EMBRYONIC OLFACTORY LEARNING IN LARVAE OF *SMILISCA PHAEOTA* (COPE, 1862) (ANURA: HYLIDAE)

APRENDIZAJE OLFATORIO EMBRIONARIO EN RENACUAJOS DE *SMILISCA PHAEOTA* (COPE, 1862) (ANURA: HYLIDAE)

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Abstract

The first demonstration of embryonic olfactory learning in a tropical anuran was documented in the Colombian treefrog *Smilisca phaeota*. It was determined that *Smilisca* tadpoles learned to recognize a specific odor and preferred it over others. Embryos were exposed to the olfactory substance (orange essence) in two different ways; first, an artificial stimulation involving injection of the substance into the egg; and second, a "natural" exposure mixing the odor in the water surrounding the developing embryo. Learning capacity was evaluated in an aquarium where two olfactory stimuli were presented, the odor experienced as embryos and a novel odor. Olfactory influence prior to hatching simultaneously created in the individuals an avoidance behavior toward the novel odor. In this case, larvae associated preferably to neutral stimuli such as water. For each method, control embryos were exposed to saline solution and water.

Key words: embryonic olfactory learning, olfactory stimuli, tadpoles, Colombia, *Smilisca phaeota*.

Resumen

La primera demostración de aprendizaje olfatorio embrionario en un anuro neotropical fue documentada en *Smilisca phaeota*, un hilido colombiano. Los renacuajos de esta especie aprendieron a reconocer un olor específico y a preferirlo en vez de otros. La esencia de naranja utilizada fue expuesta a los embriones en dos tratamientos. Primero, una estimulación artificial inyectando la sustancia en el vitelo del embrión y segundo, una exposición "natural" mezclándola en el agua donde éste se desarrolló. La capacidad de aprendizaje se evalúo en un acuario donde el renacuajo se enfrentó al mismo tiempo a dos estímulos olfatorios, el olor experimentado como embrión y otro extraño. Simultáneo al aprendizaje, la influencia olfatoria embrionaria creó en el individuo una repulsión al olor desconocido. En este caso, el renacuajo se asocia preferiblemente a un estímulo neutral como lo es el agua. Los controles para cada tratamiento fueron embriones expuestos a solución salina y agua, respectivamente.

Palabras clave: aprendizaje olfatorio embrionario, estímulos olfatorios, renacuajos, Colombia, *Smilisca phaeota*.

INTRODUCTION

The anuran capacity to learn olfactory traits from their environment has been evaluated in several laboratory assays (Grubb, 1973a, 1973b; Hepper and Waldman, 1992; Pfennig, 1990). In experiments with different frog species, Grubb (1973a, 1973b) concluded that anurans can learn and remember odors from natal pools as cues for homing to breed. In tadpoles, it has been demonstrated that they are able to learn during embryogenesis to recognize artificial odors that have been injected into the egg or added to the water surrounding the developing embryo (Hepper and Waldman, 1992; Pfennig, 1990). Later in the larval stage, they show a high preference for such odors, orienting toward the

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previously learned odor sources. Perception and processing of the cues is through of the olfactory sense, which becomes functional before other sensorial systems (Spaeti, 1978).

Assuming that the capacity for olfactory learning is a general characteristic of amphibians (Hepper and Waldman, 1992), I tested between 1998 and 1999 the effects of exposure of *Smilisca phaeota* embryos to artificial odors on their subsequent odor preferences in the tadpole stage. The methodology was virtually identical to that used by Hepper and Waldman (1992), both in terms of injecting the olfactory stimuli within the vitelline membrane and in presenting stimuli in the water surrounding the developing embryo. I expected that this species would exhibit an embryonic capacity to learn odors, as has been shows in other species.

MATERIALS AND METHODS

Study area. The study was conducted in El Danubio (Alto Anchicayá region; 3º 33' N, 76º 53' W; 300 m elevation). Dagua municipality (Valle del Cauca department), Colombia, which is located on the western slopes of the Cordillera Occidental (figure 1). This region is typical of tropical humid forest (Espinal and Montenegro, 1963), with a bimodal rainy season from March to May and from September to November, with annual precipitation exceeding 3.000 mm (data from the Corporación Autónoma Regional Valle del Cauca –CVC-, cited in Vargas-S. and Castro-H., 1999).

Study species. The species, *S. phaeota* (Cope, 1862) (Hylidae: Hylinae) is widely distributed from northeastern Nicaragua to the Pacific lowlands of Colombia and Ecuador, below elevations of 1.000 m (Duellman, 1970). This species may breed in temporary pools throughout the year, but breeding activity seems to be greatest during the rainiest months. Using five *S. phaeota* egg clutches each collected in different temporary roadside puddles (less than 1 m in diameter), two different experiments were conducted to analyze the effects of early exposure to odors on learning capacity. The objective of the first experiment, which was divided into three sub-experiments, was to evaluate the acquisition of preferences for odors injected into the egg. The second experiment also evaluated the acquisition of odor preferences, but the stimuli were mixed into the water surrounding the developing embryo.

