Responses to Hormones/biogenic amines in Invertebrates: Student Laboratory Exercises

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PURPOSE

The purpose of these experiments is to observe organisms undergoing behavioral and physiological changes due to altered hormonal status. In these experiments one will also learn ways to quantify and index behavioral repertoires for comparative purposes in order to address potential mechanistic actions to explain the behavioral changes.

PREPARATIONS

Crayfish and *Drosophila melanogaster*

INTRODUCTION

Hormones are chemical signals that are released from cells and transported within the organism to target cells where they can exert their action. Mammals have a closed circulatory system and hormones are transported in the blood to stimulate specific cells. In contrast, insects and crustaceans have an open circulatory system so called because the hemolymph is contained in a cavity called a hemocoel instead of vessels. Hemolymph, a fluid equivalent to blood, directly bathes tissue such as muscles and parts of the nervous system and contains blood cells, lymph, and interstitial fluid. Hormones travel to target tissue in the hemolymph just as they would in blood of a mammal.

In organisms, homeostasis is maintained in many ways by hormones. The nervous system and endocrine system are closely linked; hormones influence development of neurons and conversely the nervous system affects hormone secretion. For example, during stressful situations, nerves signal for an increase in secretion of certain hormones and a decrease in the secretion of others. Hormones can also affect the development of an organism. Specifically in insects and crustaceans, the hormones that regulate the process of developmental changes during metamorphosis are known as ecdysteroids. The key hormone is 20-hydroxyecdysone (20-HE), currently regarded as the active form of ecdysone (Riddford 1985; Steel and Davey 1985). The ecdysteroids are important in causing the behavioral and physical changes that occur during the developmental stages of each molt (Truman 1996; Baehrecke 1996; Cayre et al. 2000; Farkaš and Sutáková,1998,1999; Henrich et al. 1993, 1999). Even the neurons and muscle can be restructured during metamorphosis and ecdysteroids are the key player in this process.

Numerous studies have been conducted in *Drosophila* and the tobacco hornworm *Manduca sexta* (Jacobs and Weeks 1990; Levine and Weeks 1996; Truman

1996; Truman and Reiss 1995). Behavior of the *Drosophila* larva changes both immediately before and after a molt, but the possible mechanisms modulating behavior are poorly understood. In late 3rd instar, the animals slow down and begin to form a pupa. Reduced synaptic strength may contribute to their lower locomotory activity during this stage and could possibly be related to increased concentration of ecdysone (Ruffner, et al., 1999), since the highest levels of ecdysteroid have been measured during the 3rd instar larva to prepupal formation and in the pupa stage preceding the adult stage (White et al., 1997). The possible change synaptic physiology has not been studied; however, we do know that application of 20-HE will reduced synaptic transmission in *Drosophila* larvae (Ruffner, et al., 1999) and has an effect on overall development (Li and Cooper, 2001).

Serotonin (5-HT), dopamine (DA) and octopamine (OA) are well known to act as neuromodulators in insects, particularly in *Drosophila melanogaster*, which when altered can produce behavioral and developmental defects as well as organizational problems in the CNS circuits (Monastirioti, 1999; Osborne, 1996). 5-HT modulates voltage dependent potassium channels and heart rate in *Drosophila* (Johnson et al., 1997; Zornik et al., 1999). DA is known to alter sexual behavior, sensory habituation (Neckameyer, 1998a,b) and increase activity in adult flies (Friggi-Grelin et al., 2003) but depress synaptic transmission at the NMJ in larval *Drosophila* (Cooper and Neckameyer, 1999). OA expression is stress related in *Drosophila* (Hirashima et al., 2000) and OA receptors are present in mushroom bodies in *Drosophila* CNS which is a region important for learning in adults (Han et al., 1998). In fact, 5-HT, DA, and OA all have some central effects in the *Drosophila* brain related with learning or behavior (Blenau and Baumann, 2001; Monastirioti 1999). Recently, direct actions of these neuromodulators were shown to alter central neural activity (Dasari and Cooper, 2004).

These biogenic amines have broad differential effects on development and physiology in larvae as well as in adults. The development and the distribution of 5-HT immunoreactivity neurons in the CNS are established (Valles and White, 1988). 5-HT has a role in many physiological process such as regulating locomotion and cardiac output (Dasari and Cooper, 2006; Kamyshev et al., 1983; Johnson et al., 1997; Nichols et al., 1999; Zornik et al., 1999; Johnson et al., 2000). Since 5-HT alters the activity of sensory-to-motor central circuits in larval *Drosophila* (Dasari and Cooper 2004) this opens the possibility that the serotonergic system could sculpt the formation of neural circuits by altering the neural activity in the developing CNS of *Drosophila.* Activity of developing neural circuits is well established to play a major role in the patterning of the adult CNS in mammals prior to critical periods (Hubel and Wiesel, 1963a,b, 1968, 1970).

