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Embryonic Olfactory Learning in Frogs

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We examined the effect of embryonic olfactory experiences on behaviour after hatching in two species of anuran amphibians, *Rana temporaria* and *Rana sylvatica*. Odorants (orange and citral) injected into the egg were "learned" by the embryo and subsequently preferred by tadpoles after hatching. The observed preferences were specific to the odours experienced prior to hatching, and tadpoles discriminated between the odour experienced as embryos and a novel odour. The acquired preference was maintained after metamorphosis for those individuals that exhibited a preference as tadpoles. Preferences of tadpoles were also influenced "naturally" by odours present in the water surrounding the developing embryos. The experimentally induced olfactory preferences did not appear to influence the exhibition of sibling recognition abilities. The study represents the first demonstration of "embryonic" learning in amphibians, and the functions and importance of early olfactory learning for amphibians are discussed.

The mammalian olfactory system becomes functional prior to birth, as shown by behavioural and physiological experiments on rodents, sheep, and humans (e.g. Brunjes & Frazier, 1986; Hepper, 1991; Schaal et al., 1991). Odours learned in the prenatal environment subsequently influence behaviour after birth. For example, orange injected into the amniotic fluid of rat fetuses on Day 19 of gestation induces a preference for orange in pups 12 days after birth, but no preference is seen in pups who have not been prenatally exposed to orange (Hepper, 1990). Apple juice paired with

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lithium chloride, injected into the amniotic fluid of 20-day-old rat fetuses, induces an aversion to apple juice in the rat after birth (Smotherman, 1982; Stickrod, Kimble, & Smotherman, 1982), Moreover, rat fetuses are capable of discriminating between different chemical substances, such as lemon, milk, and saline, *in utero* (Smotherman & Robinson, 1987). Postnatal olfactory preferences are also influenced by more "natural" prenatal exposure. The pups of pregnant female rats fed garlic during gestation show a preference for garlic over onion, whereas pups of mothers not fed garlic failed to show a preference (Hepper, 1988). The results demonstrate that maternal diet may influence postnatal olfactory preferences.

Chemosensory responses underlie many behavioural processes, not only in mammals but throughout the animal kingdom. Yet the functioning of olfactory systems has been little studied in other animal groups. Most studies of embryonic birds have concentrated on auditory stimuli, but there is some evidence of olfactory functioning prior to birth (Tolhurst & Vince, 1976). Among invertebrates, chemical communication has been most thoroughly studied in social insects (ants, wasps, and bees) where the pre-imaginal learning of olfactory stimuli may be important for feeding, habitat, and host selection (Caubet, Jaisson, & Lenoir, this volume).

Amphibians are model vertebrates for the study of development, and olfaction clearly is important in several aspects of amphibian life history. Frog and toad larvae orient to foods by their odours (Risser, 1914), react to chemical alarm substances that are released upon a predator's attack (Pfeiffer, 1974), and discriminate between kin and non-kin on the basis of chemical cues (Waldman, 1985). After metamorphosis, many amphibians home to particular water bodies to breed by olfaction and possibly other sensory modalities (e.g. Grubb, 1973; Twitty, 1966). Among salamanders, chemical cues are known to play important roles in courtship, the maintenance of territorality, and the recognition of eggs and young (see review in Madison, 1977).

How preferences for particular odours develop in amphibians is unknown, but imprinting to cues during early development has been suggested as a possible ontogenetic mechanism that ultimately permits individuals to home (Twitty, 1966) and to recognise their kin (Waldman, 1981). In amphibians, as in other species, the development of kin-recognition abilities may be shaped by exposure to odours early during development (Hepper, 1991). Rat fetuses prefer the amniotic fluid of their mother to that of another female (Hepper, 1987), a preference that may represent the initial development of kin recognition. Newly emerged callow workers of the ants, *Cataglyphis cursor* and *Camponotus floridanus*, exhibit a preference for nestmates that is acquired during larval life (Carlin & Schwartz, 1989; Isingrini & Lenoir, 1986; Isingrini, Lenoir, & Jaisson, 1985).

Maternal odours are potentially salient cues by which amphibian larvae can recognize kin, and in some species, kin-recognition abilities are influenced by learning of these cues prior to and immediately after hatching (see reviews Blaustein & Waldman, in press; Waldman, 1991). Waldman (1981) reared tadpoles of the toad *Bufo americanus* in social isolation from the time of oviposition. Tadpoles were given a choice between full siblings (same mother and father) and paternal half-siblings (same father, different mother) or between full siblings and maternal half-siblings (same mother, different father). Individuals discriminated between full and paternal half-siblings but not between full and maternal half-siblings. This suggests that factors derived from the mother—for example cytoplasmic contributions to the egg-cell or components of the egg jelly—influence the development of sibling recognition. Maternal biases are also evident in the kin-recognition abilities of tadpoles of the frog *Rana cascadae* and the toad *Bufo boreas* (Blaustein, Chang, Lefcort, & O'Hara, 1990; Blaustein & O'Hara, 1982).

This paper examines the effect of early, embryonic exposure to odorants on the subsequent chemosensory preferences of two species of frog, *Rana temporaria* and *Rana sylvatica*, and relates olfactory learning by embryos to the development of kin recognition abilities.