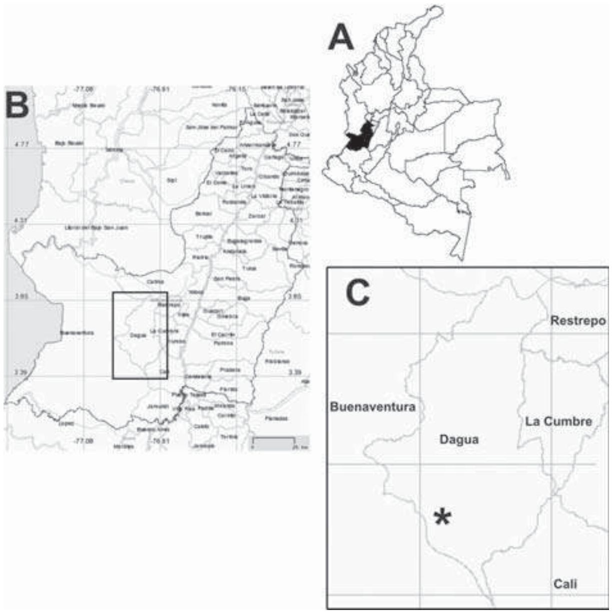

Figure 1. Maps of the Colombia (**A**), the Departamento of Valle del Cauca (**B**), and Dagua Municipality showing the study site El Danubio (*****) in Anchicayá river region (**C**). Maps courtesy of Instituto Geográfico Agustin Codazii (Colombia) and Instituto Alexander von Humboldt (Valle del Cauca and Dagua), generated from WEB (Rodriguez et al., 2004).

Embryonic olfactory learning (general methods for experiments 1 and 2)

Study subjects. Tadpoles used in the experiments were obtained from the five *S. phaeota* clutches collected from the puddles, each with an average of 300 eggs. Two clutches were collected on 20 and 24 November 1998, respectively, and the other three clutches were collected on 15 February 1999. The difference in the collection dates was due to the difficulty of finding multiples amplectant *S. phaeota* pairs on the same night, since the study was conducted during a period of low breeding activity. The egg clutches were moved to a laboratory located in a room of a residence located near the collecting sites, where conditions of light, temperature, and humidity were comparable to those in the natural environment.

The egg masses were divided into groups of 20 eggs and placed in a 1 L plastic recipient filled with fresh spring water, where they remained until the beginning of the experiment. Temperatures in the room were maintained between 24-26 °C during the day and 20-23 °C at night, with a 14:10 h photoperiod. Light was supplied with a 60 W yellow light bulb.

Procedure. Testing was performed in a 45 x 20 x 15 cm glass aquarium filled to a depth of 10 cm with fresh spring water. The aquarium was divided lengthwise into three equal parts of 15 cm by marking the test box floor with white lines with masking tape. A 4 x 4 x 4 cm box made of fine stainless-steel mesh was placed in the middle of each end of the aquarium. Each box was tightly packed with cotton wool impregnated with the odorant used in the different tests.

The odorant stimuli used in these experiments were orange and blackberry essences diluted to 20% in water. Pieces of cotton wool were impregnated with the odor substance for 12-h immediately preceding the experiments. The control stimulus was fresh water, with the piece of cotton wool impregnated in the same way for 12 h. Tadpoles were classified in the experiments as experimental or controls depending on whether they were exposed or not, respectively, to the stimulus substances.

Experiment 1: acquisition of odor preferences by embryonic *S. phaeota*. To evaluate the responses of tadpoles exposed to odor stimuli, embryos were assigned to two experimental groups and a control group. The experimental groups consisted of embryos injected with 10 ml orange essence (diluted to 20% in water) and 10 ml isotonic saline solution, respectively. These groups will be referred to hereafter as orange and saline tadpoles. The injection was conducted during embryogenesis (between stages 15-18 of development: Gosner, 1960) into the egg (within the vitelline membrane), using a 1 ml insulin syringe (Becton-Dickinson Co, USA) and 29-gauge needle (12.7 mm), with the aid of a dissecting microscope (magnification x30). The control group consisted of tadpoles reared alone in fresh water and not injected with any substance. After this process, each embryo of the experimental and control groups was placed individually into a 300 ml water-filled plastic tray and maintained there until the respective tests. The hatchling tadpoles were fed with boiled lettuce leaves cut into pieces. Each treatment group was used in a different experiment.

