J Comp Physiol A (1985) 156:565–577

Journal of Comparative Physiology A Sensory, Neural, and Behavioral Physiology

Olfactory basis of kin recognition in toad tadpoles*

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Accepted December 29, 1984

Summary. 1. Larvae of the American toad (*Bufo americanus*) preferentially associate with their siblings in laboratory tests, suggesting that they can recognize kin. The sensory basis of their kin recognition abilities was investigated by measuring the responses of individuals in a Y-maze to waterborne cues emanating from their siblings and from non-siblings.

2. When simultaneously presented with water flowing from two containers, each holding members of a different sibling group, test subjects spent significantly more time oriented toward their siblings than toward non-siblings. Similar results were obtained when the stimulus water was first passed through intermediary reservoirs. Hence, kinship cues are unlikely to be acoustic or vibratory stimuli perceived by the auditory or lateral line systems.

3. Tadpoles whose external nares were blocked with a gelatinous paste did not behaviorally discriminate between water flowing from siblings and that flowing from non-siblings. Retested after their nares were unplugged, these individuals oriented significantly toward their siblings, as did sham-treated test individuals.

4. Stimulus water conditioned by sibling groups and then stored for 24–30 h failed to elicit a discrimination response, indicating that kinship cues released by tadpoles lose their effectiveness during this period. Signals with rapid fade-out times would probably be more efficient under natural conditions than those that persisted after individuals had moved.

5. Test subjects simultaneously presented with water flowing from siblings and blank (dechlorinated tap) water showed no tendencies to discriminate between these stimuli. When individuals were exposed both to water from non-siblings and to blank water, however, they oriented significantly toward the blank water. Kin association may thus result in part from negative klinokinetic responses induced by contact with factors released by non-kin.

6. Chemical cues released by tadpoles appear sufficient to communicate their kinship identity. These cues are probably perceived and processed by the main olfactory system. Kin recognition mechanisms may be further elucidated by chemical and neurophysiological analyses.

Introduction

An ability to recognize differences among individuals or groups of individuals underlies many aspects of social behavior. While such forms of animal communication have long been of interest to investigators of sensory biology, how genetical kinship (Hamilton 1964) might be communicated among conspecifics has until recently been ignored. Empirical studies now suggest, however, that many organisms are able to behaviorally discriminate between their relatives and non-relatives (see reviews in Holmes and Sherman 1983; Waldman 1983). Although the recognition mechanisms that effect such discriminations may vary considerably, in many cases kin recognition abilities are remarkably fine-tuned.

Kinship signals, like those encoding other information about an individual's status, are likely to be conveyed in modes that have been selected to meet the constraints associated with an organism's life history and habitat. Parent-offspring identifications may be mediated either through vo-

* Presented at the Animal Behavior Society meeting, 15–19 August 1982, Duluth, Minnesota

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cal, visual, or chemical signals (see Colgan 1983). Less is known about recognition of collateral or non-descendent kin (e.g., siblings, cousins), but chemical signals appear to be used by many organisms. Young woodlice (*Hemilepistus reaumuri*) can identify siblings by their genetically determined secretions, which combine to form a 'family badge' (Linsenmair 1972). Sweat bees (*Lasioglossum zephyrum*) learn genetically determined odors of their sisters; by comparing smells of unfamiliar conspecifics with this learned odor, a sweet bee can subsequently assess its degree of relatedness to unfamiliar individuals (Kukuk et al. 1977; Greenberg 1979; Buckle and Greenberg 1981; Smith 1983). Kin recognition is also likely mediated by chemical cues in other social insects (e.g., Jaffe and Marcuse 1983; Pfennig et al. 1983). Sibling association patterns of juvenile coho salmon (*Oncorhynchus kisutch*) may result from the production and detection of chemical cues (Quinn and Busack, 1985). Among mammals, spiny mice (*Acomys cahirinus*) made anosmic through zinc sulfate treatment lose the ability to discriminate between siblings and non-siblings (Porter et al. 1978), as do similarly treated ground squirrels (*Spermophilus tridecemlineatus*) (Holmes 1984). Albino rats can discriminate between siblings and non-siblings based on odors (Wills et al. 1983). Olfactory cues also appear to be important in kin recognition mechanisms as they function to facilitate inbreeding avoidance and the attainment of optimal outbreeding (e.g., in mice, Gilder and Slater 1978; D'Udine and Partridge 1981). In other species, however, vocal cues (e.g., bank swallows, *Riparia riparia,* Beecher and Beecher 1983) or visual cues (e.g., pigtail macaques, *Macaca nemestrina,* Wu et al. 1980; Fredrickson and Sackett 1984; Japanese quail, *Coturnix coturnix japonica,* Bateson 1982) appear sufficient to elicit sibling recognition.

Larvae of many anuran amphibians preferentially associate with their siblings under a variety of testing conditions (*Bufo americanus*: Waldman and Adler 1979; Waldman 1981, 1982, 1985; *Bufo boreas:* O'Hara and Blaustein 1982; *Rana cascadae:* Blaustein and O'Hara 1981, 1982b, 1983; O'Hara and Blaustein 1981; *Rana sylvatica:* Waldman 1984). Anuran larvae are in several respects model subjects for studying the ontogenetic and sensory mechanisms underlying kin recognition abilities: they show consistent sibling preference responses, are readily available in large numbers, and can be easily subjected to experimental manipulations. In this report, I present evidence that waterborne chemical cues may be used by American toad (*Bufo americanus*) tadpoles in communicating kinship identity, and describe experiments that reveal how such cues might be processed by individuals when making kinship assessments.

Materials and methods

Study animals. Twenty-seven breeding pairs of American toads (*Bufo americanus*) were collected from various ponds near Ithaca, New York, prior to initiating oviposition. Pairs were transported to the laboratory, and each was placed in a separate 10 l plastic bucket, half-filled with dechlorinated tap water. Toads generally spawned in these buckets within 24 h. The egg mass of each pair was then moved to a separate 75 l glass aquarium, where embryos hatched, and in which larvae were reared until testing. Tadpoles were fed spinach daily. Tanks were illuminated by overhead fluorescent room lamps under an LD 14:10 photoperiod. Water was continuously aerated, and approximately two-thirds of it was changed with fresh dechlorinated tap water every other week, or as needed.