EMBRYONIC OLFACTORY LEARNING IN *RANA TEMPORARIA*

GENERAL METHOD

Subjects. Subjects used in Experiments 1–3 were derived from 15 breeding pairs of the common frog *Rana temporaria*, obtained from a natural breeding colony in Northern Ireland established from adult frogs caught locally. Each pair was placed into a glass aquarium (60 × 45 × 30 cm) and left to spawn. After spawning the egg mass was removed from its aquarium, divided into clumps of 20 eggs, and placed into 1-l plastic containers filled with fresh spring water. The eggs were then left alone until the start of the experiment. Temperature in the laboratory was maintained between 21–23°C and a 14-hr light : 10-hr dark photoperiod was observed.

Procedure. Testing was performed in a 60 × 20 × 20-cm glass aquarium. This was filled to a depth of 8 cm with spring water when testing tadpoles (Experiments 1 and 2) and left damp when testing juvenile frogs (Experiment 3). At 1 cm from each end of the aquarium, on the middle of the floor, was placed a 2-cm box made of fine stainless-steel mesh. This was tightly packed with cotton wool impregnated with the odorant to be tested. The olfactory stimuli used in these experiments, supplied by Unilever, were "fresh orange" (502.366.A) and "natural strawberry" (52.124), both concentrated natural extracts. The cotton wool was impregnated by placing it in the odour solution for 12 hr immediately preceding the experiment. Fresh spring water was used as a control stimulus (termed "blank" water) and the cotton wool was similarly impregnated by being placed into fresh water for 12 hr. Observations using dye instead of the odorant revealed a decreasing concentration of dye from the mesh box to the centre of the glass test box. A white line was drawn across the floor of the test box, dividing the box into two 30 × 20 × 20-cm halves.

EXPERIMENT 1: ACQUISITION OF ODOUR PREFERENCES BY EMBRYONIC *RANA TEMPORARIA*

Experiment 1A

In Experiment 1A we examined (1) the response of "naive" tadpoles—that is, tadpoles that had not previously experienced this odour—to the orange stimulus, and (2) the effect of exposing *R. temporaria* embryos to orange on the later preferences of tadpoles.

Two hundred individuals, from the spawn of 10 pairs of adult frogs, were tested. Each pair provided twenty individuals for testing, half of which were used in the experimental group ($n=100$) and half in the control group ($n=100$). For experimental embryos 1 μl of pure orange essence was injected into the egg (within the vitelline membrane) using a 10-μl microsyringe (Harvard Instruments, 52-3803) and needle (26 guage). Injections were carried out after gastrulation between Stages 15 and 18 of development (Gosner, 1960). Injections were performed under a magnifying glass (magnification ×15) to ensure precise needle placement. After this injection, each embryo was placed individually into a 330-ml water-filled plastic tray, where it remained until testing. The ten embryos comprising the control group were likewise removed from the 1-l container and placed into the individual containers. These individuals received no injection. The day of hatching was noted, and after hatching the tadpoles were fed on a combination of fish food and spinach. A single batch of food was made at the start of the experiment and used for all experiments.

The tadpoles were all tested between Stages 26 and 36 of development, as proposed by Gosner (1960). We found no evidence of variation in the responses of younger and older tadpoles in any experiment, and thus we report pooled data across these ranges.

The two stimulus boxes were placed into the test apparatus, one containing the orange essence and the other blank water. The side of the orange stimulus was counterbalanced across trials such that for each group the orange was on the left for half and the right for the other half of the trials. The tadpoles were placed individually into the middle of the testing apparatus and left for 5 min. During this period the time spent in each half of the test box (orange or blank water side) was observed. The side on which the individual spent the greatest amount of time was recorded. After each trial the water in the aquarium was removed, the aquarium flushed out, and fresh water replaced for the start of the next trial. Fresh stimuli were also used for each trial.

Results and Discussion

The results from experimental and control tadpoles were analysed separately using a two-tailed binomial test (Seigel, 1956) to compare the number of individuals that spent the greatest amount of time in the half containing the orange with those that spent the greatest amount of time in the half containing the blank water.

A significantly greater number of the control (orange naive) tadpoles showed a preference for the water side ($p=0.04$; see Table 1). In addition to individual preferences being analysed, the preferences exhibited by each family were assessed to eliminate the possibility that responses of siblings were correlated and thus not completely independent. Each family was

TABLE 1
Odour Preferences of *Rana temporaria*

		No. of Individuals Preferring			*No. of Sibships Preferring*		
Experiment	*Treatment*	*Stimulus*	*Water*	p^*	*Stimulus*	*Water*	p^*
1a	Orange injected	72	28	0.0001	10	0	0.002
	Control	39	61	0.04	3	7	n.s.
1b	Injected controls	15	35	0.007	1	9	0.02
1c	Orange injected	14	36	0.003	1	9	0.02
2	Orange exposed	65	35	0.004	10	0	0.002
	Control	34	66	0.002	1	9	0.02
3	Orange injected	29	21	n.s.			
	As tadpoles						
	Orange preference	22	10	0.05			
	Water preference	7	11	n.s.			

*Binomial probabilities, two-tailed.

classified as either preferring orange or water, depending on the side preferred by most individuals in that family, and the numbers of families preferring orange and water were compared by two-tailed binomial test. No significant preference was observed for control families; 7 of the 10 exhibited a preference for the water side. Naive tadpoles may be considered to be either neutral or slightly aversive to the orange odour.

