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IMPRINTING ON NATIVE POND ODOUR IN THE POOL FROG, RANA LESSONAE CAM.

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INTRODUCTION

Imprinting on environmental chemical stimuli in early development is a common feature of vertebrates. While in mammals imprinting is directed to a mother (Hudson, 1999), in lower vertebrates (fish, reptiles) which grow without maternal care it is addressed to stimuli marking the place of birth (Hasler and Scholz, 1978; Grassman, 1993).

The froglets of the pool frog Rana lessonae keep near the native pond after metamorphosis and this pattern of behaviour is, at least partly, based on the preference for native pond odour, which is, presumably, learnt during larval development (Bastakov, 1986, 1992). The goal of the study was to see whether natural and artificial chemical markers, which were present in the water at different stages of larval development, would be able to modify the behaviour of adult animals.

MATERIALS AND METHODS

The study was performed with the pool frog (Rana lessonae Cam.). All frogs and eggs used in experiment were collected in ponds of Moscow region.

To reveal the natural reaction of wild individuals of the pool frog to native pond water frogs (Group 1) were caught near their native pond at the end of metamorphosis, on stages 43-46 (Gosner, 1960), and were tested in a chamber 76 cm long, 12 cm wide and 15 cm high, made of white plastic and covered with a transparent glass. A pair of familiar and unfamiliar odorants was placed in Petri dishes, 20 ml each, in the opposite sides of the chamber (Figure 1). 4-11 frogs were placed simultaneously in the starting position in the centre of the chamber and were left walking freely in the testing apparatus. To describe the spatial distribution of frogs between two odorants the test-chamber was conditionally divided on 5 sections as shown in Figure 1. The number of frogs in each section was counted with 5 min intervals during a 40 min test. Thus 8 blocks of five numbers were

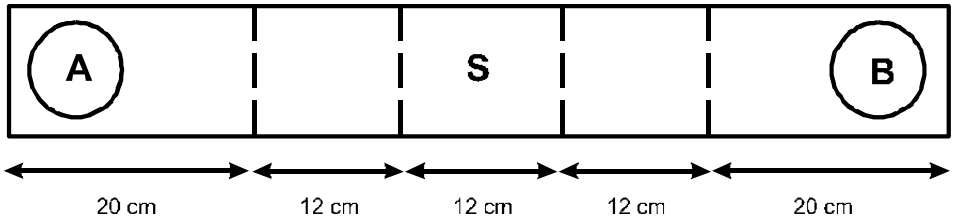

Figure 1. Scheme of a test-chamber, view from above. A and B – Petri dishes with chemical stimuli, S – starting position of frogs.

obtained in a test for further analysis. That means each frog was counted several times during a test. To prevent frogs from the selection of a particular side of the chamber half of the frogs in each group was tested with an altered position of odorants in the chamber. The results of such tests in each group were then combined for further analysis according to the position of a familiar odorant. After each test the chamber was washed with tap water. The tests were conducted at night and a 40 W incandescent lamp placed 40 cm from the middle of a long wall provided diffuse illumination from one side of the chamber. The reaction to odorants was determined as asymmetry in the distribution of frogs along the test-chamber. We compared the number of frogs first in two utmost sections and then in two «left» against two «right» sections using nonparametric Wilcoxon matched pairs test. A test of a group on a certain odorant was regarded as a sample and each registration of frogs' distribution obtained after 5 min intervals as a single observation.

In tests wild frogs of Group 1 were offered native pond water paired with tap water. In our study we used only tap water settled for 2-3 days. A separate test (test 2 of Group 1) was conducted to find out whether wild frogs discriminate between water from the native pond and water taken from a strange pond. This test could show the specificity of frogs' reaction to pond water .

To find out when the memorization of chemical stimuli could occur we reared tadpoles in a chemically marked environment and tested them after metamorphosis in the reaction to odorants, which were present in the water during larval development (Groups 2-12). The oviposited eggs for rearing tadpoles were collected in ponds before hatching and the larvae were grown in aquaria in the laboratory. On stages 21-43 (from the beginning of active feeding) all groups of tadpoles were fed leaves of boiled common nettle. In different periods of larval development aquaria contained the chemical markers such as water solutions of artificial stimuli (morpholine - 10^{-8} mole/l or β-phenylethanol - 10^{-7} or 10^{-8} mole/l), natural pond water and boiled nettle, tadpoles' foodstuff. These odorants were used later to test frogs after metamorphosis.

