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Once a Loser Always a Loser? Using Crayfish to Teach Behavioral Endocrinology

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Introduction

Using live animals to address questions about evolutionary and proximate mechanisms of behavior is often avoided in high-enrollment introductory biology labs because of the difficulties involved in maintaining a large animal colony. However, by working with live animals students can understand the relationship between behavior and the endocrine mechanisms that drive it. We present a behavioral endocrinology lab using live crayfish to examine aggressive behavior and the role of serotonin in the maintenance of a "loser effect," i.e. the likelihood that an individual that has lost an aggressive encounter in the past will be defeated in future confrontations. The students aim to determine whether serotonin can reverse the loser effect by comparing aggressive interactions of crayfish before and after the losers have been injected with serotonin. During the course of the lab students learn how to handle animals, observe and score behavior, and generate an ethogram based on their observations. While multiple hormones are involved in aggression, we chose to use serotonin based on primary literature showing its role in aggression and establishment of social status. Additionally, serotonin is fast acting and it is present in invertebrate and vertebrate species. Because serotonin is an evolutionarily conserved hormone, it is easy to draw the connection between its effect on crayfish behavior and its role in human behavior. Based on the content knowledge of the student body and the focus of the course, this lab can be easily modified to incorporate a variety of discussion topics of varying complexity levels.

Student Outline

Experimental protocol

1) Prepare a fighting arena by filling up a Tupperware © container with deionized/ pond water and placing gravel on the bottom. Use a divider to separate the arena into equal halves (you can use the container lid).
2) Pick two size-matched crayfish of the same sex. If more than one animal is housed per tank, make sure that opponents do not come from the same tank.
3) Place the animals in different halves of the arena and allow 10 minutes to acclimate.
4) At the end of acclimation period, lift the divider to allow interaction.
5) As the crayfish interact, score each behavior using the ethogram in Table 1 and record it in the data sheet (Figure 1).

Table 1. Crayfish ethogram (adapted from Moore, 2007)

Behavior	Score
Tailflip away from opponent	-2
Retreat	-1
Ignore	0
Approach	1
Threat (Meral spread)	2
Approach and threat	3
Pushing / Boxing	4
Pulling at opponent's claws and body parts (will not see if animals are banded)	5

It is easiest if each pair of students within a group only scores the behavior of one of the crayfish, while the other pair scores the behavior of the opponent.

6) Allow the interaction to persist for a set amount of time (e.g. 20 minutes or until a winner is established).
7) Separate the crayfish and place each opponent into separate isolation containers.
8) Based on crayfish availability, you can decide whether to use (a) winners from the first interaction as opponents for the losers in the next interaction or (b) use naïve individuals that have not fought before.

   a. Using winners from previous interactions may confound new interactions (i.e. winners tend to win future interactions); however, you should still see a difference between the interactions of serotonin vs. saline injected losers and saline injected winners.

   b. If you intend to use naïve individuals, then place them in the isolation containers and return the winners to the respective holding tanks.
9) Inject the winning/naive crayfish with 100 µl of saline and loser crayfish with 100 µl of serotonin/saline (your instructor will specify which solution you will be injecting).
10) Since crayfish communicate chemically as well as visually, you need to reset the fighting arenas before starting the next set of interactions.
11) Depending on how many crayfish you have available, find either a size-matched naïve opponent, or a previous winner from a different group to interact with the loser crayfish that has been injected with serotonin.
12) Place the injected loser and saline-injected opponent into the fighting arena that is divided into equal halves (as in step 3).
13) Lift the divider and score the interaction.
14) At the end of the experiment return the crayfish to their respective tanks.

15) Compare the behavior of the loser before and after injection.

a. If your instructor did not tell you what you injected (i.e. serotonin or saline), can you figure it out by comparing your results with your classmates?

b. Plot the scores of each opponent.

Figure 1. Sample recording sheet for fighting crayfish.

Sheet 1: Agonistic behavior of crayfish 1
Sex _____
Total Length (TL) _____cm Length of cheliped_____cm Weight_____g

Time	Behavior	Notes

Materials

Supplies and ordering information are listed in Table 1.

Notes for the Instructor

Crayfish maintenance

1) General holding tanks should be filled with about 3-4" of pond/stream water. If pond water is not available, just put tap water in the tank about 24 hours before putting live animals. You can also use deionized water.
2) Place mud or gravel on the bottom of each tank and PVC tubes for crayfish to hide in. Alternatively, you can use clay pots, bricks or plants.
3) Use an aerator for each tank. Depending on how many tanks you may have, a large air pump with multiple connectors might be more practical (and economical) than multiple small pumps.
4) For fighting arenas and isolation containers, use fresh pond or deionized water. Do not use water from holding tanks.

Table 1. Supplies and ordering information.