For the experimental group, individuals exhibited a significant preference (see Table 1) for the side containing the orange essence ($p<0.0001$). Similarly, all 10 experimental families showed a preference for the orange side ($p=0.002$). The results clearly show that exposure to orange prior to hatching influences the preference for orange after hatching.

Experiment 1B

One difference between the experimental and control groups above was that whilst the experimental group received an injection of orange, the controls received no injection at all. It may be that the injection per se, or the increase in fluid in the egg rather than the contents of the injection, influenced the tadpoles' preference. To control for this a further group of 50 tadpoles, obtained from the egg masses of 10 breeding pairs (5 individuals from each) was examined. These individuals were treated as the experimental group in Experiment 1A above, but instead of receiving an injection of orange essence they received an injection of 1-μl isotonic saline. Individuals were then raised and tested as described above.

Results and Discussion

The results (see Table 1) demonstrated that the tadpoles exhibited a preference for the water stimuli over the orange ($p=0.007$). This indicates that neither the injection per se nor the increase in fluid in the egg resulting from the injection influenced the tadpoles' preference. These results are supported by analysis of the family data; 9 families showed a preference for the water stimuli ($p=0.02$). In conjunction with Experiment 1A the results demonstrate that injection of orange essence into the egg prior to hatching leads to the development of a preference for orange after hatching.

Experiment 1C

We next examined the specificity of the altered preference induced by prenatal injection of odorant. It may be that exposure to orange prior to hatching results in a non-specific preference for olfactory stimuli other than water, rather than in a specific preference for orange. To examine this, 50 tadpoles from 10 breeding pairs were used. These were treated identically to the experimental animals in Experiment 1A. Embryos were injected with orange at Developmental Stages 15–18 and then tested as tadpoles between Developmental Stages 26 and 36. In this case, however, individuals were tested for their preference between strawberry (impregnated onto the cotton wool and thus a novel but unfamiliar olfactory stimulus) and water. Testing was otherwise identical to that reported for Experiment 1A.

Results and Discussion

The results (see Table 1) indicated that individuals exhibited a significant preference for the water side of the aquarium rather than that containing the strawberry ($p=0.003$), and this was supported by analysis of family preferences where 9 of the 10 families preferred the side containing the water ($p=0.02$). This result suggests that the acquired preference is specific to the olfactory stimuli experienced whilst in the egg and does not lead to a generalized preference for novel olfactory stimuli.

EXPERIMENT 2:
THE INFLUENCE OF ODOURS IN THE ENVIRONMENT ON EMBRYOS

The above experiments have demonstrated that the preferences of tadpoles may be altered by olfactory stimuli experienced before hatching. However, whether this occurs naturally or is restricted to the experimental situation remains an open question. Whilst an alteration in preference can be elicited by injection of an odorant into the egg, we wished to determine whether later preferences could be influenced by a more "natural" means. In its natural environment the developing embryo may be exposed to odours in the egg or to olfactory stimuli in the surrounding water that permeates the membranes of the egg. In this experiment we examined whether alterations in preferences may be obtained by introducing olfactory stimuli into the water surrounding the developing embryo.

Two hundred individuals were examined, derived from 10 pairs of breeding adults; 100 individuals formed the experimental group and 100 the control group. Embryos in the experimental group were housed in groups of 10 and placed in a 1-l plastic container with 750 ml of fresh spring water and 100 ml of orange essence. Close watch was kept on these individuals, and they were removed from the container immediately upon hatching. After removal tadpoles were housed individually as described above in fresh water with *no* orange. Thus tadpoles were only exposed to the orange via its presence in the water surrounding the eggs. The control group was treated similarly to the experimental group but had no orange essence added to their water prior to, or after, hatching. Individuals were again tested between Developmental Stages 26 and 36 in a procedure identical to that reported in Experiment 1A.

Results and Discussion

The results (see Table 1) indicate that tadpoles that had been reared as embryos in "orange-flavoured" water significantly preferred the orange side of the aquarium to the control side ($p=0.004$). Control tadpoles exhibited a preference for the water side of the aquarium ($p=0.002$). Analysis of the family data supported this; all 10 experimental families preferred the orange side ($p=0.002$), whereas 9 of the 10 control families preferred the water side ($p=0.02$). Thus exposure to olfactory stimuli via the water surrounding developing embryos can influence the later preferences of tadpoles, suggesting a "natural" route by which learning can occur prior to hatching.

EXPERIMENT 3:
EMBRYONIC LEARNING AND METAMORPHOSIS

Tadpoles undergo a major change, metamorphosis, to adulthood (Duellman & Trueb, 1986). In this experiment we examined the preference of juvenile frogs, after metamorphosis, for olfactory stimuli to which they had been exposed as embryos. Fifty individuals (5 from each family of the experimental group in Experiment 1A) were tested 2 weeks after metamorphosis. Individuals had been housed individually until this time. Frogs were tested in the glass aquarium described above. Orange-impregnated cotton wool was packed into the stainless steel mesh box and placed at one end of the aquarium and water-impregnated cotton wool into a similar box at the other end. The froglets were placed individually into the aquarium and left for 10 min. The time spent on either side of the aquarium was recorded, and the side on which they had spent the greatest amount of time was determined. After each trial the aquarium was thoroughly flushed out with clean water. Fresh stimuli were used for each trial.