5-HT is associated with modulation of eating/digestion in crustaceans (Shuranova et al., 2006) and humans (Aubert et al., 2000) as well as in motor unit coordination (LeBeau et al., 2005; Dasari and Cooper, 2004; Strawn et al., 2000; Weiger, 1997) and behavior (Bicker 1999; Toth et al., 2005; Barnes and Sharp, 1999) in a wide variety of animals. MDMA (ecstasy), a drug of abuse, modulates the homeostasis of the serotonergic system in humans and animal models (Green et al., 2003). Research is scant on the effects of MDMA in the developing CNS of mammals as well as in insects (Dasari et al., 2007).

Crayfish behavior is commonly studied in the laboratory in order to control the multitude of variables within natural field settings. However, there are variables that impact behavioral studies of crayfish when the animals are caught in the wild and brought into the laboratory setting. For example, life history, social ranking, and past injuries can be difficult to assess in wild caught crayfish. Within the intact crayfish a few hormones (neurotransmitters) have been studied to address regulation of behaviors. By pooling information in various studies of crustaceans some headway is being made. For example, Sneddon et al., (2000) noted that the neurotransmitters octopamine, dopamine and serotonin (5-HT) all increase in the hemolymph when a crab is exercised. Melatonin is known to have a cyclic pattern in many crustaceans but it varies depending on the species and their circadian/rhythmic patterns (Tilden et al., 2003a,b). The effect of neuromodulators on complex circuits is being tackled in crustaceans (Grashow et al., 2009; Marder and Eisen, 1984; Marder and Thirumalai, 2002; Pagé and Cooper, 2004; Shuranova et al., 2006; Strawn et al., 2000; Swensen and Marder, 2001) as well as in *Drosophila* (Dasari and Cooper, 2004; Dasari et al., 2009).

Crayfish are good for studying a threat response, such as from a predator, since crayfish behaviors in social groups have been studied and the behavioral repertories cataloged (Bovbjerg 1953, Rubenstein and Hazlett 1974, Pavey and Fielder 1996), Stein and Magnuson 1976). Behaviors in the repertoire include chelipeds spread out to ward off a potential predator or a tail flip response, either forward are backward, to rapidly escape a predator. It is suggested that such behaviors are regulated by neuromodulators, such as 5-HT (Huber and Delago 1998; Livingston et al., 1980; Yeh et al. 1996); however, the general notion of 5-HT's action for aggressive behavior is debated (Listerman et al., 2000; Strawn et al., 2000; Tabor and Cooper, 2002).

Likewise, other environmental and internal factors influence behaviors such as a temperature, satiety, reproductive status, and diet. These and other factors can have a multitude of effects on various hormones, neurotransmitters and peptides, and various substances may counteract actions of others or even increase the effect of particular compounds (Djokaj et al., 2001). Thus, it may seem to be a hopeless myriad of factors to address and characterize the effects of hormones on behavior, but some behaviors are tightly linked to the molting process and new research is being conducted on the hormonal regulation related to these behaviors.