First we divided the period of embryonic and larval development on two parts: before and after stage 21, the beginning of active feeding. Groups 2 and 3 were exposed to chemical markers during these periods correspondingly. Then each of the two periods was also subdivided in two parts, thus presenting 4 intervals for exposure in chemical markers: stages 1-18, eggs before hatching (Group 4), 18-21 (Groups 5 and 6), 25-31, up to the beginning of hind limb toes differentiation (Group 7) and 32(40)-43, at metamorphosis (Groups 8-10). After metamorphosis frogs were kept in terrariums and had no contact with the odorants used for exposure. For the first time frogs were tested in a chamber described above within 1 month after metamorphosis. Small groups of 6-7 frogs were tested twice with odorants in different positions in the chamber. When tested frogs were offered a native odorant paired with tap water (in groups for which morpholine, β-phenylethanol or boiled nettle served as native odorants) or with strange pond water (in Groups 2 and 4 for which native pond water served as native odorant). Frogs were also tested in the reaction to native foodstuff - water extract of boiled nettle (1 boiled leaf left for 30 min in 200 ml of water) which was paired with tap water.

Groups of frogs exposed to morpholine or β-phenylethanol during larval development were also tested on a series of concentrations of a native odorant with 1-2 day intervals between testes. We checked the concentrations from 10^{-4} to 10^{-9} mole/l for β-phenylethanol and from 10^{-7} to 10^{-9} mole/l for morpholine with a step of 10 times.

To see whether frogs can learn two different stimuli present separately in two different periods of larval development the following exposure was used. Group 3 was exposed to β-phenylethanol on stages 18-21 and to boiled nettle on stages 21-43, Group 8 was exposed to native pond water on stages 1-21 and to morpholine on stages 32-43. Frogs of these groups were tested on each native odorant.

On stages 21-43 water in aquaria always contained the foodstuff and thus the stimulus used at this period for exposure was always complex – chemical marker together with foodstuff, boiled nettle. To find out whether frogs discriminate single components in a scent mixture, and thus whether our tests on a chemical marker alone were adequate, we exposed one group of tadpoles on stages 32-43 to a mixture of morpholine (10^{-7} mole/l) and β-phenylethanol (10^{-8} mole/l). After metamorphosis this group (Group 10) was tested on a mixture of odorants and on single components paired with tap water. Additionally we compared the results of tests of Group 8, exposed to morpholine on the same stages, when tested on morpholine alone and on morpholine mixed with foodstuff.

To reveal whether frogs possess a natural preference for the chemical markers used in experiment animals which did not contact with these stimuli during larval development were used as a control. Thus Group 11 of wild frogs served as a control in tests with laboratory foodstuff, boiled nettle. Group 12 grown in settled tap water with foodstuff, boiled nettle, only was a control in tests with morpholine and β-phenylethanol. This group was also tested on a series of concentrations of the stimuli as described for other groups.

To describe the dynamics of frogs' reaction to native odorants groups were tested during the following periods of terrestrial life: 1-4, 7-9 and 19 months after metamorphosis. The first period represents the time before wintering, the second – after first wintering, and the last one – after second wintering.

RESULTS

Wild frogs caught at the end of metamorphosis near the native pond exhibited a significant preference for water from the pond paired with settled tap water (test 1 of Group 1 in Table 1). The froglets also preferred native pond water paired with strange pond water (test 2 of Group 1 in Table 1).

Frogs reared in laboratory conditions and exposed to native pond water before stage 21 or exposed to boiled nettle after stage 21 also revealed preference for the native odorants (Group 2 and 3 in Table 1). Thus exposure to chemical markers in both periods of larval development, presumably, created a preference for these stimuli. To determine whether there is a stage when exposure does not lead to a preference for the native odorant both periods of larval development were subdivided in further experiments. Group 4 exposed to pond water only before hatching (stages 1-18, nearly 4-6 days) failed to discriminate between native and strange pond water. But incubation of tadpoles of Groups 5 and 6 in water solutions of morpholine or β-phenylethanol between hatching and the beginning of active feeding (stages 18-21) caused the formation of preference for these odorants paired with tap water (see Table 1). Groups 5 and 6 were exposed to odorants for about 4-7 days only. Group 7 exposed to β-phenylethanol for 24 days on stages 25-31 was indifferent to this stimulus after metamorphosis. On the contrary, all the frogs reared after stage 32 or 40 in contact with β-phenylethanol or morpholine or with mixture of

them exhibited preference for the stimuli used, such as Groups 8-10 of Table 1. The minimum time of exposure was 12-15 days in Group 9.