The first sub-experiment used both the orange tadpoles and the control group. Twenty larvae from each of the five clutches were taken (50 of which were from the orange group and the remainder from the control group). Each group was presented simultaneously with the orange and water stimuli. The second sub-experiment was conducted with 50 larvae obtained from five clutches (10 individuals each), all injected with isotonic saline solution. Here, the larvae were presented simultaneously with orange and control stimuli. The third sub-experiment was conducted with another 10 embryos from each family (n = 50) injected with the orange essence. This experiment examined whether the pre-hatching exposure to orange odor resulted in a non-specific preference for olfactory stimuli other than water, rather than a specific preference for orange. The larvae were tested for their preferences for either a totally unknown odor stimulus (blackberry essence) vs. water.

For the odor preference assays, each group was examined in the previously described aquarium between stages 26-36 of development (Gosner, 1960) or 30-35 days of age. The larvae were placed individually into the middle of the test aquarium and given two minutes of acclimatization. Then, their odor preference was evaluated by recording the area of the aquarium where the tadpole spent the greatest amount of the total test time (300 s). After each test, the water was replaced in the aquarium and the stimuli boxes.

Experiment 2: the influence of environmental odors on embryos. In this experiment, it was determined whether preferences for odors may be established by a mechanism of presentation that is more natural than injection. The goal was to examine whether odor preferences might be established by introducing olfactory stimuli into the water surrounding the developing embryo, since such substances apparently are permeable to the membrane of the embryo (Hepper and Waldman, 1992).

I used 100 individuals from five clutches (20 embryos each); half constituted the experimental group and other half the control group. The embryos in the experimental group were separated into five groups of 10 embryos each and placed in 1 L plastic beakers with 750 ml of fresh water mixed with 100 ml of orange essence. The embryos were monitored and immediately after hatching, tadpoles were moved individually into 300 ml fresh water-filled plastic trays (without any odor mixtures), thereby ensuring that odor stimulations were only received during the embryonic phase. The control group was treated in the same way, except they were never exposed to the orange essence. The assays with the tadpoles were conducted between stages 26-36 of development (Gosner, 1960), as described in experiment 1.

Statistical analysis. A two-tailed binomial test with a significance level of = 0.05 (DeGroot, 1988) was used to compare the number of both experimental and control individuals that spent time in the end of the aquarium containing the familiar-stimulus versus those that spent time in the end containing water or a novel (blackberry) stimulus. A significant difference between the two groups would imply a preference for either end of aquarium. In order to eliminate any possible correlations between responses of siblings, the odor preferences for each family were analyzed with the sign-test, comparing the number of families that showed a preference for a familiar orange stimulus versus water or unfamiliar blackberry stimulus. Families were classified as either preferring orange or water, depending on the side preferred by most individuals in that family.

RESULTS

Experiment 1. Individuals exposed as embryos to the orange essence showed a significant preference for this stimulus in the larval stage versus the water stimulus control ($p \ll 0.01$, table 1). All five families from the experimental group also exhibited preferences for the orange stimulus, although the differences were not significant (table 1). For the control group, the larvae showed a greater preference for the water stimulus. The number of tadpoles that associated with the end with the water stimulus was significantly different ($p \ll 0.01$, table 1) to the number of individuals that remained near the orange stimulus. Similarly, all families of the control group showed a preference for the water stimulus, but the differences were not significant (table 1).

The tadpoles exposed to the saline solution exhibited a preference for the water stimulus over the orange stimulus ($p \ll 0.01$, table 1). The number of families that showed a preference for the water stimulus was greater than the number of families that associated to the orange stimulus, but the difference was not significant (table 1). The tadpoles exposed to the orange odor exhibited a significant preference for the water stimulus over the blackberry stimulus ($p \ll 0.01$, table 1). All five experimental families showed a preference for the water, but the differences were not significant (table 1).

Experiment 2. The tadpoles reared in the water mixed with the orange stimulus exhibited a significant preference for the stimulus to which they had been exposed ($p \ll 0.01$, table 1). The tadpoles in the control group showed a significant preference for the water stimulus ($p \ll 0.01$). In both groups, the five families showed a preference for the stimulus learned during embryogenesis, but the differences were not significant (table 1).