Results and Discussion

The results (see Table 1) indicated no significant preference for either side of the aquarium. However, the test group included individuals that as tadpoles had preferred the orange side ($n=32$) and those that had spent the greatest time on the water side ($n=18$). When analysed separately, no preference was observed for the frogs that as tadpoles had preferred water. However, tadpoles that exhibited a preference for orange maintained this after metamorphosis (see Table 1). This suggests that preferences acquired as embryos and exhibited as tadpoles are maintained after metamorphosis.

EMBRYONIC OLFACTORY LEARNING IN *RANA SYLVATICA*

The above experiments indicate that the behavioural preferences manifested by tadpoles of the common frog (*R. temporaria*) are influenced by olfactory stimuli experienced before hatching. In the next series of experiments we sought to replicate and extend these findings. (1) A different species of frog, the wood frog (*R. sylvatica*), was examined to determine whether embryonic olfactory learning could be found in more than one species. (2) A number of refinements in methodology were implemented regarding the apparatus and testing procedure. The time spent in association with the various stimuli, in addition to the side of greatest preference, was used in the analysis. (3) A second olfactory stimulus was used to ensure embryonic learning was not specific to orange. (4) The time of embryonic exposure was altered. In these experiments the developing embryo was injected with the stimuli before Stage 10, prior to the onset of gastrulation. (5) We sought to examine the effects of experimental exposure of embryos to olfactory stimuli on the subsequent abilities of these individuals, as tadpoles, to recognize their siblings.

GENERAL METHOD

Subjects. Wood frog (*R. sylvatica*) egg clutches obtained from pairs spawning in a breeding pond, in Carlisle, Massachusetts, U.S.A., were used in Experiments 4–6. Twelve egg masses were collected immediately upon oviposition and were transported to the laboratory, where they were kept separately, in containers of spring water, at 5°C for 6–36 hr. As breeding pairs were observed prior to, and during oviposition, we were able to determine unambiguously the parentage of each clutch. Egg masses comprised distinct sibling groups, and no two shared either maternal or paternal parents.

Procedure. Embryos from each sibling group were assigned in random sequence to one of three treatments—orange, citral, or control. Three to five days after the eggs were laid, and prior to gastrulation (Stage 10, Gosner, 1960), embryos were injected with small quantities of odorants (orange or citral) in isotonic saline solution (20% Holtfreter's solution, Rugh, 1962), or for controls, with the saline solution alone. Either 10 μl "natural-flavour orange" (IFF 135-98295) or 1 μl "citral refined" (IFF 03-4262), prepared by serial dilution in 0.1 ml solution, was injected just within the vitelline membrane of each embryo using a 1-ml tuberculin syringe (26-gauge needle). Control embryos were injected in precisely the same manner as in the experimental condition, but without odorants. Injections were done while the embryos were being examined under a dissecting microscope (magnification ×30) to ensure precise needle placement. The orange odorant was extracted and concentrated from natural oranges, whereas citral, a synthesized compound, has an extremely strong lemon-like flavour. International Flavors and Fragrances Inc. (Union Beach, New Jersey, U.S.A.) supplied both substances.

Experimental groups were reared in groups of 10, with siblings, in 1-l beakers filled with spring water. Additional individuals from each sibling group were left untreated (received no injections) but were also housed in groups of 10. Beakers were maintained between 20–23°C on a 14 hr L:10 hr D photoperiod. Tadpoles were fed daily on a preparation of pulverized rabbit chow, agar, and gelatin. A large quantity was made up and frozen at the beginning of the experiment, so that just the necessary daily rations were thawed when needed. This procedure ensured that a uniform and consistent supply of food was available throughout the experimental period. Water in beakers was replaced twice weekly with fresh spring water.

As in Experiments 1 and 2, tadpoles were tested between the ages of 20 and 50 days, corresponding to Gosner (1960) Developmental Stages 26–36. As in the previous experiments, we found no evidence of variation in responses of younger and older tadpoles, and thus we report pooled data across this range. Subjects in the following experiments were used in one test only.

Subjects were tested in a 45 × 35 × 15-cm plastic tank, filled to a depth of 8 cm with 12 l of fresh spring water. Two pipettes were affixed to each of two ring stands, located 45 cm apart on opposite sides of the tank. The pipette tips were positioned 5 cm above the water surface. Beakers at the top of each ring stand were filled with stimulus solution or spring water, as dictated by the specific experimental protocols (see below). Stimuli (odorants or water) flowed from these beakers through the pipettes into the test tank at 10 ml/min. Flow rate was regulated by clamps on plastic tubes that siphoned fluid into the pipettes. The test tank was divided into thirds with black lines drawn on the tank bottom and strings positioned above it.

