Pheromonal Control of Male Courtship Behavior in *Ambystoma mexicanum*: A Laboratory Exercise in Comparative Neurobiology

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An important premise of my philosophy of teaching is that interest in science arises from *doing*, as opposed to *being taught* science, and to this end I try to involve students in my own research whenever possible. Among the courses I teach is an upper-level elective course in comparative neurobiology, which includes a laboratory exercise that uses ethological methods to test the hypothesis that pheromonal cues produced by female axolotls are the principle cues used by males to identify receptive females and to initiate courtship. The exercise complements my own research, which concerns structure/function relations among the gonadotropin releasing (GnRH) neuronal populations of vertebrates. I have focused on urodele amphibians, including *Ambystoma mexicanum*, because their simple brains and stereotyped behaviors make them excellent model systems for investigating basic questions in vertebrate reproduction. There is considerable indirect and inferential evidence that urodele amphibians, including ambystomids, make extensive use of pheromonal signals during reproduction, but the question has not been much studied. The laboratory exercise thus presents a genuine research problem, as opposed to the typical undergraduate lab exercise, in which the techniques are thoroughly worked out and the answers known. Just as we scientists can appreciate the difference between exploring the unknown and simply repeating the work of others, students likewise may be unimpressed by "canned" lab projects, but become genuinely excited by the real thing.

However, incorporating real science into the undergraduate curriculum presents certain challenges. Undergraduate students lack sophisticated technical skills, the cost of fully equipping a student laboratory with state-of-the-art equipment is usually prohibitive, and the standard academic schedule of a 3-4 hour lab period that meets once a week does not lend itself to many types of endeavors. Research designs that utilize non-invasive behavioral measures to elucidate physiological processes circumvent many of these problems. It is my experience that a brief introduction to ethological approaches and to the behaviors in question is often sufficient to produce keen, insightful observations on the part of even inexperienced students. From a pedagogical point of view, the less time needed for technical training, the more time is available for more intellectually challenging and creative aspects of research, including exploring the background literature, designing experiments, and analyzing data. It is not necessary to justify invasive, potentially deleterious surgical procedures or euthanasia to institutional animal use committees, or to the students, many of whom have strong feelings about animal suffering. Finally, if we are to judge by the number of popular articles and TV programs featuring animals, observing the natural behaviors of animals is intrinsically interesting and enjoyable.

The laboratory hand-out is reproduced below. Following this, I will briefly present the experimental protocol that my 1994 class designed, their results, a few technical comments and suggestions, and a few words on some preliminary experiments I am conducting with students on the neurochemical basis of male axolotl courtship.

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A. Social Signals and Reproduction. In order to reproduce sexually, animals must overcome a number of problems. First, males and females must be able find and recognize each other. Then, they must be in synchrony with regard to reproductive condition and willingness to mate, they must be able to recognize members of the opposite sex that are in this condition, and/or they must possess the ability to bring the potential mate into a state of reproductive readiness. Finally, they must coordinate the fertilization of gametes.

In many species, this coordination is achieved through an exchange of social signals between males and females. Reproductive social signals span a vast variety of behaviors that influence every sensory modality. They include the visual displays of male peacocks, the bright coloration patterns of coral reef fish, the chorus of frogs, amplexus in urodeles, tongue flicking in snakes, and release of chemical pheromonal cues in many aquatic and terrestrial organisms. In the language of ethologists, each of these signals constitutes a *fixed action pattern*, i.e., a species-specific, stereotyped behavior. It is common for mating to involve an exchange of social signals as the individuals move through the various phases of reproduction: Each behavior by the male, for example, elicits a response from the female, which in turn elicits a different behavior by the male.

B. Introduction to the Lab. During the next four weeks, we will study reproductive behavior and the species-specific stimuli by which it is controlled. The focus of our experiments will be courtship behavior by male axolotls (*Ambystoma mexicanum*). You will first perform an ethological study, identifying and characterizing the various behaviors that make up the courtship and mating ritual of this species. You will then design and carry out experiments to test the hypothesis that pheromone released by female axolotls triggers male courtship.