Tadpoles were tested between Gosner (1960) developmental stages 26 and 38 ($\bar{x}=33$), which span the period between hindlimb bud formation and complete toe differentiation. Body lengths of test subjects ranged from 15 to 24 mm. In most tests, the behavioral responses of sibship groups that were obtained from pairs collected in the same pond were compared (cf. Waldman 1981). Except where otherwise noted, each subject was tested only once.

Testing apparatus. Tendencies of individual tadpoles to behaviorally discriminate between various stimulus groups (e.g., siblings versus non-siblings) were measured by recording the durations of time spent by test subjects oriented toward water simultaneously flowing from two sides of a Y-shaped glass tube ('Y-maze'), where each arm was connected to a different stimulus container. Water from stimulus containers (10 l plastic buckets) was strained and siphoned through 4 mm (internal diameter) Tygon tubing (intake opening covered with fine nylon mesh) at a rate of 1 drop/s (regulated by clamps) to a pair of three-way stopcocks (see Fig. 1). Each stopcock was connected to the two ends of the Y-shaped tube by 7 mm (i.d.) Nalgene tubing, which was grooved in places to allow trapped air to escape. Water flow from the stimulus containers could thus be directed to either side of the Y-maze. The Y-shaped tube, made of Kimax glass (135 mm longitudinal length, stimulus arms each 73 mm long, 10 mm i.d. walls), served as the actual testing device. Water flowed through the tube and out a rubber stopper inserted in tubing (14 mm i.d.) at the base of the Y-maze; the rate of flow was regulated by additional clamps on tubing leading out of the stopper. Because the stimulus containers were positioned above the test apparatus, water flow was driven by gravity.

During preliminary trials, food dyes were placed in the stimulus containers and their dispersion patterns in the glass tube were observed. No intermixing within the arms of the tube was apparent.

Testing procedure. Each test was begun by adjusting the flow rates from the stimulus containers. A test subject was removed from its rearing tank and placed within the tubing at the base of the Y-maze. After water accumulated so that the Y-maze was approximately three-quarters full, clamps at the base of the apparatus were opened, allowing water to flow through the outlet tubing at a rate of 2 drops/s.

The test subject was allowed 5 min to acclimate to the apparatus. The individual's movements in the central section

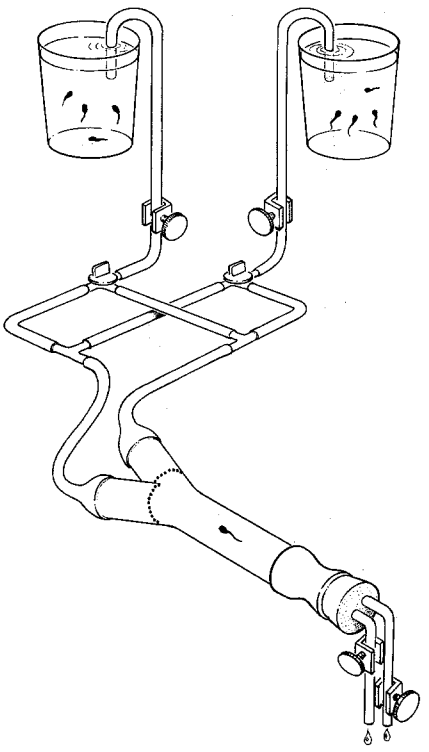

Fig. 1. Diagrammatic representation of testing apparatus for basic choice tests. Twenty-five tadpoles of each of two sibships were placed in separate stimulus containers filled with dechlorinated tap water. Water flowed from the stimulus containers through tubing to a pair of three-way stopcocks; water from stimulus containers could thus be shunted to either side of the actual testing device, a Y-shaped glass tube. Flow rates were controlled by clamps where indicated. In each test, an individual tadpole was inserted at the base of the Y-shaped tube; it was considered to be oriented toward either of the stimulus groups if its snout extended past the criterion (dotted) lines. The apparatus is not drawn to scale. See text for further details

of the Y-maze ('neutral') and into the two arms from which stimulus water flowed were then timed and recorded for 10 min. An individual was considered to be oriented toward one of the stimuli if its head was in the corresponding arm of the Y-maze; observations were facilitated by markings placed on the glass that precisely delineated the junctions of the central section of the maze with the arms.

At the completion of the initial test period, the original stimulus directions were reversed by adjusting the stopcocks so that water previously flowing through the left side of the apparatus was shunted to the right and vice versa. Water in the Y-maze was released by opening a tube at the base of the stopper, and flow rates were all recalibrated. The test subject was then allowed 5 min for reacclimation, and its movements were recorded for an additional period of 10 min. Each individual was thus tested with the same stimuli flowing from both sides of the apparatus. Moreover, consecutive test subjects were presented these stimuli in different sequences. The testing apparatus was completely drained between tests of different subjects, and all tubes and tubing were thoroughly cleaned between tests of different experimental groups.

In the apparatus, test subjects must swim against the current to move into the stimulus arms. Indeed, most subjects actively moved among the three sections of the apparatus throughout test periods. At times, however, some individuals became inactive, and then tended to rest in the central section of the Y-maze. In these cases, after 1 min the base of the glass tube was gently tapped with a rubber strip, inducing the tadpole to start swimming. Although this procedure does not bias the preference results toward either stimulus group, the amount of time spent in the neutral area does not necessarily reflect accurately lack of preference between the stimulus groups.

Baseline distributions of test subjects within the sections of the testing apparatus were obtained. Forty subjects were individually tested following these procedures, with simultaneous exposure only to dechlorinated water from both stimulus arms.

Testing protocol and rationale. In the first series of experiments, tadpoles were tested for a preference to orient toward water flowing from a container holding their siblings versus water flowing from a container holding members of another sibship. At least 3 h prior to the beginning of each test, two buckets were filled with dechlorinated tap water, and 25 tadpoles from each of two sibships were transferred from their rearing tanks to these containers. While the stimulus groups were held therein, water flowed from the containers directly through the connecting tubes and stopcocks to the Y-maze. Six pairs of different sibships were used as stimulus groups for these tests, and 10 members of each sibship were tested with each pair of stimulus groups. Therefore, 20 different individuals served as subjects in each of these 'basic choice' tests, as in all series of tests reported in this paper.