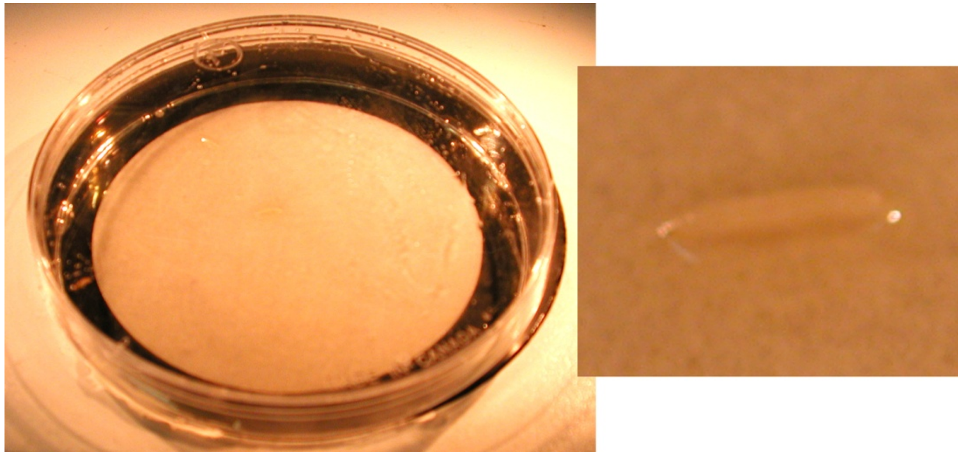
MATERIALS AND METHODS

Drosophila

Item	**Number Needed Per Group**
Dissecting microscope	1
Novaflex lamp	1
Drosophila larvae (3rd instar stage)	6
Filter paper (No. 10)	2
Large Petri dishes (10 mm)	2 halves
Small weigh boats	2
Cotton tip wooden dowels	2
Dilute apple juice	4 drops
Serotonin solution (100 nM)	2 drops
Yeast solution	2 drops

1. The wild type fruit fly, *Drosophila melanogaster*, Canton-S, will be used in this study. The methods used to stage the fly larvae have been described previously (Campos-Ortega and Hartenstein, 1985). The early 3rd instar stage of larvae will be used in these studies; these larvae will still be burrowing in their food. All the animals will be housed in room temperature (25°C) on cornmeal-agar-dextrose-yeast medium.

2. Take 6 fly larvae out of the stock container. Place three in the labeled Petri dish containing yeast solution made with 5-HT (100 nM) and three others in the labeled Petri dish containing yeast solution without the 5-HT. Allow the larvae to eat for 15 minutes. During this time, label the bottom of two Petri dishes that contains apple juice filter paper; label one 5-HT and the other control. Using separate dishes keeps the control larvae form being contaminated with residual 5-HT on the surface or from excretion of the larvae.



3. After 15 minutes place the control Petri dish on the microscope and count the number of mouth hook movements (MHM) for 30 seconds for each individual larva. Mouth hooks are black stripes on the front side of *Drosophila* that move every time the *Drosophila* larva eats. Alternate measuring one control larva and then one 5-HT fed larva. Repeat the procedure for all the larvae. Be careful not to recount the same larvae. Record the MHM per minute for indexing with reports in the literature.

4. After counting the MHM gently pick up the larvae with a small wooden dowel and move to the appropriate dish containing the apple juice moistened filter paper. Place a lid on the Petri dish to encourage the larvae to crawl before placing the dish on the microscope. Count the number of body wall movements (BWM) for 30 seconds. One body wall movement is a complete contraction in the mid-length of the larvae while it is crawling. Alternate measuring one control larva and then one 5-HT fed larva. Repeat the procedure for all the larvae. Report the body wall movements per minute for indexing with reports in the literature.

Data Table 1: Behaviors in Control *Drosophila*

Trial	Body Wall Movements / min	Mouth Hook Movements / min
1		
2		
3		
Average		

Data Table 2: Behaviors in 5-HT *Drosophila*

Trial	Body Wall Movements / min	Mouth Hook Movements / min
1		
2		
3		
Average		

Crayfish

Item	Number Needed Per Group
Crayfish	2
Plastic tub filled with distilled water	2
Electronic balance	1
10-mL plastic or glass pipette	1
1-mL syringe	2
Serotonin solution (100 nM)	Volume varies
Crayfish saline solution	Volume varies

5. Select two crayfish of approximately equal size and weight. Carefully blot dry a crayfish with paper towel and label this experimental crayfish with a number 1 on the dorsal thorax using quick drying fingernail polish. Weigh the experimental crayfish and calculate the amount of 5-HT needed to obtain a 100nM 5HT hemolymph solution. Approximately 30% of the weight for a blue crab or a lobster is hemolymph (Gleeson and Zubkoff, 1977; Guirguis and Wilkens, 1995); assume this percentage also holds true for crayfish in these experiments. The 5-HT stock solution is 1 μM. Determine the amount of stock solution (V_2) to be injected into the crayfish for a circulating concentration of 100 nM 5-Ht using the following calculations:

crayfish weight x .30 = hemolymph volume

$$M_1V_1 = M_2V_2$$

$$(0.100\ \mu M)\ (\text{hemolymph volume}) = (1\ \mu M)\ (V_2)$$

(Make sure to see how many ml to inject (10 gram crayfish obtains a 0.3 ml injection)

6. Double check the calculation with the teaching assistant/ instructor and obtain a syringe and needle containing the stock 5-HT. A saline control syringe will be also provided. Saline will be used as a control for handling and injection volume. The control saline is composed of crayfish saline (mM: 205 NaCl; 5.3 KCl; 13.5 $CaCl_2.2H_2O$; 2.45 $MgCl_2.6H_2O$; 5 HEPES adjusted to pH 7.4) which is a modified Van Harreveld (1936) saline. The saline will also have a microfilter attached to keep out bacteria.