Table 1. Treatment conditions during larval development and further distribution of frogs in a chamber when tested after metamorphosis.

Group	Treatment [1]		Test number	Odorants tested	Score for [3]		p [4]	No. of frogs
	Stages	Odorants [2]			Odorant	Water		
1	1-46	PW	1	PW	92	7	<0,00001	21
			2	PW	80	15	<0,0001	25
2	1-21	PW	1	PW	31	10	<0,05	18
3	21-43	Nt	1	Nt	93	61	<0,05	28
4	1-18	PW	1	PW	21	30	n.s.	17
5	18-21	M	1	M	34	9	<0,01	6
6	18-21	P	1	P	24	3	<0,001	6
7	25-31	P	1	P	14	15	n.s.	8
8	32-43	M+Nt	1	M	47	23	**<0,05**	12
			2	M+Nt	24	7	<0,05	13
9	40-43	P	1	P	55	37	<0,05	17
10	32-43	M+P	1	M+P	49	19	**<0,05**	7
			2	M	18	20	n.s.	7
			3	P	20	23	n.s.	7
11	Control	PW	1	Nt	83	94	n.s.	21
12	Control	Nt	1	M	10	12	n.s.	7
			2	P	8	6	n.s.	12
			3	M+P	12	12	n.s.	11

[1] Control – frogs which did not contact in larval development with the odorants they were tested at

[2] PW - native pond water (tested in pair with strange pond water, except test 1 in group 1), M – morpholine, P - β-phenylethanol, Nt - boiled nettle, «+» – two odorants were present simultaneously

[3] All observations during a test are summarized, thus each frog was counted several times in a test

[4] Normal type – strong reaction, comparison of the number of frogs in utmost sections of the chamber, boldface type – weak reaction, significant when the number of frogs are compared in 2 «left» and 2 «right» sections only

Thus exposure of tadpoles to chemical markers on stages 18-21 and 32(40)-43 created preference for the markers in froglets. Exposure to odorants on stages 1-18 and 25-31 led to indifference for the native odorants. Frogs of Groups 3 and 8 which were exposed to one stimulus during early larval development (including stages 18-21) and to another stimulus during late larval development (including stages 32-43) managed to reveal preference for both stimuli. The reaction of these groups to odorants with which they contacted in early development was similar to such observed in Groups 2 and 6 of Table 1.

Frogs which contacted with morpholine or β-phenylethanol on stages 18-21 or 32-43 preferred these odorants in concentrations of 10^{-7}-10^{-8} mole/l, equal or lower than that used for incubation (nearly 70% of registrations of frogs were in 3 sections close to native odorant). High concentrations of β-phenylethanol, such as 10^{-4}-10^{-5} mole/l, were rejected by the frogs which contacted with it on the mentioned stages (70% in 3 sections close to tap water). Frogs were indifferent to intermediate concentrations of β-phenylethanol (70% in 3 central sections). Indifferent reaction was observed in all

groups of frogs to the lowest concentration used in tests - 10^{-9} mole/l, which is, presumably, an olfactory threshold.

To determine whether frogs discriminate single odorants in a scent mixture Group 10 was exposed to a mixture of morpholine and β-phenylethanol. When tested it showed preference for the mixture but not for morpholine or β-phenylethanol alone (see Table 1). In larval development Group 8 was exposed to morpholine in the presence of foodstuff, boiled nettle. When tested on morpholine alone it showed weak preference, significant only when the number of frogs were compared in 2 «left» and 2 «right» sections. That means most frogs moved only one section towards the native stimulus and during the second half of the test they distributed randomly in the chamber (test 1 of Group 8 in Table 1, boldface type). When the same group was tested on morpholine together with native foodstuff we observed a strong preference for the native scent mixture, significant when the number of frogs were compared in utmost sections. That means the majority of frogs moved to the very section containing the native odorant and stayed there during the whole test (test 2 of Group 8 in Table 1, normal type).