Waterborne cues that might be important in influencing tadpoles' orientation in the basic choice tests may be chemical, but the procedures do not exclude all other possible cues (e.g., sound). To eliminate non-chemical cues, the experiment was repeated incorporating a modification to the design of the apparatus. Instead of flowing directly to the Y-maze, water from the stimulus containers was siphoned at a rate of 1 drop/s to intermediary buckets. Small reservoirs thus accumulated in these buckets, from which water was fed into the tubing leading to the Y-maze (Fig. 2). Because water dripped from the stimulus containers into the reservoirs, individuals in the test apparatus were exposed only to cues that could be transferred through non-connecting bodies of water. Eight pairs of sibships were used as stimulus groups, and 10 members of each sibship served as subjects in each test.

To more specifically examine the role of olfaction in detecting waterborne cues, basic choice tests were repeated with tadpoles whose external nares were occluded with a gelatinous paste prior to testing. Small amounts of Orabase ('plain formulation', Hoyt Laboratories, Needham, Mass.) were applied with a blunt needle inside both nares of test subjects, as they were cushioned in moist cotton under a dissecting microscope. After the application, individuals appeared to swim in a normal manner, suggesting that the treatment did not cause them great trauma. The blocking compound has no pharmacological effect, but creates a physical barrier that prevents water (and odorants) from passing through the external nares. Some odorants might still reach olfactory receptors within the nasal cavity by passing from the oral cavity through the choana.

Within 30 min of treatment, test individuals were placed into the base of the Y-maze and tested for their tendencies to discriminate between water flowing directly from a stimulus container holding siblings and from one holding members of another sibship, using procedures identical to those described for the basic choice tests. Three tests, each involving different sibship pairs, were conducted. After being tested, all subjects from two of these tests were individually retained in marked

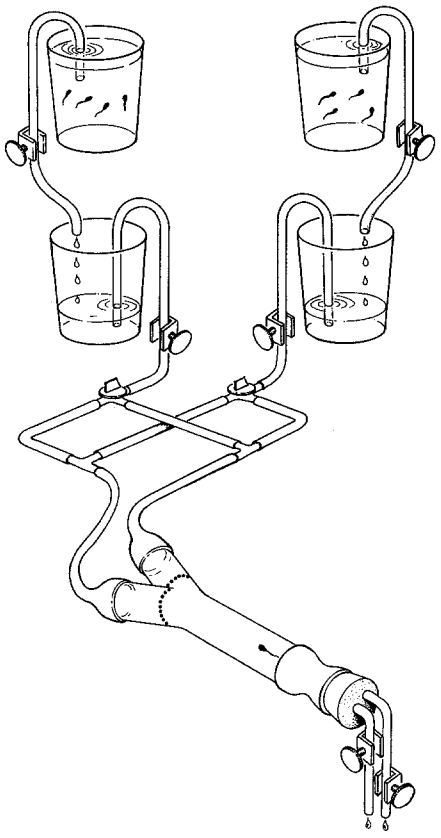

Fig. 2. Diagrammatic representation of testing apparatus for experiments in which the effects of chemical waterborne cues were examined. The design is similar to that employed in the basic choice tests (Fig. 1); however, water from the stimulus containers, instead of directly flowing to the three-way stopcocks, first dripped into intermediary reservoirs. Water from these containers was then siphoned into the tubing leading to the stopcocks and testing apparatus. Flow rates were controlled by clamps where indicated. Testing procedures were identical to those employed in basic choice tests. The apparatus is not drawn to scale. See text for further details

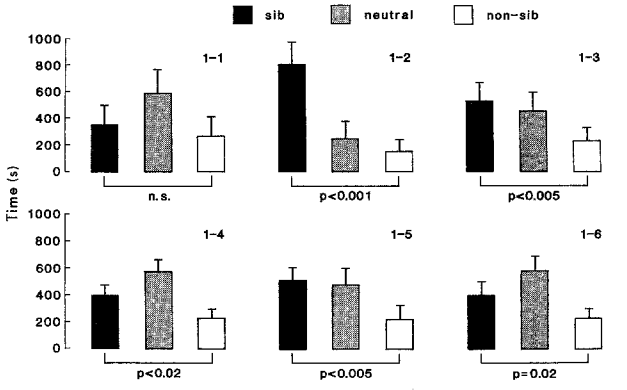

Fig. 3. Mean times spent by test individuals in a Y-maze oriented toward water flowing from a container holding their siblings, in a neutral region (expressing no preference), and oriented toward water flowing from a container holding non-siblings (see Fig. 1). Each test reflects measurements of the movements of 20 different individuals over 20 min periods; bars denote 95% confidence limits. Times spent oriented toward siblings and non-siblings were compared for each test group by a Wilcoxon signed-ranks test; two-tailed probabilities are indicated

petri dishes, and were held for a period of 24–48 h. They were then retested. During the holding period, the blocking compound absorbed water and became dislodged from the nares; no evidence of occlusion of the nares was found for any of the individuals upon microscopic examination prior to retesting. As an additional test of the effect of the handling procedures during treatment, in two additional tests (with two different sibship pairs), a needle was briefly inserted within the nares of test individuals but the compound was then applied to skin on the ventral surface of the head of each subject. All other procedures for testing these sham-treated individuals were identical to those used in the basic choice tests.

The influence of elapsed time on the saliency of possible recognition cues released by tadpoles was examined in two additional tests. Groups of 25 siblings were allowed to swim freely for at least 24 h in buckets containing fresh water, after which they were removed. The water, thus 'conditioned' by the sibships, was held at room temperature for an additional period of 24–30 h. These containers were then used as the test stimuli by directly attaching them (through the stopcocks and tubing) to the Y-maze. Subjects were tested for their tendencies to orient toward water in which their siblings had been held versus water in which members of another sibship had been held. Different sibship pairs were used in each test.

Discrimination between cues provided by one's siblings and those provided by non-siblings could result either from recognition of one's siblings (e.g., as similar to oneself) or recognition of one's non-siblings (as different from oneself). To evaluate these possibilities, two series of tests were conducted. Tadpoles were tested for their tendencies to discriminate between water in which their siblings were currently held and fresh dechlorinated tap water (six tests, involving six sibships). They were also tested for their tendencies to discriminate between water in which members of another sibship were currently held and fresh dechlorinated tap water (five tests, involving five sibships as stimulus groups). Twenty tadpoles from each sibship were tested. Procedures were otherwise identical to those used in the basic choice tests.