EXPERIMENT 4: ACQUISITION OF ODOUR PREFERENCES BY EMBRYONIC *RANA SYLVATICA*

Experiment 4A

Some odour stimuli may be intrinsically attractive or repulsive to tadpoles, even in the absence of any previous exposure. To investigate how *R. sylvatica* react to orange and citral odours, we examined the tendencies of control tadpoles, injected as embryos just with saline solution, to discriminate between orange odour and blank water, and between citral and blank water. Three tadpoles from each of 12 sibships were tested in each experiment. Subjects were individually removed from their beakers with a large spoon and were placed alone into the centre of the tank. For 5 min subjects were allowed to acclimatize to the tank with spring water ("blank" water) dripping onto both sides of the tank. Tests were begun as water streams were switched to stimulus streams. This was accomplished by halting flow through the first set of pipettes and simultaneously initiating flow from the stimulus beakers through a second set of pipettes. Orange and citral stimulus solutions used in these experiments, and in the other experiments that follow, were prepared by serial dilution in spring water (1.0% orange and 0.01% citral, by volume). Fresh spring water served as the alternative blank stimulus.

During 10-min test periods, we recorded the movements of subjects into each section of the tank. Total times spent in the left, centre, and right thirds were then computed for each subject. Based on preliminary trials following the procedures described above but using food dyes in place of odorants, we found that the dispersion of odorants over 10-min periods was generally limited to that third of the tank into which they are introduced.

Results and Discussion

R. sylvatica tadpoles showed no preference between the novel orange stimulus and blank water. Of 36 individuals tested, 17 spent more time in the tank section into which orange was released and 19 spent more time in the tank section into which blank water was released. As 3 individuals were tested in each family, we conducted statistical analyses on mean responses of each family to eliminate the possibility that responses of siblings were correlated and thus not completely independent (Table 2). Neither the average times spent in orange and blank water sections, nor the number of families that preferred one stimulus over the other, differed significantly.

When tested for a preference between citral and blank water, however, subjects appeared to avoid citral: 11 of 36 subjects preferred the citral side, but 25 preferred the blank water. Considering family means, the average time spent near citral was significantly less than that spent near the blank water (Table 2). Likewise, most families showed a mean preference for blank water. In the absence of prior exposure, orange thus appears to be a neutral stimulus, whereas citral represents a potentially aversive stimulus.

TABLE 2
Odour Preferences of *Rana sylvatica* Tadpoles

Experiment	*Treatment*	*Odour Stimulus*	*Mean Time (sec)*	p^*	*Number of Sibships Preferring*	p^{**}
4a	Injected controls	Orange	218		6	
		Blank	212	n.s.	6	n.s.
	Injected controls	Citral	138		3	
		Blank	307	0.005	9	n.s.
4b	Orange injected	Orange	253		12	
		Blank	186	0.05	0	0.0004
	Citral injected	Citral	224		6	
		Blank	232	n.s.	6	n.s.
5	Orange injected	Orange	218		10	
		Citral	170	0.05	2	0.04
	Citral injected	Orange	142		0	
		Citral	275	0.005	12	0.0004

*Wilcoxon signed-ranks test, two-tailed.
**Binomial probabilities, two-tailed.

Experiment 4B

We next tested whether subjects that had been injected with each odorant showed an altered response to that stimulus when it was later presented to them. Subjects that had been exposed to orange odour as embryos were tested for their tendency to orient toward orange odour in preference to blank water. Similarly, subjects that had been injected with citral were tested for their possible discrimination between citral and blank water. Three tadpoles from each of 12 sibships were tested in each experiment.

Results and Discussion

R. sylvatica tadpoles that had been injected with orange showed a subsequent preference for this stimulus over the blank water: 26 of 36 subjects tested spent more time in the orange side of the tank, and the other 10 spent more time in the blank water side. Considering mean family preferences for each side, this difference was significant ($p<0.05$, Table 2). Moreover, this response was consistent for all 12 families, as none demonstrated a preference for the blank water side.

Unlike orange-injected subjects, *R. sylvatica* tadpoles that had been injected as embryos with citral failed to show a preference for either stimulus: 17 of 36 subjects spent more time in the citral side of the tank, and the other 19 spent more time in the blank water side. Times spent on citral and blank sides did not significantly differ, and equal numbers of families spent more time near each stimulus (Table 2). This apparent lack of preference contrasts strongly, however, with the results of control tests in which subjects showed a strong aversion to citral. Variation in the numbers of families preferring citral and water between the experimental and control tests approached significance ($p=0.08$; probability computed in 1000 iterations following the Monte Carlo method of Roff & Bentzen, 1989).

As individuals injected with orange subsequently demonstrated a positive affinity for this stimulus, individuals injected with citral appear also to have acquired a tolerance, if not a preference, for this otherwise aversive stimulus. Both tests suggest that tadpoles learn odours to which they are exposed as embryos and respond preferentially to these stimuli throughout larval development.

EXPERIMENT 5: ODOUR DISCRIMINATION IN *RANA SYLVATICA*

To examine the specificity with which individuals learn odours introduced into their embryonic environment, we tested subjects that had been injected with orange or citral odours for their tendency to discriminate between these two stimuli. Unlike in previous tests, in which single odours were presented together with blank water, we now presented test subjects with orange and citral odours simultaneously through the pipettes on either side of the test tank. Five tadpoles from each of 12 sibships were tested in each experiment.

Results and Discussion

R. sylvatica tadpoles clearly discriminated between the two odorants. Subjects that had been injected with orange odour preferred orange, and those that had been injected with citral preferred citral. Of the 60 orange-treated subjects, 37 spent more time in the orange side of the tank, and 23 spent more time in the citral side. Even when comparing mean family values, time spent near orange was significantly greater than that spent near citral ($p<0.05$, Table 2). Ten of 12 families showed consistent preferences for orange ($p<0.04$).