7. Hold the experimental crayfish behind its claws and wrap it in a paper towel. Position your hand to hold the tail spread out with your pinky finger or have your partner hold the tail down. Inject the experimental crayfish with the appropriate amount 5-HT by inserting the needle in the clear space toward the edge of the abdomen. Do not inject into the midline of the abdomen because this is the location of the central nerve cord. Hold the needle in place for approximately 30 seconds to avoid the solution from leaking out when withdrawing the needle. Repeat the procedure with saline in the control crayfish.

8. Place the crayfish in the observation tanks; one crayfish per tank. Crayfish are very visual so minimize the visual disturbance over the crayfish. Observe the crayfish without stimulus for 5 minutes. After the 5 minutes, reach over with the plastic rod and gently tap the experimental crayfish on the telson. If the telson is tucked under the animal, tap the side of the abdomen close to the telson. Observe and record the behavior due to the tap as well as any behavior between the taps. Examples of behavior: no response, tail flip, defensive posture, retreat. Repeat this every 2 minutes for 10 minutes (5 times total); more often may cause the crayfish to become habituated to the stimulus. Record data in Table 3.

9. After the 5 taps, carefully touch the water in front of the crayfish and record the behavior. This should be done just far enough away that the crayfish will not be able to grab your hand with its chelipeds.

Response:

10. Repeat the procedure for the control crayfish. Make sure to treat the control and experimental animals in a similar manner when conducting these experiments. Record data in Table 4.

TAKE CAUTION as a crayfish pinch or tail flip can cause injury. It is not the strength of the muscle pinching that hurts but the fine teeth like structure at the tip that can pierce the skin. If your skin is broken by a pinch or tail flip wash well with soap and water and inform the teaching assistant/ instructor.

Data Table 3: Behaviors in 5-HT Crayfish

Trial	Retreat	Tail Flip	Defensive Posture	Total Responses
1				
2				
3				
4				
5				
Average				

Number of chelipeds present: ______________ (0, 1 or 2)

Data Table 4: Behaviors in Control Crayfish

Trial	Retreat	Tail Flip	Defensive Posture	Total Responses
1				
2				
3				
4				
5				
Average				

Number of chelipeds present: ______________ (0, 1 or 2)

DISCUSSION

Such laboratory investigations can provide pertinent information because they can initiate one to start to address cellular mechanisms of compounds to account for the observed behaviors.

Parallel investigations on the well-characterized neuromuscular junctions (NMJs) of these organisms would be beneficial in examining roles of 5-HT to compliment potential commonalities. The crayfish neuromuscular preparations offer an advantage over the *Drosophila* preparations because the motor nerve terminals are large enough to place intracellular electrodes within the axons for assessing effects on the properties of the action potential shape and size that can directly affect the amount of calcium that enters the nerve terminal, thus influencing the amount of vesicles to dock and release

transmitter. In addition, one can readily address if action is affecting evoked calcium entry within the nerve terminal by using calcium sensitive indicators and confocal microscopy (Cooper et al., 1995). The interactions of compounds altering presynaptic transmission and 5-HT enhancing presynaptic release is worthy of investigation for a number of reasons. First, the mechanism of action of neuromodulators is not understood and the interactions of signaling pathways would reveal novel avenues of compounds on neuromodulatory actions within nerve terminals. Secondly, 5-HT is a normal substance released into the crustacean hemolymph at various times in behaviors. The actions on behavior are still an area plagued with phenomenon logical assessment without mechanistic driven questions. With the *Drosophila* mutations, such as *Rutabaga* and *Dunce* that are available which produce altered levels of second messengers (Zhong et al., 1992), future studies on the mechanistic actions and biochemical cascades induced by neuromodulators can also be investigated.

ALTERNATE PROCEDURES / EXTENSIONS:

Students can work in groups of 4, two counting mouth hook movements and two counting body wall movements, sharing the 10 larvae.

Drosophila: The following day, check the different larvae to determine if length of time larvae are allowed to eat in the yeast solution could affect the length of time before the pupae stage forms.

Crayfish: The following week check molting of the control and experimental crayfish. Repeat the experiment to determine if there is a difference in behavior of the control and experimental crayfish.

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