Statistical methods. The total amounts of time each of the 20 subjects spent in each of the arms of the Y-maze (oriented toward the two stimulus groups) and in the central section of the maze (neutral) were computed for every test. A preference score for each subject was determined as the difference in time it spent orienting toward the two stimulus groups. The null hypothesis that the median of these scores equaled zero was tested by the Wilcoxon signed-ranks test (Weiss and Hassett 1982). This procedure is computationally equivalent to comparing times spent by each individual oriented toward the two stimulus groups in a matched-pairs design (e.g., Siegel 1956). Pooled results from each test series were subjected to the same analysis, but in addition, comparisons between times spent in the central section and in each of the two stimulus arms were made.

These data were further analyzed to better illustrate response variability among individuals. The numbers of individuals spending more than half of their response time (excluding time spent in the neutral section) oriented toward each of the two stimulus groups were determined for each test and for each test series. Frequencies were compared with those expected under a binomial distribution (binomial test; Siegel 1956).

Table 1. Summary of test results

Treatment	Number of tests	Total number of individuals	Time (s)			Sib–Non-sib			Sib–Neutral			Non-sib–neutral		
			Sib mean (SD) median	Neutral mean (SD) median	Non-sib mean (SD) median	I[a] (Z)	N^b	P^c	T^a (Z)	N^b	P^c	T^a (Z)	N^b	P^c
Basic choice tests	6	120	495 (302) 484	484 (292) 495	220 (204) 191	1,200.5 (6.28)	119	<0.0001	3,417.5 (0.09)	117	ns	1,034 (6.20)	114	<0.0001
Chemical waterborne factor tests	8	160	410 (296) 390	644 (280) 645	145 (127) 125	1,888 (7.76)	160	<0.0001	3,466 (4.98)	159	<0.0001	100 (10.66)	156	<0.0001
Plugged nares tests	3	60	366 (223) 352	421 (323) 371	413 (273) 410	706	56	ns	803.5	60	ns	910	60	ns
Plugged nares controls	2	40	539 (289) 580	395 (256) 437	266 (240) 230	157	39	<0.001	270	40	ns	191.5	40	<0.005
Plug shams	2	40	273 (145) 260	727 (244) 730	200 (141) 206	197.5	40	<0.005	31	40	<0.001	15	40	<0.001
Conditioned (>24 h) water tests	2	40	362 (255) 392	499 (369) 418	339 (300) 353	338	37	ns	332.5	40	ns	289.5	40	ns
			Sib	Neutral	Water	Sib–Water			Sib–Neutral			Water–Neutral		
Sib vs blank water	6	120	288 (230) 256	614 (280) 645	298 (223) 272	3,326 (0.50)	118	ns	1,149.5 (6.34)	118	<0.0001	1,160.5 (6.47)	120	<0.0001
			Non-sib	Neutral	Water	Non-sib–Water			Non-sib–Neutral			Water–Neutral		
Non-sib vs. blank water	5	100	213 (199) 151	654 (272) 655	332 (250) 284	1,556	98	<0.005	385	100	<0.001	820.5	100	<0.001

[a] Wilcoxon signed-ranks test, normal approximations were used when $N>100$
[b] Excludes ties
[c] 2-tailed; ns denotes $P>0.05$

The preceding tests focus on subjects' responses to stimulus groups rather than on their overall distributions within the testing apparatus. Analyses of these distributions can also be useful in assessing whether movement patterns of experimental subjects differ from those expected if tadpoles move randomly. Under the testing conditions used here, movement patterns of individuals presented only with dechlorinated water as stimuli provide some measure of a random response. Numbers of individuals spending the largest proportion of the test period (including neutral, non-response time) in each section of the apparatus were determined for each test series. These distributions were then compared with that shown by subjects presented only with dechlorinated water (18 in neutral section, 11 in each stimulus arm). For each comparison, a 3×2 contingency table was constructed; differences in overall response patterns were determined by a chi-square test (Siegel 1956).

All statistical inferences were based on two-tailed probabilities.

Results

Basic choice tests

In five of the six tests of tadpoles exposed to water flowing from stimulus containers holding siblings

Table 2. Results of tests in which the duration of time spent by individual tadpoles orienting toward water in which their siblings were currently held was compared with that spent orienting toward water in which non-siblings were currently held. Individuals showing no preference are not included in this analysis

Test number	Number of test individuals preferring		P^a
	Sibs	Non-sibs	
1–1	12	8	ns
1–2	18	2	0.0004
1–3	16	3	0.004
1–4	15	5	0.04
1–5	18	2	0.0004
1–6	14	6	ns

[a] Binomial probabilities, 2-tailed; ns denotes $P>0.05$

and non-siblings, test subjects spent significantly more time oriented toward their siblings than toward non-siblings ($P\leq 0.02$; Fig. 3). Results pooled from all tests indicate that tadpoles swam in the arm of the Y-maze oriented toward siblings over twice as long as in that toward non-siblings (495 vs 220 s, $P<0.0001$; see Table 1). No difference is evident between the duration of time spent oriented toward siblings and that spent in the neutral section at the base of the Y-maze (495 vs 484 s, $P=0.40$). But tadpoles apparently preferred to remain in the neutral section rather than orient toward non-siblings (484 vs 220 s, $P<0.0001$). Overall, 93 of the 120 test individuals spent more than half of their response time oriented toward siblings; 26 were mostly oriented toward non-siblings ($P<0.0001$; see Table 2). If time spent in the neutral section is included, 52 individuals oriented toward siblings, 58 remained in the neutral section, and 10 oriented toward non-siblings ($\chi^2=10.38$, 2 *df*, $P=0.006$).