Even more dramatically, 46 of the 60 citral-treated individuals spent more time in the citral side of the tank, whereas just 14 spent more time in the orange side. Time spent in the citral side was significantly greater than that spent in the orange side ($p<0.005$, Table 2). Every family displayed a preference for citral rather than orange odour ($p<0.0004$).

These results indicate that the acquired odour preference is highly specific. Even though both orange and citral can be classified as citrus odours, embryos exposed to either of these stimuli appear not to generalize their odour preferences. Rather, the affinity of citral-injected subjects for citral was intensified by its presentation simultaneously with orange odour. The developing nervous system of anuran embryos thus seems sufficiently plastic to "imprint" on a variety of odorants. Even slight intraspecific variation in the chemical cues to which embryos are exposed in principle could lead to the subsequent recognition of large numbers of labels, as might be necessary for individual or kin-recognition systems (Lacy & Sherman, 1983; Waldman, 1987).

EXPERIMENT 6: THE INFLUENCE OF LARVAL OLFACTORY EXPOSURE ON LATER SIBLING RECOGNITION

Although we know that tadpoles recognize their siblings on the basis of chemical cues (e.g. Waldman, 1985, 1986), obviously the odours we chose for our experiments are very unlike those used by tadpoles to recognize their kin under normal conditions. We were thus curious whether the larval experiences that we effected through the injection of odorants into the embryonic environment supplanted the normal development of kin-recognition abilities (Cornell, Berven, & Gamboa, 1989; Waldman, 1984) or simply supplemented it. Even though *R. sylvatica* tadpoles previously exposed to orange or citral odours subsequently show preferences for these odours, they may also still recognize and associate with untreated siblings in preference to untreated non-siblings.

For each experimental treatment, we thus tested 3 individuals from each of 12 families for their tendencies to discriminate siblings from non-siblings. Experimental tanks identical to those used in previous tests were used for this study, but rather than pipetting odours into each side, we placed 10 siblings of the test subject into a removable fibreglass mesh enclosure on one side, and 10 non-siblings of the test subject (but all siblings of one another) into an enclosure on the other side. After 5 min of acclimatization, the movements of single subjects among all three compartments were recorded for 10 min. Then, to control for side preferences, we rapidly moved the test subject to a second clean tank in which the positions of the two stimulus compartments were reversed. After 5 additional min of acclimatization, we tested subjects for another 10-min period. Stimulus groups were all untreated tadpoles—i.e. they had never received any injections—but they were otherwise reared under the same regimen as the experimental groups. Neither sibling nor non-sibling stimulus groups had previously been exposed to test subjects as larvae. Siblings, however, were all originally obtained from the same egg mass and may have derived similar chemical cues from that egg mass. Non-sibling stimulus groups were comprised of sibships that were chosen at random for each test.

Results and Discussion

Control *R. sylvatica* tadpoles that had been injected with saline solution as embryos spent significantly more time in the section of the test tank near their siblings than in the non-sibling section (comparison based on mean family values, see Table 3): 25 of 36 subjects spent more time near their siblings, and in 11 of 12 families, more individuals preferred the sibling compartment to the non-sibling compartment. Subjects that had been injected as embryos with orange showed weaker sib preferences. Although 20 of 36 subjects spent more time near their siblings, the mean time spent in the sibling compartment was only slightly greater than that spent in the non-sibling compartment (Table 3). Neither this preference nor the selection by 8 of 12 families for the sibling compartment was statistically significant. Citral-treated subjects, in contrast, showed preferences for their siblings that were comparable to those shown by control subjects. These individuals spent significantly more time near siblings than non-siblings: 24 of 36 subjects preferred the sibling compartment, and 10 of 12 families demonstrated an overall preference for siblings. A chi-squared test on the distribution of preferences shown by the families for

TABLE 3
Sibling Association Preferences of Treated *Rana sylvatica* Tadpoles

	Treatment		
	Injected Controls	*Orange Injected*	*Citral Injected*
Mean time(s) oriented toward			
siblings	494	448	489
non-siblings	394	420	387
p^*	(0.005)	(n.s.)	(0.05)
Number of sibships preferring			
siblings	11	8	10
non-siblings	1	4	2
p^{**}	(0.006)	(n.s.)	(0.04)

*Wilcoxon signed-ranks test, two-tailed.
**Binomial probabilities, two-tailed.

the two experimental and control condition revealed no difference between their preferences for siblings ($\chi^2(2)=2.482$, n.s.).

The results are equivocal but suggest that tadpoles that are imprinted with experimentally applied odours can respond both to these odours and to the odours of their untreated siblings. Natural odours that are presumably present in the embryonic environment may serve as cues directing the development of individual or kin recognition in a manner similar to the imprinting we have demonstrated to citrus odours. Many specific chemical labels may be involved, so that recognition might be based on the additive perception of these cues, consistent with the findings of this experiment.

