* of these fish? Do you think some or all of your results can be applied to wild populations? Which ones, if any, and why?
- You may or may not be aware that even species that can distinguish different wavelengths of light as "color" may not see colors the same way we do. An animal's **spectral sensitivity** is the range of wavelengths of light that elicit a response in the visual system. **Hue discrimination** is the ability to distinguish different wavelengths of light as different stimuli ("colors"). If you plan on doing anything with color, then you must familiarize yourself with the literature on *Betta splendens* spectral sensitivity, hue discrimination, and other aspects of vision.
- What other environmental factors might affect the agonistic display of *Betta splendens*? Of what evolutionary and/or ecological significance are these factors?

III. Choosing the Appropriate Statistical Test

There are many variations one could choose to examine behavior in our fish model.

- multiple fish, each subjected to one of two different types of stimuli
- multiple fish subjects, each subjected to two different types of stimuli, in sequence
- multiple fish, each subjected to more than two different types of stimuli
- etc.

Your team must determine whether your data

- fall along a normal ("bell shaped") distribution
- are parametric or non-parametric
- are independent or paired

This information will help you choose the appropriate statistical test for your analysis.

A. Independent or Paired Parametric Data

Timed duration of behavior is usually considered parametric, continuous numerical data. However, because of the limitations of our recording devices, you are unlikely to be able to record data at intervals other than seconds. Because of this, even timed durations of behavior are best considered ordinal, non-parametric data.

B. Independent Non-parametric Data

The **Mann-Whitney U test** is used to compare differences between two independent groups if the dependent variable is ordinal, but is not normally distributed. If multiple fish are each being subjected to a single stimulus (e.g., either treatment or control), then trials should be considered **unpaired data**. The **Mann-Whitney U** is appropriate for the analysis.

C. Paired Non-parametric Data

The **Wilcoxon signed-rank test** can be used to compare repeated measurements on a single subject (paired data) to assess whether their population mean ranks differ. If a single fish is being subjected to two different stimuli, then the trials should be considered **paired data**. The **Wilcoxon Test** is appropriate for this analysis.

D. Non-parametric Data, More Than Two Variables

The **Friedman Test** is a non-parametric version of the Analysis of Variance (ANOVA) test, used when data are ordinal. If a fish is subjected to more than two different classes of stimulus, this test would be appropriate for the analysis. Before embarking on this more complex project, be sure you and your team members are ready and willing to learn and use the appropriate statistical test, which is "off label" for BIL 161.

IV. Paired or Independent Samples?

In your project examining *Betta splendens* behavior, you will perform paired trials. Your team will choose two different stimuli--of your team's choice--and present them to each of 24 experimental subjects (12 fish each week, for two weeks).

You will measure the **same parameters**--of your team's choice--**for all subjects**.

The **paired trials** will allow you to compare the reaction of the same subject to two different stimuli while avoiding the confounding effects of individual differences in temperament, health, hormone levels, or any other factor that could affect the fish's behavior.

The **large sample size** will allow you to test whether there is a statistically significant tendency in your population to react to one stimulus or the other in particular ways.

V. Behavior Trials: Timing and Protocol

Developing a good protocol requires that you understand the set-up of experimental stations that everyone will be using during the data collection sessions. An overview is provided here. But it would be wise of you to read next week's lab manual to get full details about how the trials will be conducted.

Because all teams will be using the same subjects, you will be moving from station to station, and not simply using a single pair of fish at your own station.

There will be **six numbered stations**. Each will have

- One **male fish** in a bowl on the **left** (the **left fish**)
- One **male fish** in a bowl on the **right** (the **right fish**)
- One **female fish** in a rectangular container
- All fish have **ID codes** written on their containers in wax pencil.

Your paired test will employ two different stimuli. For simplicity, we'll refer to them here as **stimulus A** and **stimulus B**. But your team may name the stimuli any way you see fit.

One **station round** will consist of your team running a single one-minute trial on each of two fish at the station. You will flip a coin to determine the order of the stimuli.
Let's say the coin toss said use **stimulus A** on **left fish**, and **stimulus B** on **right fish**.

First Station:

Trial 1: **left fish** receives **stimulus A**
Five minute rest period begins at end of timed stimulus.
Cover the fish bowl and begin preparing for Trial 2.

Trial 2: **right fish** receives **stimulus B**
Five minute rest period begins at end of timed stimulus.
Cover the fish bowl and wait for instructor signal to change stations.

At the end of two trials, your instructor will tell teams when to move to the next station.