Effects of chemical waterborne factors

In each of the eight tests in which water flowed from the stimulus containers through intermediary reservoirs to the arms of the testing apparatus, tadpoles oriented toward their siblings more often than toward non-siblings ($P<0.05$; Fig. 4). Time spent oriented toward siblings was greater than that oriented toward non-siblings (410 vs 145 s, $P<0.0001$; Table 1). Tadpoles spent significantly more time in the neutral section (644 s) than in either of the stimulus arms ($P<0.0001$). In all, 119 of the 160 test individuals spent more than half of their response time oriented toward siblings;

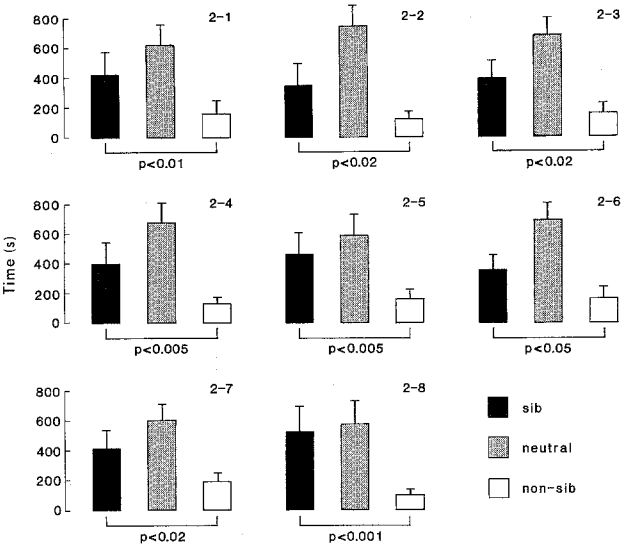

Fig. 4. Mean times spent by test individuals in a Y-maze oriented toward water flowing through an intermediary reservoir from a container holding their siblings, in a neutral region (expressing no preference), and oriented toward water flowing through an intermediary reservoir from a container holding non-siblings (see Fig. 2). Data are presented as in Fig. 3

Table 3. Results of tests in which the duration of time spent by individual tadpoles orienting toward water conditioned by their siblings was compared with that spent orienting toward water conditioned by non-siblings

Test number	Number of test individuals preferring		P^a
	Sibs	Non-sibs	
2–1	15	5	0.04
2–2	14	6	ns
2–3	14	6	ns
2–4	16	4	0.01
2–5	15	5	0.04
2–6	13	7	ns
2–7	14	6	ns
2–8	18	2	0.0004

[a] Binomial probabilities, 2-tailed; ns denotes $P>0.05$

41 were mostly oriented toward non-siblings ($P<0.0001$; see Table 3). The distribution of subjects in the testing apparatus significantly varied from the baseline pattern (54 individuals oriented toward siblings, 102 remained in the neutral section, and 4 oriented toward non-siblings; $\chi^2=28.93$, 2 *df*, $P<0.0001$).

Effects of blocking the external nares

Tadpoles whose external nares were blocked with a gelatinous paste showed no significant preference

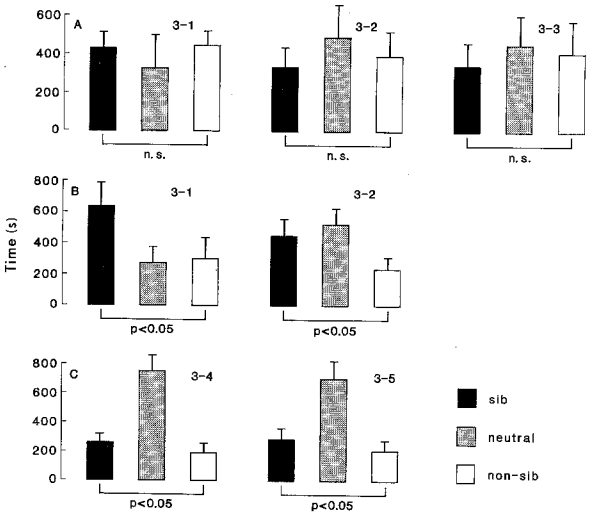

Fig. 5A–C. Mean times spent by test individuals, with blocked nares or sham treatment, in a Y-maze oriented toward water flowing from a container holding their siblings, in a neutral region (expressing no preference), and oriented toward water from a container holding non-siblings. Three groups (tests 1–3, series **A**) of tadpoles had a gelatinous paste applied within their external nares, and their responses in the testing apparatus were subsequently recorded. Twenty-four hours after testing, the paste had become dislodged from the test subjects' nasal passages, and two of the groups were retested (tests 1–2, series **B**). Individuals in two additional groups (tests 4–5, series **C**) had the paste applied on their skin outside of their nares, and were then tested in the Y-maze. Data are presented as in Fig. 3

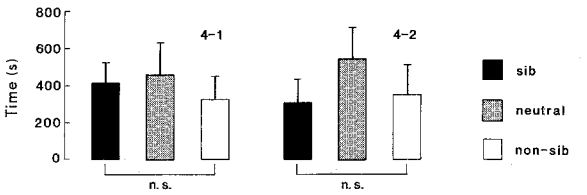

Fig. 6. Mean times spent by test individuals in a Y-maze oriented toward water flowing from a container that had previously held their siblings, in a neutral region (expressing no preference), and oriented toward water flowing from a container that had previously held non-siblings. Tadpoles had been removed from the stimulus containers 24–30 h before subjects were tested. Data are presented as in Fig. 3

for either of the stimulus arms in any of the three tests (Fig. 5A). Pooled results indicate that tadpoles spent approximately equal amounts of time oriented toward their siblings (366 s), toward non-siblings (413 s), and in the neutral section (421 s). No significant differences are evident among these measures (Table 1). An examination of individual responses shows that 27 of the 60 subjects spent more than half of their response time oriented toward siblings; 29 were mostly oriented toward non-siblings ($P=0.89$). Preferences were not apparent in any of the individual tests (9 vs 11, 8 vs 9, and 10 vs 9). Moreover, overall movement patterns did not differ from random (15 individuals oriented toward siblings, 26 remained in the neutral section, and 19 oriented toward non-siblings; $\chi^2=0.21$, 2 *df*, $P=0.90$).

Subjects retested after the paste had become dislodged from their nares showed a preference to orient toward their siblings. This effect was significant for each of the two test groups ($P<0.05$; Fig. 5B), and for the combined results ($P<0.001$; Table 1). Tadpoles spent, on average, less time oriented toward non-siblings (266 s) than either toward siblings (539 s) or in the neutral section (395 s, $P<0.005$). Overall, time oriented toward siblings did not significantly differ from that spent in the neutral section (see Table 1). Of the 40 subjects tested, 28 spent more than half of their response time oriented toward siblings; 11 were mostly oriented toward non-siblings ($P=0.009$) (individual test results: 12 vs 8, 16 vs 3). The distribution of subjects in the testing apparatus did not significantly differ from the baseline pattern (17 individuals oriented toward siblings, 18 remained in the neutral section, and 5 oriented toward non-siblings; $\chi^2=3.54$, 2 *df*, $P=0.17$).