GENERAL DISCUSSION

Our experiments demonstrate, for the first time, that amphibians exposed to odorants while still embryos subsequently develop specific preferences for these odours. The acquired preference is expressed by tadpoles during the larval stage and after metamorphosis by juvenile frogs. Although more species need to be studied, the overall consistency of results between our two study species, *R. temporaria* and *R. sylvatica*, suggest that embryonic learning may be a general characteristic of amphibians. The acquisition of odour preferences that we have demonstrated parallels recent reports of prenatal learning in mammals (e.g. Brunjes & Frazier, 1986; Hepper, 1991). Because frogs fertilize their eggs externally and the embryos are easily examined and manipulated, amphibians may prove convenient subjects for further investigations of the timing and specificity of embryonic learning. Instances of embryonic learning might also be found in other vertebrates, such as fishes whose ability to imprint on both natural and artificial odours after hatching has been investigated (reviewed in Stabell, 1984).

The ability of amphibian embryos to learn particular odours present in the egg may confer important adaptive advantages. Previous research has demonstrated that kin recognition systems of frogs and toads have a strong maternal component (Blaustein & O'Hara, 1982; Blaustein et al., 1990; Waldman, 1981). Maternal cues are present in highest concentration during early developmental stages, and individuals, while still very young, can presumably learn these cues most readily. Our results further demonstrate that embryos can learn odours in the external environment that permeate cell membranes. Natural pond odours, thus learned, may serve as cues by which larvae can return to their oviposition site, should this be advantageous for growth or survival (Pfennig, 1990). Moreover, our results suggest that odour preferences that develop during embryonic stages might be retained after metamorphosis, providing a possible means for homing to natal sites to breed (Grubb, 1973).

In anuran larvae, the olfactory sense generally becomes functional before other sensory systems, and in *R. temporaria* it is the only specialized modality known to function immediately after hatching (Spaeti, 1978). Our experiments raise the distinct possibility that amphibian olfactory systems begin functioning considerably earlier. Further work is necessary, however, to clarify the precise developmental stages at which olfactory learning can occur. Taken together with evidence of prenatal learning in mammalian species, our results suggest an important role for olfactory experiences before birth/hatching on subsequent behavioural development.

REFERENCES

Blaustein, A.R., Chang, K.S., Lefcort, H.G., & O'Hara, R.K. (1990). Toad tadpole kin recognition: recognition of half siblings and the role of maternal cues. *Ethology, Ecology and Evolution, 2*, 215–226.

Blaustein, A.R., & O'Hara, R.K. (1982). Kin recognition in *Rana cascadae* tadpoles: maternal and paternal effects. *Animal Behaviour, 30*, 1151–1157.

Blaustein, A.R., & Waldman, B. (in press). Kin recognition in anuran amphibians. *Animal Behaviour*.

Brunjes, P.C., & Frazier, L.L. (1986). Maturation and plasticity in the olfactory system of vertebrates. *Brain Research Review, 11*, 1–45.

Carlin, N.F., & Schwartz, P.H. (1989). Pre-imaginal experience and nestmate brood recognition in the carpenter ant, *Camponotus floridanus*. *Animal Behaviour, 38*, 89–95.

Cornell, T.J., Berven, K.A., & Gamboa, G.J. (1989). Kin recognition by tadpoles and froglets of the wood frog *Rana sylvatica*. *Oecologica, 78*, 312–316.

Duellman, W.E., & Trueb, L. (1986). *Biology of amphibians*. New York: McGraw-Hill.

Gosner, K.L. (1960). A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica, 16*, 183–190.

Grubb, J.C. (1973). Olfactory orientation in *Bufo woodhousei fowleri, Pseudacris clarki* and *Pseudacris streckeri*. *Animal Behaviour, 21*, 726–732.

Hepper, P.G. (1987). The amniotic fluid: an important priming role in kin recognition. *Animal Behaviour, 35*, 1343–1346.

Hepper, P.G. (1988). Adaptive fetal learning: Pre-natal exposure to garlic affects post-natal preferences. *Animal Behaviour, 36*, 935–936.

Hepper, P.G. (1990). Foetal olfaction. In D.W. Macdonald, D. Müller-Schwarze, & S.E. Natynczuk (Eds.), *Chemical signals in vertebrates, 5* (pp. 282–288). Oxford: Oxford University Press.

Hepper, P.G. (1991). Recognizing kin: Ontogeny and classification. In P.G. Hepper (Ed.), *Kin recognition* (pp. 259–288). Cambridge: Cambridge University Press.

Isingrini, M., & Lenoir, A. (1986). La reconnaissance coloniale chez les hyménoptères sociaux. *Année Biologique, 25*, 219–254.

Isingrini, M., Lenoir, A., & Jaisson, P. (1985). Preimaginal learning as a basis of colony-brood recognition in the ant *Cataglyphis cursor*. *Proceedings of the National Academy of Sciences, USA, 82*, 8545–8547.

Lacy, R.C., & Sherman, P.W. (1983). Kin recognition by phenotype matching. *American Naturalist, 121*, 489–512.

Madison, D.M. (1977). Chemical communication in amphibians and reptiles. In D. Müller-Schwarze & M.M. Mozell (Eds.), *Chemical signals in vertebrates* (pp. 135–168). New York: Plenum Press.

Pfeiffer, W. (1974). Pheromones in fish and amphibia. In M.C. Birch (Ed.), *Pheromones* (pp. 269–296). Amsterdam: North-Holland.

Pfennig, D.W. (1990). "Kin recognition" among spadefoot toad tadpoles: a side-effect of habitat selection? *Evolution, 44*, 785–798.

Risser, J. (1914). Olfactory reactions in amphibians. *Journal of Experimental Zoology, 16*, 617–652.