Organism/ Item	Description	Company	Product #	Price/ unit	Quantity*
Live crayfish	Medium, pack of 50	Carolina Biological	142514	$99.95	1
Live crayfish	Medium,pack of 12	Carolina Biological	142512	$36.00	2
Preserved crayfish	Pail of 25	Biologyproducts.com	CF0406P	$16.25	1
BD Tuberculin syringe	1cc,; pack of 100	Fisher Scientific	14-829-10D	$21.11	1
Needles	26.5 or 31.5 gauge	Fisher Scientific	305111	$7.71	1
Serotonin creatinine sulfate complex	250 mg	Sigma-Aldrich	H7752	$20.60	1
Eppendorf tubes	2ml, for serotonin and saline	Fisher Scientific	13-698-792	$34.53	1
Fighting arenas	Any container (Tupperware © works well)	Any home goods store (Home Depot, Target, Wal-Mart, etc.)			6
Small containers for isolation	Tupperware container with lids work well	Any home goods store (Home Depot, Target, Wallmart, etc.)			12
Gravel	To place on the bottom of fighting arenas	Home Depot			
PVC Tubes	1/ crayfish	Home Depot			62
Small rubber bands	To band the crayfish (optional)	CVS/Walgreens etc.			124
Tank lids		Any pet store			30
Holding tanks	10 gallons	Any pet store			30
Thick protective gloves	Only needed if you will not band the crayfish.				
**Aerator	For each tank	Any pet store			
**Air pump		Any pet store			
**Tubing	Specific for the air pump	Any pet store			

* Quantities are based on 24 students per class, with 4 students per group.

** See crayfish maintenance.

Solutions

If you choose to run a blind experiment, where your students don't know whether they are injecting serotonin or saline into the loser crayfish, then you will need some sort of a coding system to distinguish between saline and serotonin without making it obvious to the students.

For a blind experiment, randomly assign which loser crayfish in the class will be injected with serotonin and which with saline. Do NOT tell your students what substance they are injecting, but remember what you assign.

Solutions must be prepared in advance. Saline can be stored refrigerated for up to a year. Serotonin aliquots can be kept at -20°C indefinitely. Do NOT thaw and refreeze serotonin; use each aliquot once and discard the remainder.

Table 2. Ingredients for crayfish saline solution.

Chemical	Molarity (mM/L)	Formula Weight (g/mol)	Grams needed for 1000 ml solution
NaCl	210	58.44	12.27
KCl	5.4	74.50	0.402
CaCl2	10	147	1.47
MgCl2	2.4	203.30	0. 487
NaHCO3	2	84.006	0.168

Crayfish Saline

1) Combine all the chemicals (Table 2).
2) Add 1000 ml of deionized water
3) Adjust pH to 7.4
4) Make 50, 100 µl aliquots and freeze.

   These aliquots should either be in differently colored tubes or marked with a symbol that does not readily identify the tube contents.
5) Distribute the remaining saline between in a beaker and store at 4°C.

Serotonin (5-Hydroxytryptamine)

1) You will need to dissolve serotonin with saline and create aliquots.
2) To get the stock concentration of 0.5mM/L, dilute 250 mg of 5-HT in 122 ml of crayfish saline
3) Aliquot serotonin into eppendorf tubes, at 200 µl of 0.5 mM/L, store at -20°C until ready to use.
4) On the day of the experiment, dilute serotonin aliquots to the desired concentration (0.05mM/L) by mixing it with 1800 µl of crayfish saline (or dilute 100 µl of serotonin in 900 µl of saline). One 1000 µl tube should be enough for an entire class. You might need to test this concentration with your particular batch of animals, but this is a good starting point.

Handling and injecting live crayfish

1) To pick up a live crayfish, approach it from behind and gently grasp the carapace. Never grab the extremities. The crayfish may flail its legs and chelipeds.

   a. If the chelae have not been banded, provide protective gloves for the students that will hold the crayfish during the injection (see below). If the crayfish have been banded, then you don't need gloves.
2) To inject, one student should hold the crayfish by the carapace, and place a gloved finger (if the chelae are not banded) right in front of the chelae. While one student is holding the crayfish, the other student should gently insert the needle into the space between 2nd and 3rd walking leg.
3) Once the needle is in (it does not need to be in all the way), the student should slowly expel 100ul of either saline or serotonin into the hemolymph. This needs to be done slowly, for about 10 seconds.
4) Injected crayfish should be placed gently back into a holding tank and given 10-15 minutes to recover and for serotonin to take effect.

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About the Authors

Anna Goldina received her B.S. in biological sciences from Florida International University in 2003 and is currently a PhD candidate at Florida International University. Her dissertation research examines the role of hormones in the evolution of communication behavior in electric fish. As a teaching assistant, she teaches introductory biology laboratories and really enjoys developing inquiry-based laboratories that challenge and inspire students.

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