Second Station:

Trial 3: **left fish** receives **stimulus B**
Five minute rest period begins at end of timed stimulus.
Cover the fish bowl and begin preparing for Trial 2.
Trial 4: **right fish** receives **stimulus A**
Five minute rest period begins at end of timed stimulus.
Cover the fish bowl and wait for instructor signal to change stations.

Notice that you have changed the order of stimuli at the second station.

This controls for the effects of a fish behaving differently simply because a stimulus is the *second one* to which it is exposed, and not because the stimulus was *different*.

Third Station:

Before you begin, flip a coin again to determine the stimulus order for Trials 5 – 8.
Let's say this time the coin said to use **stimulus B** on **left fish**, and **stimulus A** on **right fish.**
Trial 5: **left fish** receives **stimulus B**
Five minute rest period begins at end of timed stimulus.
Cover the fish bowl and begin preparing for Trial 2.
Trial 6: **right fish** receives **stimulus A**
Five minute rest period begins at end of timed stimulus.
Cover the fish bowl and wait for instructor signal to change stations.

Fourth Station:

Trial 7: **left fish** receives **stimulus A**
Five minute rest period begins at end of timed stimulus.
Cover the fish bowl and begin preparing for Trial 2.
Trial 8: **right fish** receives **stimulus B**
Five minute rest period begins at end of timed stimulus.
Cover the fish bowl and wait for instructor signal to change stations.

Fifth Station:

Flip a coin again to determine the stimulus order for Trials 9 – 12.
Let's say this time the coin once again said to use **stimulus B** on **left fish**, and **stimulus A** on **right fish.**
Trial 9: **left fish** receives **stimulus B**
Five minute rest period begins at end of timed stimulus.
Cover the fish bowl and begin preparing for Trial 2.

Trial 10: **right fish** receives **stimulus A**
Five minute rest period begins at end of timed stimulus.
Cover the fish bowl and wait for instructor signal to change stations.

Sixth Station:

Trial 11: **left fish** receives **stimulus A**
Five minute rest period begins at end of timed stimulus.
Cover the fish bowl and begin preparing for Trial 2.
Trial 12: **right fish** receives **stimulus B**
Five minute rest period begins at end of timed stimulus.
Cover the fish bowl and wait for instructor signal to change stations.

When your team has completed 12 trials, you will be halfway done.

At the next change, you should be back at the station where you started.
Review the stimuli you presented to each of these fish in the first round.
You will now present each fish with the ***alternative*** stimulus, the one it did not receive in the first round. This will comprise your paired sample.

For example:

If you did this in the first round (first station):

Trial 1: **left fish** receives **stimulus A**
Five minute rest period begins at end of timed stimulus.
Cover the fish bowl and begin preparing for Trial 2.
Trial 2: **right fish** receives **stimulus B**
Five minute rest period begins at end of timed stimulus.
Cover the fish bowl and wait for instructor signal to change stations.

Then you will do this in the second round (first station):

Trial 13: **left fish** receives **stimulus B**
Five minute rest period begins at end of timed stimulus.
Cover the fish bowl and begin preparing for Trial 2.
Trial 14: **right fish** receives **stimulus A**
Five minute rest period begins at end of timed stimulus.
Cover the fish bowl and wait for instructor signal to change stations.

Do this alternation of stimuli for another 12 trials. At the end, you will have two trials for each fish, each with a different, paired stimulus.

You might wish to devise a table or spreadsheet something like that shown in Table 1. It is provided as a sample. Your team will likely need to make a spreadsheet specific to its experimental protocol.

Table 1. A sample spreadsheet for experimental trial organization.

ROUND ONE				
Trial #	**left or right fish**	**fish ID Code**	**Station #**	**stimulus**
1a				
2a				
3a				
4a				
5a				
6a				
7a				
8a				
9a				
10a				
11a				
12a				
ROUND TWO				
Trial #	**left or right fish**	**fish ID Code**	**Station #**	**stimulus**
1b				
2b				
3b				
4b				
5b				
6b				
7b				
8b				
9b				
10b				
11b				
12b				

VI. Peer Review and Critique

When all teams have a preliminary protocol ready, each team should give a brief presentation their project proposal to the class. Include:

- observation
- question
- overall hypothesis
- null and alternative hypotheses
- prediction
- statistical test to be used
- brief description of methods

This should take no more than about ten minutes per team, but it is a critical part of project development. Critique by interested/expert peers (your instructor and your classmates) will help you find weaknesses in your experimental design and correct them before you waste time and energy on something that will not work well.

After each team has presented their project and been critiqued, teams should meet again for about 15-20 minutes to edit and refine their projects.

Once your team has a fully developed research project, complete the **Research Project Template** linked to this week's syllabus section.

Submit it to your instructor electronically before you leave lab today.