Sham-treated individuals, which were handled similarly to the experimental subjects but whose external nares remained unoccluded, spent significantly more time oriented toward their siblings than toward non-siblings in each of two experimental tests ($P<0.05$; Fig. 5C). Overall, the amount of time spent oriented toward siblings (273 s) was significantly greater than that oriented toward non-siblings (200 s, $P<0.005$), but tadpoles spent most of their response time in the neutral section (727 s; Table 1). Although the mean differences appear small, of the 40 subjects tested, 31 spent more than half of their response time oriented toward siblings; 9 were mostly oriented toward non-siblings ($P=0.0006$) (individual test results: 16 vs 4, 15 vs 5). When neutral time is included, a tendency to remain in the base of the apparatus is apparent: 4 individuals oriented toward siblings, 35 remained in the neutral section, and 1 oriented toward non-siblings ($\chi^2=17.05$, 2 *df*, $P=0.0002$).

Saliency of waterborne cues with time

Tadpoles showed no clear preference to orient toward water in which their siblings had previously been held rather than toward water that had held non-siblings. In one test, subjects appeared to weakly (nonsignificantly) prefer siblings, but in the other they weakly preferred non-siblings (Fig. 6).

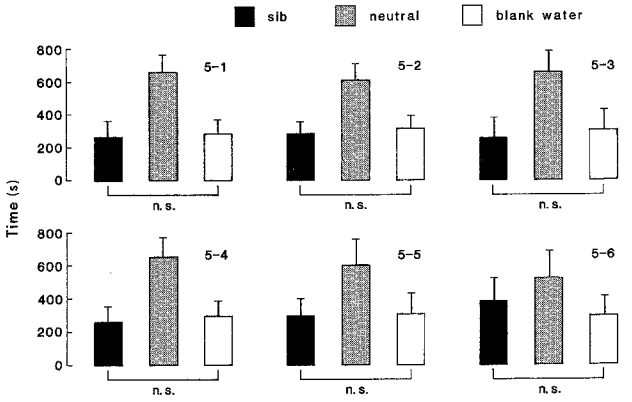

Fig. 7. Mean times spent by test individuals in a Y-maze oriented toward water flowing from a container holding their siblings, in a neutral region (expressing no preference), and oriented toward water flowing from a container filled with fresh dechlorinated tap water ('blank water'). Data are presented as in Fig. 3

Overall, no significant differences emerge among durations of time spent in the arm toward siblings (362 s), the arm toward non-siblings (339 s), and in the neutral section (499 s) (Table 1). Of the 40 subjects tested, 17 spent more than half of their response time oriented toward siblings; 20 were mostly oriented toward non-siblings ($P=0.74$) (but note variation in the individual test results: 11 vs 7, 6 vs 13). The distribution of subjects in the testing apparatus conformed to the baseline pattern (12 individuals oriented toward siblings, 15 remained in the neutral section, and 13 oriented toward non-siblings; $\chi^2=0.48$, 2 *df*, $P=0.79$).

Orientation toward siblings versus blank water

Tadpoles did not prefer to orient toward water in which their siblings were currently held when this stimulus was paired with fresh dechlorinated tap water. No significant trends were found in any of the tests (Fig. 7). Tadpoles spent approximately equal time in the arms of the maze oriented toward their siblings (288 s) and oriented toward blank water (298 s), but they spent more time in the neutral section than oriented toward either of these stimuli (614 s, $P<0.0001$; Table 1). Overall, 54 of the 120 subjects tested spent more than half of their response time oriented toward siblings; 64 were mostly oriented toward blank water ($P=0.40$). By these testing criteria, a significant preference for blank water emerges in one test (test 5; Table 4). Because of the large amount of time spent in the neutral section, the distribution of subjects in the apparatus departed from the baseline pattern (20 individuals oriented toward siblings, 81 remained in the neutral section, and 19 oriented toward blank water; $\chi^2=6.45$, 2 *df*, $P=0.04$).

Table 4. Results of tests in which the duration of time spent by individual tadpoles orienting toward water in which their siblings were currently held was compared with that spent orienting toward fresh ('blank') water. Individuals showing no preference are not included in this analysis

Test number	Number of test individuals preferring		P^a
	Sibs	Water	
5–1	11	8	ns
5–2	10	10	ns
5–3	11	8	ns
5–4	8	12	ns
5–5	5	15	0.04
5–6	9	11	ns

[a] Binomial probabilities, 2-tailed; ns denotes $P>0.05$

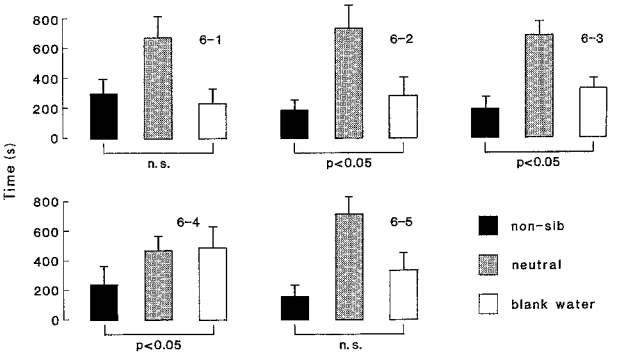

Fig. 8. Mean times spent by test individuals in a Y-maze oriented toward water flowing from a container holding non-siblings, in a neutral region (expressing no preference), and oriented toward water flowing from a container filled with fresh dechlorinated tap water ('blank water'). Data are presented as in Fig. 3

Orientation toward non-siblings versus blank water

In three of the five tests conducted, time spent by tadpoles oriented toward non-siblings significantly differed from that spent oriented toward blank water. In each of these cases, tadpoles preferred fresh dechlorinated tap water to the water in which non-siblings were currently held (Fig. 8). Overall, time spent in the arm of the maze oriented toward blank water (332 s) was significantly greater than that spent oriented toward non-siblings (213 s, $P<0.005$). Subjects spent significantly more time in the neutral section (654 s) than in either of the stimulus arms ($P<0.001$; Table 1). Thirty-four of the 100 subjects tested spent more than half of their response time oriented toward non-siblings; 64

Table 5. Results of tests in which the duration of time spent by individual tadpoles orienting toward water in which non-siblings were currently held was compared with that spent orienting toward fresh ('blank') water. Individuals showing no preference are not included in this analysis

Test number	Number of test individuals preferring		P^a
	Non-sibs	Water	
6–1	13	6	ns
6–2	4	15	0.02
6–3	4	16	0.01
6–4	6	14	ns
6–5	7	13	ns

[a] Binomial probabilities, 2-tailed; ns denotes $P>0.05$

were mostly oriented toward blank water ($P=0.003$). In one test, there was a nonsignificant trend in the opposite direction (test 1, Table 5). If time spent in the neutral section is included, 11 individuals oriented toward non-siblings, 70 remained in the neutral section, and 19 oriented toward blank water ($\chi^2=8.75$, 2 *df*, $P=0.01$).