During **Week 1** of the project, we will observe and videotape the normal axolotl courtship ritual. You will prepare a catalog of the male courtship behaviors, using the film record or your own notes. In **Week 2**, you will present your ethological analyses, and we will discuss the study questions. The study questions for this project focus on techniques, experimental design, and data analysis for studying the role of sex pheromones. You will develop a detailed protocol using behavioral procedures to test the specific hypothesis of this project and apply this procedure during **Week 3,** when you will work on your own gathering data. In **Week 4** we will discuss procedures for statistical analysis of the data. You will turn in a written **Laboratory Data Analysis** for this project.

C. Ethological analysis of behavior. Neuroethological studies begin with ethology. The behavior(s) that are the object of the study must be clearly and accurately described (why?). Early ethologists frequently used the phrase "objective study of behavior" to refer to their methodological approach. That scientists strive to be objective in their methodology seems self-evident. In practice, however, this is seldom a trivial task. Some common problems that beginning investigators encounter are:

(1) Problems identifying and distinguishing among subsets of motor activities that make up an entire behavioral sequence. To say that behaviors of an animal are stereotyped does not meant that they are completely invariant. Most complex behaviors are comprised of a number of much smaller motor acts, which may or may not be linked together in a fixed sequence. We can think of these smaller units, operationally, as "behavioral units": A single behavioral unit, which itself is made up of the activities of many muscles, is an all-or-none phenomenon. For example, prey capture by a frog consists of several steps that seem to meet the definition of "behavioral units": the frog first orients toward the prey, he may then walk or hop toward the prey, followed by the final step in the sequence which is a rapid extrusion by the tongue which captures the insect. However, there is a lot of variation possible: How the frog orients towards the prey, i.e., whether just the head or the whole body moves, seems to depend upon the location of the prey in the animal's visual field. Whether or not stage 2 occurs frequently depends upon the distance to the prey: frogs frequently move from orientation directly to capture without the intervening locomotion step if the insect is near. The amount of time intervening between the three stages is also variable. Currently, there is a debate among neuroethologists about whether terms like "fixed action pattern," which by classical definition implied a high degree of behavioral stereotypy, may no longer be useful (see letters for Gerhard Roth and Russell Fernald). It should also be noted that attempts to define "behavioral unit" in strictly objective and quantitative terms have proven to be problematic.

(2) Problems clearly and accurately defining the various acts that make up a complex behavioral sequence. The principle difficulty here lies in the tendency to be anthropomorphic, that is, to assign human behavioral definitions, or motivations, or emotional states to animals, or to assume that someone else will understand your behavioral analysis because of a shared understanding of culturally defined words. For example, use of the word "stroll" to describe a category of locomotion may have meaning in a particular cultural context of human movement, but there is no *a* *priori* reason to think that animals "stroll." You could, however, use the word stroll as a short-hand descriptor of an animal behavior *after you have clearly and objectively defined what you mean by this term.*

Similarly, it is important to avoid terms like "wants" or "is trying" to do something. For example, in your observations you will probably infer that a male engages in a particular behavior because he is trying to attract the female. However, in the natural setting male displays are often used in other contexts, for example, territorial defense. Singing by male songbirds is a good example of a behavior that may have more than one meaning.

(3) Problems establishing an appropriate context for the behavior to take place. Most animals are reluctant to engage in reproductive behaviors if they are mishandled or otherwise stressed in any way, if they are in a strange environment, if they have recently been moved, etc. It is very important that you obey specific instructions about handling the animals: above all, be gentle. Approach them slowly with the net, transport them slowly, lay them gently into the test tub. Males should be transferred to the testing arena at least 30 minutes prior to behavioral observations.