Table 1. Odor preferences in *S. phaeota*. Odor and water were the stimuli presented in the aquarium during the test. Orange 1 treatment is the tadpole group exposed to blackberry stimulus in the aquarium. In the comparisons between individuals that chose either odor or water end of the aquarium or the center, n is the number of tadpoles recorded more time in the center of apparatus (columns 5 and 7). The differences between families preferring odor stimulus or water in all treatments were not significant (sign-test, p > 0.05) († = two-tailed binomial test; **ns** = not significant)

Experiment 1									
	# individuals preferring			**center - odor**		**center - water**		**# families preferring**	
Treatments	**odor**	**water**	**p†**	**n**	**p†**	**n**	**p†**	**odor**	**water**
Orange	33	9	< 0.01	8	< 0.01	8	ns	5	0
Control	11	35		4	ns	4	< 0.01	0	5
Saline	8	30		12		12		1	4
Orange 1	8	38		4		4		0	5
Experiment 2									
	# individuals preferring			**center - odor**		**center - water**		**# families preferring**	
Treatments	**odor**	**water**	**p†**	**n**	**p†**	**n**	**p†**	**odor**	**water**
Orange	34	11	< 0.01	5	< 0.01	5	ns	5	0
Control	10	35		5	ns	5	< 0.01	0	5

Individuals in the center of the aquarium. In the different tests conducted on both experimental and control tadpoles, it was observed that some individuals chose to spend the greatest amount of time in the center of aquarium (table 1). After pooling the data obtained from each test, I observed that there were significant differences ($p < 0.01$) between the number of individuals that chose the learned odor stimulus (or water) and those that stayed in the center of the aquarium (205 vs. 38 individuals, respectively). The number of tadpoles that remained in the center of the aquarium was not significantly different ($p > 0.05$) from those that spent the majority of time in the end where the unknown stimulus or the water was presented (38 vs. 57 individuals, respectively).

DISCUSSION

The results obtained suggest that *S. phaeota* tadpoles exposed to olfactory substances during embryogenesis formed a significant preference for the learned odors. Embryonic exposure to the orange odor exerted a strong effect on learning and caused the larvae to express a specific attraction toward this odor. In addition, it simultaneously created an avoidance behavior towards a novel odor (blackberry essence), preferring instead a neutral stimuli when presented with a choice between neutral stimulus or a novel odor. The preference for the water stimulus in the larvae injected with saline solution also may be considered as either neutral or as avoidance behavior to the orange odor (Hepper and Waldman, 1992). Thus, I have demonstrated a "natural" mechanism whereby embryonic olfactory learning can occur, since such odors obviously permeate the egg membranes (Hepper and Waldman, 1992).

Previous studies with vertebrates also have demonstrated that a prenatal o pre-hatching exposure to a determined substance modifies odor preferences in the post-natal stage (Griffiths, 2003; Hepper, 1990; Hepper and Waldman, 1992; Saidapur and Girish, 2000; Smotherman, 1982), as well as the attraction of tadpoles toward known environmental stimuli (Gamboa et al., 1991; Pfennig, 1990). Pfennig (1990) working with frogs and Stabell (1984) working with salmonids attributed their findings to philopatry, arguing that an ability to return to the natal site (a demonstrated suitable reproductive habitat) elevates growth and survivorship rates of offspring.

However, it is doubtful that selection has favored the development of a natal homing mechanism in *S. phaeota* that involves learning and remembering odors from their natal puddles after metamorphosis, as others have speculated is a possible means for homing to natal sites to breed in other species (Grubb, 1973a, 1973b). It seems improbable that ephemeral puddles, like those where *S. phaeota* breeds, would retain their aromatic traits from one breeding period to the next (Grubb, 1973b). Prior to this study, the capacity to recall odor preferences after metamorphosis had been described only in species that breed in more permanent ponds (Blaustein et al., 1984; Hepper and Waldman, 1992) where such abilities may be adaptive.

The design of the aquarium, divided into three regions, allowed for inspection of whether the larvae were really making a choice for a stimulus, whereas an aquarium only divided into two regions may hide the fact that some tadpoles may not be responding at all to the stimuli (B. Waldman pers. comm.). There were some individuals that spent the majority of the test period in the center (the neutral area) of the aquarium, which may indicate that these tadpoles were for some reason less prone to show a preference for a particular olfactory stimulus. Indeed, the results demonstrated that there were genuine preferences acquired by the tadpoles involved in the different assays and not simply random movements. However, the differences among the number of individuals that chose the known odor (or water in some cases) and those that remained in the center of the aquarium suggests that embryonic exposure to odors has a significant effects on subsequent behavior, with specific preferences exhibited for the known stimulus.

In conclusion, I have shown that *S. phaeota* embryos have the capacity to learn odors either introduced through the vitelline membrane or present in the surrounding external environment. This olfactory experience as embryos simultaneously produces two subsequent behaviors in tadpoles: a specific attraction toward the learned odor and repulsion to any other novel odor. These findings are consistent with those of Hepper and Waldman (1992), in which they stated that embryonic learning might be a general characteristic of amphibians.

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