Discussion

Chemical signals appear sufficient to elicit a sibling recognition response among toad tadpoles. Tadpoles exposed only to waterborne cues released by their siblings and non-siblings could discriminate between them and orient toward their siblings. As they could not see the stimulus groups, visual cues were not required. Tadpoles showed a strong orientation response even when stimulus water was passed through an intermediary reservoir. Thus individuals can perceive cues released by conspecifics even when they lack direct exposure to them; moreover, the signals apparently do not decay immediately after their emission. These properties suggest that tadpoles were responding to chemical cues, rather than to acoustic or vibratory stimuli, or to other possible waterborne signals (e.g., electrical).

The results do not preclude the possibility that additional sensory modalities also play a role in effecting kin discriminations; however, in larval anurans the olfactory sense generally becomes functional before other sensory systems, and in *Rana temporaria*, for example, it is the only specialized modality to function immediately after hatching (Spaeti 1978; also see Roberts and Hayes 1977; Roberts 1980 for a description of the somatosensory system). The kin recognition system of *Bufo americanus* tadpoles appears to become crystallized early in development, shaped in part by social interactions with conspecifics (Waldman 1981). Once traits of these individuals, usually siblings, become incorporated into a recognition 'template', tadpoles may identify kin by comparing traits of conspecifics with those stored in this template (and with their own traits, which might also be present therein). In laboratory pools, *B. americanus* tadpoles associate with their siblings in preference to non-siblings soon after larvae begin free-swimming, and the response continues until they begin metamorphosis (Waldman 1981). The early development of chemosensory abilities may facilitate the rapid expression of kin recognition abilities after hatching.

Tadpoles whose external nares were blocked with a gelatinous paste failed to discriminate between water flowing from siblings and water flowing from non-siblings. When the plugs subsequently became dislodged, these same test subjects oriented toward their siblings. This is consistent with the hypothesis that individuals identify their siblings by olfaction. Some odorants potentially could have entered the nasal cavity through the choana from the buccopharyngeal cavity; the flow rate across the sensory receptors within the nasal cavity would, however, be greatly reduced (cf. Wysocki 1982). Though shams appeared unaffected, individuals with blocked nares might simply have been unmotivated to respond to the stimuli presented them in the testing apparatus. In a study of the responses of *B. americanus* tadpoles to various odorants, Risser (1914) obtained essentially this result with tadpoles whose nostrils had been filled with petrolatum. Tadpoles thus deprived of olfaction were unable to discriminate between food and non-food packets, but their general swimming movements also seemed to be less vigorous after their nares were occluded. With the procedures used in the experiments reported here, swimming movements appeared unaffected. Indeed, subjects with blocked nares spent less time in the neutral section of the Y-maze than those tested in any of the other experiments in which tadpoles were exposed both to sibling and non-sibling stimuli (see Table 1). Nonetheless, experiments on individuals with severed olfactory nerves would be useful.

Chemosensory responses could result from stimulation of either the main or accessory olfactory systems. Receptors for both systems lie within sacs of the nasal cavity: the olfactory epithelium and the vomeronasal (Jacobson's) organ, respectively. The olfactory epithelium is formed and most

likely innervated quite early, before larvae become free-swimming (see Spaeti 1978), but the vomeronasal organ does not become fully differentiated until tadpoles are moderately developed (in *Bufo regularis*, simultaneously with limb bud formation; Khalil 1978). Most test subjects used in this study were developed to the stage that both olfactory systems could presumably have been functional. Generally, however, very young larvae could rely only on the main olfactory system for perceiving kinship cues. In contrast, Spaeti's (1978) findings suggest that the gustatory sense may not begin functioning until metamorphosis is complete. The visual and auditory senses also appear to be of little importance to tadpoles. Young larvae fail to orient directly to visual stimuli, and though the optic system becomes more complex through larval development, it remains relatively simple, evidently unable to resolve detailed images prior to metamorphosis (Spaeti 1978; but see Reuter 1969). Similarly, tadpoles apparently cannot perceive sound until midway through the larval period (Spaeti 1978; also see Witschi 1949). The aquatic medium is particularly suitable for chemical communication, so it should not be surprising that olfaction serves as a dominant modality for larval anurans, supplemented by a well-developed lateral line system (Russell 1976) which may also be sensitive to some chemical stimuli (Onoda and Katsuki 1972). The possible chemical sensitivity of 'free' nerve endings in the skin of young larvae (Roberts 1980) has not been investigated.

What chemical cues might be used by individuals in discriminating siblings from non-siblings is unknown; indeed, little is known in general about chemical communication in anurans (see review in Madison 1977). Although the effectiveness of the signal is not diminished by passing the stimulus water through an intermediary reservoir, if water conditioned by sibling groups is stored for 24–30 h, tadpoles appear no longer to be able to distinguish between sibling and non-sibling stimuli. Additional tests are needed, but based on these data the cues appear to be sufficiently volatile that they deteriorate within this period. Cues that persisted in the environment long after individuals had moved to a new location would constitute poor kinship signals, especially if they function to facilitate the aggregation of siblings in schools (Waldman 1982). Chemical signals are often characterized by slow fade-out times, effectively acting as markers of locations individuals have occupied; however, they can also be selected to have fast fade-out times and limited active space to accommodate rapid rates of information transfer (Wilson 1968). Kinship cues emitted by toad tadpoles appear to be of this latter sort.