D. Evidence for reproductive pheromones in urodele amphibians. Urodele amphibians use olfactory cues in a variety of contexts, including territorial marking, recognition of breeding sites, nests, and egg clutches, and individual recognition. Although it is widely believed that urodeles also use sex pheromones, most of the evidence is either indirect or based on qualitative or anecdotal observations, or does not directly measure reproductive behavior (for a review, see Verrell, 1988). For example, most species possess a variety of well-developed exocrine glands, including several in the cloacal region that are controlled by gonadal secretions, and it is inferred that these are likely the source of sex pheromones. Male newts showed a preference for water from a female in a Y-maze test (Dawley, 1984). Madison (1975) showed that odor-based preferences are different in male and female salamanders, and that these preferences vary with breeding condition. Extracts from male mental (chin) glands stimulated sexual receptivity in a female plethodontid salamander (Houck and Reagan, 1990), providing direct evidence of that male chemical signal affects female reproductive behavior. However, the question of whether sexual activity or responsiveness of male urodele amphibians is influenced by chemical secretions of females has, to our knowledge, not been explicitly addressed.

E. Reproductive biology of the Axolotl. The neotenic salamander, *Ambystoma mexicanum* (axolotl), offers a number of advantages for studying the use and significance of amphibian sex pheromones. Axolotls mate readily in the laboratory during most of the year, and both males and females actively participate in courtship. Since axolotls have been popular models for embryology experiments for many years, techniques for maintaining axolotls in captivity and for inducing natural spawnings have been developed, and courtship and mating behaviors have been described (cf. Eisthen, 1989; Armstrong and Malacinski, 1989).

Axolotls mature at about one year of age, at which time obvious sexual dimorphisms appear. Adult males can be identified by the presence of large bulbous cloacal glands located bilaterally at the base of the tail. Mature females lack external cloacal glands and, as a result of the large eggs masses in the ovaries, have much rounder abdomens than males

For your projects, we have available 3 adult females and 8 adult males. We also have animals of a unique mutant strain called "*eyeless.*" The gene *e*, which arose spontaneously at the Indiana University Axolotl Colony, is a single locus, autosomal, nonlethal recessive allele. The homozygous (*e/e*) phenotype is characterized by lack of eyes, a dark or freckled pigmentation, and sterility, the basis of which appears to be abnormal development of gonadotropin-releasing hormone (GnRH) neurons in the hypothalamus. In vertebrates, GnRH, released into the circulation at the median eminence, stimulates release of gonadotropins (luteinizing hormone, follicle stimulating hormone) from the anterior pituitary, which in turn stimulate gonadal maturation and secretion of sex steroids. In *eyeless*, axons of GnRH neurons fail to develop connections with the median eminence (Eagleson and Malacinski, 1986; Maccagnan and Muske, 1992). Thus, while male and female gonads are present and appear normal, they remain immature. Although it has not been experimentally determined, it is probably safe to assume that *eyeless* axolotls lack sex pheromones, which in most vertebrates are controlled by gonadal steroids.

Except for the absence of eyes, the absence of morphological secondary sex characteristics, and the differences in pigmentation, *eyeless* individuals appear indistinguishable from normal individuals of the same age with regard to size, locomotor activity, and behavioral ability.

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G. Questions and Exercises: For Discussion in Week 2

1. Develop a catalogue of the behavioral units you observe during male axolotl courtship.

2. What experimental approaches might be used to test the hypothesis that male courtship behavior is stimulated by a pheromone produced by reproductively active females? Think of both non-invasive and invasive methods. In all cases, discuss appropriate control procedures.

3. Using only non-invasive procedures, design an experiment to test the hypothesis that male axolotl courtship is stimulated by a female pheromone. Your experimental design should incorporate the use of sexually mature males and females, and sexually immature *eyeless* axolotls. Include in your design:

   (1) Discussion of how you will quantify your results.

   (2) Control experiments. Be sure you can explain what variable(s) are being controlled for.

   (3) Appropriate statistical tests of your data.