Roff, D.A., & Bentzen, P. (1989). The statistical analysis of mitochondrial DNA polymorphisms: χ^2 and the problem of small samples. *Molecular Biology and Evolution, 6*, 539–545.

Rugh, R. (1962). *Experimental embryology*. Minneapolis: Burgess Publishing.

Schaal, B., Orgeur, P., Lecanuet, J.-P., Locatelli, A., Granier-Deferre, C., Poindron, P. (1991). Chimioréception nasale in utero: expériences préliminaires chez le foetus ovin. *Comptes Rendes de l'Academie des Sciences, Série III, 113*, 319–325.

Siegel, S. (1956). *Non-parametric statistics for the behavioral sciences*. Tokyo: McGraw-Hill.

Smotherman, W.P. (1982). Odor aversion learning by the rat fetus. *Physiology and Behavior, 29*, 769-771.

Smotherman, W.P., & Robinson, S.R. (1987). Pre-natal expression of species-typical action patterns in the rat. *Journal of Comparative Psychology, 101*, 190–196.

Spaeti, U. (1978). Development of the sensory systems in the larval and metamorphosing European grass frog (*Rana temporaria* L.). *Journal für Hirnforschung, 19*, 543–575.

Stabell, O.B. (1984). Homing and olfaction in salmonids: A critical review with special reference to the Atlantic salmon. *Biological Reviews, 59*, 333–388.

Stickrod, G., Kimble, D.P., & Smotherman, W.P. (1982). In utero taste/odor aversion conditioning in the rat. *Physiology and Behavior, 28*, 5–7.

Tolhurst, B.E., & Vince, M.A. (1976). Sensitivity to odours in the embryo of the domestic fowl. *Animal Behaviour, 24*, 772–779.

Twitty, V.C. (1966). *Of scientists and salamanders*. San Francisco: W.H. Freeman.

Waldman, B. (1981). Sibling recognition in toad tadpoles: the role of experience. *Zeitschrift für Tierpsychologie, 56*, 341–358.

Waldman, B. (1984). Kin recognition and sibling association among wood frog (*Rana sylvatica*) tadpoles. *Behavioural Ecology and Sociobiology, 14*, 171–180.

Waldman, B. (1985). Olfactory basis of kin recognition in toad tadpoles. *Journal of Comparative Physiology, A, 156*, 565–577.

Waldman, B. (1986). Chemical ecology of kin recognition in anuran amphibians. In D. Duvall, D. Müller-Schwarze, & D.M. Silverstein (Eds.), *Chemical signals in vertebrates, 4: Ecology, evolution, and comparative biology* (pp. 225–242). New York: Plenum Press.

Waldman, B. (1987). Mechanisms of kin recognition. *Journal of Theoretical Biology, 128*, 159–185.

Waldman, B. (1991). Kin recognition in amphibians. In P.G. Hepper (Ed.), *Kin recognition* (pp. 162–219). Cambridge: Cambridge University Press.

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Apprentissage olfactif à l'étape embryonnaire chez la grenouille

Les effets d'expériences olfactives lors de la période embryonnaire ont été étudiés, après éclosion, chez deux espèces d'amphibiens anoures *Rana temporaria* et *Rana sylvatica*. Des substances odorantes (orange et citral) injectées dans l'oeuf étaient "apprises" par l'embryon et étaient ensuite préférées par les têtards après l'éclosion. Les préférences observées étaient spécifiques aux odeurs connues avant l'eclosion et les têtards discriminaient entre l'odeur dont ils avaient eu l'expérience avant l'éclosion et une odeur nouvelle. La préférence acquise perdure après la métamorphose pour les individus qui avaient manifesté une préférence quand ils étaient têtards. Les préférences des têtards étaient également influencées "naturellement" par les odeurs présentes dans l'eau où se trouvaient les embryons en cours de développement. Les préférences olfactives expérimentalement induits n'apparaissent pas avoir d'influence sur la capacité à reconnaître les congénères issus des mêmes ascendants. Cette étude représente la toute première mise en évidence d'un apprentissage "embryonnaire' chez les amphibiens et les fonctions et l'importance de l'apprentissage olfactif précoce pour les amphibiens sont discutées.

Aprendizaje olfatorio embrional en ranas

Se examinaron los efectos de experiencias olfatorias embrionarias en el comportamiento posterior a la eclosión en dos especies de anfibios anuros, *Rana temporaria* y *Rana sylvatica*. Substancias odoríferas (naranja y extracto cítrico) inyectadas en el huevo fueron 'apprendidas' por los embriones y luego preferidas por los renacuajos. Las preferencias observadas fueron específicas a los olores experimentados antes de la eclosión y los renacuajos discriminaron entre el olor experimentado como embriones y un olor desconocido. La preferencia adquirida se mantuvo luego de la metamorfosis en aquellos individuos que habían mostrado preferencias como renacuajos. Las preferencias de los renacuajos fueron asimismo afectadas 'naturalmente' por olores presentes en el agua alrededor del embrión en desarrollo. Las preferencias olfatorias inducidas experimentalmente no parecieron afectar la habilidad de reconocimiento entre hermanos. Esta es la primera demostración de aprendizaje 'embrionario' en anfibios. Se discuten la función e importancia del aprendizaje embrionario en anfibios.