Kinship discriminations might occur if siblings are directly attracted to one another, or conversely, if individuals avoid conspecifics they perceive to be non-siblings (Waldman and Adler 1979). When simultaneously presented with water in which their siblings were swimming and blank water, test subjects seemed not to discriminate between these stimuli. Tadpoles did, however, orient toward blank water in preference to water in which non-siblings were swimming. This trend also emerges in the results of the basic choice tests: whereas the amount of time tadpoles were oriented toward non-siblings significantly differed from that spent in the neutral section of the maze, time spent oriented toward siblings did not significantly differ from that spent in the neutral section (Table 1). The effect is less pronounced in the other test series, but together with the stimulus versus blank water data, the results suggest that avoidance of non-siblings may be one component of a recognition system that facilitates the association of siblings. Alternatively, tadpoles might generally orient toward fresh, fully oxygenated water (e.g., see Costa 1967), but this effect may be counteracted by an attraction toward siblings.

The hypothesis that sibling association among anuran larvae results, in part, from non-sibling dissociation is consistent with results from previous laboratory tests. Groups of 50 individuals were released in a pool, and their nearest-neighbor distances subsequently measured. When 25 members of each of two sibling groups were marked and tested, tadpoles assorted with their siblings rather than with non-siblings (experimental tests). When 50 members of a single sibling group were randomly divided into two subgroups, marked, and tested, the individuals did not assort by their different mark-colors (control tests). Yet in most experiments, distances between nearest-neighbor siblings (of either subgroup) in the control tests were greater than distances between nearest-neighbor siblings in the experimental tests. Still, mean distances in the control tests tended to be less than those between non-siblings in the experimental tests (data in Waldman and Adler 1979; Waldman 1981, 1984). This result would be expected if tadpoles recognize and move away from non-siblings, regardless of whether they also respond to their siblings. When all individuals in a pool were members of the same sibship, nearest-neighbor distances among them thus tended to increase.

Conceptually the kin recognition process might be very simple: tadpoles may release chemical cues,

and habituate to their own smell (and, as suggested above, to odors of individuals surrounding them early in development). Contact with an individual that matches these traits may thus fail to elicit a change in behavior. But contact with an individual that has a different odor may cause the tadpole to move away, or more simply, just to continue swimming. Behavioral recognition may then be accomplished by a process of 'phenotype matching' (see Alexander 1979; Waldman 1981; Holmes and Sherman 1982) in which a response (e.g., swimming) is elicited if an individual is encountered that does not match one's own traits or those previously learned from known conspecifics. The finding that maternal half-siblings are not discriminated from full-siblings, but paternal half-siblings are (Waldman 1981), is consistent with this notion if the chemical odor derives in part from some contribution of the maternal parent, such as cytoplasmic contributions to the eggs or factors present in the jelly enveloping the eggs during early development.

How then do toad tadpoles form sibling schools under natural conditions? When *B. americanus* tadpoles, reared in the laboratory and marked by sibship, are released in outdoor ponds, they aggregate, often in densely packed schools which consist largely of members of single sibships (Waldman 1982). Although all individuals in a pond are not necessarily present in schools at any particular time, tadpoles are rarely found dispersed throughout a pond. If kin association results from the mechanism just proposed, one might expect to find statistically clumped pockets of siblings, but hardly these well-defined sibling schools. Aside from olfaction, though, other modalities are probably involved in the formation and maintenance of tadpole schools. In particular, visual cues may direct tadpoles to areas of a pond where schools will form (Beiswenger 1977), and they may serve to attract individuals to one another (Wassersug and Hessler 1971; Wassersug 1973; Wassersug et al. 1981; but see Foster and McDiarmid 1982). Visual cues were not available to test subjects in the present experiments. When they are available, visually mediated behavior may be accompanied by a negative klinokinetic olfactory response to non-siblings, as evidenced in this study. Kin association may thus result from two opposing but not mutually exclusive processes: a visual component, effecting social attraction among conspecifics, and an olfactory component, effecting selection of siblings by rejection of non-siblings (also see discussion in Waldman 1982).

Results of a recent study on *Rana cascadae* tadpoles suggest that they, too, may use chemical cues for the communication of kinship identity; visual cues alone appear ineffective (Blaustein and O'Hara 1982a). Although kin recognition has not been studied in other amphibians, some terrestrial plethodontid salamanders are able to distinguish between their own odors and those of conspecifics. *Plethodon jordani* behaviorally discriminate between neighbors and non-neighbors based on airborne cues (Madison 1975). *Plethodon cinereus* can distinguish between substrates they have occupied and those occupied by unfamiliar conspecifics (Tristram 1977; McGavin 1978; Jaeger and Gergits 1979), and apparently can learn odor differences between familiar and unfamiliar individuals (McGavin 1978; Jaeger 1981). Where populations are philopatric, neighbors are likely to be kin, and these effects may be confounded with genetic relatedness.

Behavioral studies can provide a broad framework upon which the properties of kin recognition systems can be considered. Ultimately, however, the application of the techniques of analytical chemistry (to characterize the signals) and neurophysiology (to characterize the sensory processing apparatus) will be required for a full understanding of how toad tadpoles and other animals make kinship discriminations. Such integrative analyses have already been initiated on the problem of how individuals identify conspecifics of their own population (Nordeng 1971). Arctic salmon (*Salmo alpinus*) and Atlantic salmon (*Salmo salar*) orient toward siblings or other members of their own population in laboratory testing devices (Selset and Døving 1980; Stabell 1982); chemical (Stabell et al. 1982) and neurophysiological (Døving et al. 1974; Fisknes and Døving 1982) correlates of this response have now been identified. The chemical and physiological substrates of anuran kin recognition systems will undoubtedly be further delineated as similar paradigms are developed to study their discrimination abilities.

Acknowledgements. I thank H. Cohen, S. Egan, S. Jan de Beur, V. Katz, J. Molofsky, E. Rainey, H. Waksman, and L. Wierzbicki for assistance conducting the experiments, P. Kempthorne for statistical discussions, and P. Bateson, D.K. Dawson, R.G. Jaeger, and T.J. Pitcher for their comments on the manuscript. The research was supported by grants from NSF (DEB-7909119), the Gaige Fund (American Society of Ichthyologists and Herpetologists), and Sigma Xi. Supplementary funds were provided by NSF grant BNS-7924525 (to K. Alder). While writing the manuscript, I was supported by a NATO postdoctoral fellowship at the University of Cambridge. I thank P. Bateson and R.A. Hinde for making facilities available at the Sub-Department of Animal Behaviour, Madingley, and the University Computing Service for providing computer resources.

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