4. Is the hypothetical female pheromone more likely to be a primer or a releaser? Explain your views.

Results of the 1994 Project

In Week 2 we viewed the videotape of normal axolotl courtship, and students were asked to identify and define the behavioral units that make up male courtship. Based upon their analyses, and my previous observations, we identified two distinct stages of courtship, a preliminary phase, which seems to be exploratory in nature, followed by a much more active phase, and a total of six distinct, easily recognizable behaviors.

A. Catalog of Male Axolotl Courtship Behavior

Stage 1: Preliminary Courtship

1. Approach. Either the male or the female slowly approaches the partner. To be counted, the approach behavior must be directed, and must result in the animals being positioned within 1/2 body length of each other

2. Contact usually directly follows "Approach": The male or female gently contacts the torso, cloaca or tail with his or her snout, often slowly moving the snout over this area. This may elicit approach and/or contact by the partner, or the partner may move away, or there may be no response at all.

During preliminary courtship, episodes of approach-contact by both sexes are often interrupted by lengthy periods of inactivity, in which the male and female remain stationary, at variable distances from one another.

Stage 2 Active Courtship.

The second stage of courtship is characterized primarily by a dramatic increase in male locomotor activity, both random and female-directed, and by the expression of behaviors not usually observed in preliminary courtship. In between bouts of female-directed behavior, the male may rapidly, and seemingly randomly, move about the container.

3. Push: the male vigorously butts his snout at the head or torso of the female and moves forward, pushing the female around the container with his snout, or he attempts to move forward. The female usually remains passive during this maneuver.

4. Following. Movement of the female away from the male elicits rapid following by the male.

5. Over-the-Head. Further on in Stage 2, the male approaches the female from behind or the side, and slides his entire body over her head or upper body, often bringing his cloacal gland into contact with her nostrils.

6. Tail wave. The male stands erect usually a few inches in front of the female's head, with tail lifted and head pointed away from the female, exposing his cloaca. With limbs and upper torso rigid, the male slowly waves his tail from side to side.

B. Experimental Design. The protocol developed by the class to test the hypothesis that a female pheromone stimulates male courtship behavior consisted of a classical randomized blocks design, in which each male was tested once in four different conditions in which either the partner or the type of water in the test arena was varied. (In all cases, "water" refers to dilute Holtfreter's solution, of the same composition and concentration as used by the Axolotl Colony). For each trial, students first placed a male in the test arena for 30 minutes, then introduced the partner, and for the next 20 minutes recorded incidences of each of the six preliminary and active courtship behaviors, as well as general activity, measured by counting the number of grids crossed by the male. Just prior to the student trials, I pre-tested the eight males for willingness to court responsive females and found six to be responsive.

Condition 1: female partner, fresh water
Condition 2: *eyeless* partner, fresh water
Condition 3: *eyeless* partner, water from tub housing females.
Condition 4: *eyeless* partner, water from tub housing another male.

C. Experimental Results. Of the six males identified as sexually responsive in the pre-trials, one was not tested in Condition 1 at all, due to confusion in the scheduling, three more showed no response to a female, leaving only two males which exhibited courtship in Condition 1. For Condition 3, three of six males, including one who was unresponsive in Condition 1, exhibited strong courtship to the *eyeless* partner in female water. One of the six was not tested (another scheduling error), and the remaining two failed to court the *eyeless* partner. Males tested with the *eyeless* in Conditions 2 and 4 (fresh water and male water), exhibited virtually no courtship. These results are displayed graphically in Fig. 1. Non-specific locomotor activity (not shown) generally paralleled courtship activity.

As the error bars and the foregoing discussion indicate, there was a great deal of variance among males. Some of the variance can probably be reduced by improving the experimental design. Since the latency between introduction of the partner, and the first courtship overtures of males can vary by as much as 30 minutes, it is best not to commence event recording until after the male makes his first approach. In most cases this will also increase the number of courtship events occurring in a 20 minute period.

A large component of the variance appears to be due to short-term fluctuations in male sexual responsivity. As noted above, fully 50% of the males who were responsive to females during pre-trials did not respond to females under identical conditions during the trials. The short-term variation in male responsivity can to some extent be controlled for by pre-testing males on the same day of the experimental trials. It is possible, for example, that the two males identified as unresponsive in

Fig. 1: Pheromonal Control of Male Courtship

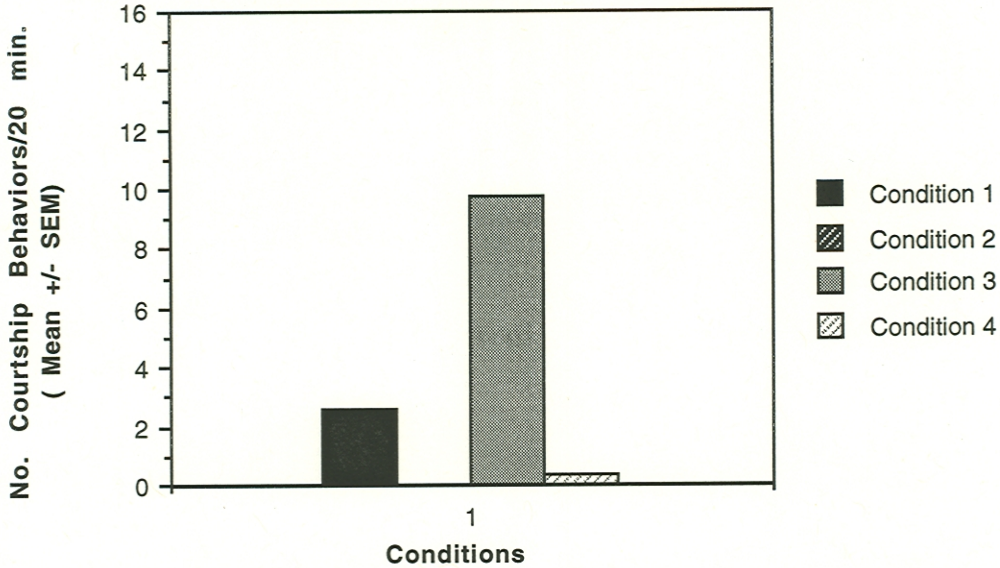

Figure 1. Average number of male courtship behaviors per 20 minute trial as a function of partner and type of water. Males were most responsive to an *eyeless* partner when tested in water from a tub housing females (Condition 3). There was a moderate response to female partner in fresh water (Condition 1). None of the males responded to the *eyeless* partner in fresh water (Condition 2), and only one of 6 males exhibited a slight response to the *eyeless* in male water (Condition 4).

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pre-trial tests would have responded to one or more of the experimental conditions had they been studied on a different day.

The basis of variability among sexually mature males in their responses to females is not known. We are presently conducting experiments on the efficacy of certain neuropeptides and hormones, which can facilitate sexual behavior in other species, to enhance sexual responsiveness of male axolotls. In a pilot study, we found that chicken gonadotropin-releasing hormone II (cGnRH II), injected i.p. at 100 μg/100 gm body weight can dramatically enhance a male's willingness to court. Whether the stimulatory effect of GnRH interacts with the normal sensory cues, chemical or otherwise, that receptive females provide, or whether GnRH might be acting "downstream" of sensory systems, i.e., directly on motor centers in which courtship behaviors are organized, is not known.

We are also interested in knowing whether acute treatments with GnRH or other hormones or neurotransmitters can restore normal sexual activity, including spawning, to chronically unresponsive males, i.e., males that do not respond to females for many months. Recently, Toyoda et al. (1994) showed that prolactin and human chorionic gonadotropin enhanced the preference shown by male Japanese newts to a chemical attractant of females. Apart from basic research questions about the neurochemistry of sex behavior, information on the behavioral action of hormones and neuroactive substances are of potential value to axolotl breeding programs. Such studies can also be incorporated in student laboratory exercises. The injections are easy to perform and have no apparent deleterious effects on the animals.