Frogs were indifferent to chemical stimuli which were not present in the water during their larval development. Thus control Group 11 of wild frogs was indifferent to an unfamiliar laboratory foodstuff, boiled nettle. Control Group 12 was indifferent to morpholine, β-phenylethanol and the mixture of them. When tested on a series of concentrations of artificial odorants frogs of this group showed indifference to all, even high concentrations (10^{-4} mole/l for β-phenylethanol) used. It is also worth mentioning that frogs which revealed preference for native odorants visited the utmost sections of the test-chamber more frequently than did frogs from the control groups (40% versus 20% of all visits during a test, $p<0,01$), as if a familiar odour increased their locomotor activity.

The reaction of frogs that revealed preference for native odorants changed with time. 1-4 months after metamorphosis the majority of groups preferred native stimuli, though Group 1 of wild frogs became indifferent to native pond water on the third month of terrestrial life. 7 months after metamorphosis two groups of frogs reared in laboratory conditions still showed preference for the native stimuli, but 8-9 month after metamorphosis the majority of groups were indifferent to native odorants. At the same time Group 1 of wild frogs and Group 3 of frogs reared in laboratory conditions revealed weak negative reaction to native odorants. Frogs of control groups and of experimental groups 4 and 7, which were indifferent to the stimuli when tested after metamorphosis, were still indifferent to them on the ninth month of the terrestrial life. The only group, Group 9, tested after the second wintering period, 19 months after metamorphosis, showed preference for the native odorant (β-phenylethanol). When tested on a series of concentrations of the odorant these frogs revealed preference for the very concentration which was used in this group for exposure.

DISCUSSION

Froglets of the pool frog (Rana lessonae Cam.) keep close to the native pond after metamorphosis. When caught near the pond and tested in laboratory these young frogs reveal strong preference for the pond water. After all, they are able to discriminate between water from the native pond and water from a strange pond. The latter means that reaction to native pond odorants is specific.

The present data was obtained while testing frogs in groups of several individuals simultaneously. Preference of wild froglets for a native odorant was shown earlier in individual tests also (Bastakov, 1986). The fact that individual and group tests provide the same results indicates that our testing method is adequate.

A series of experiments with frogs reared in laboratory conditions showed that they could memorize various chemical stimuli dissolved in water during larval development. Frogs learn not only natural stimuli such as pond water or boiled nettle, but also artificial ones such as morpholine and β-phenylethanol. Learning took place on stages 18-21 and 32(40)-43 and led to preference for the familiar odorants. In our study the minimum time of exposure for chemical stimuli was 4 days comparing to nearly 2,5 months of the whole larval period. After all, frogs could memorize two different odorants one of which was present in the water during the first period and the other during the second period. Frogs exposed to stimuli on stages 1-18 and 25-31 did not learn the odorants in our study and reacted indifferently to them. The same was true for the frogs which had never contacted with any of the odorants used in our experiments. Thus learning is possible during two separate periods of larval development. It is worth mentioning that morphological development of frog's olfactory system is also 2-staged (Spaeti, 1978). Periods of learning, as discovered in our study, and stages of active neurogenesis appear to be correlated.

The fact that frogs, which memorized chemical stimuli, reacted to very small concentrations of odorants (10^{-8} mole/l) speaks for the use of olfactory reception rather than skin chemical sensitivity. Preferring low and rejecting high concentrations of the familiar stimulus frogs are able to choose an optimal position in a concentration gradient of an odorant. While frogs, which memorized chemical stimuli through exposure in larval development, reacted differentially to various concentrations of the familiar odorants, frogs which never contacted with the same stimuli were indifferent to all concentrations tested. The situation resembles the phenomenon of experience-induced sensitivity in young mammals (Hudson, 1999).

We showed that if frogs are reared in a mixture of chemical stimuli during larval development, they react better on the scent mixture than on each of its components alone. That explains why in our study the reaction of frogs to such odorants as morpholine and β-phenylethanol is statistically more significant when we expose them to these odorants on stages 18-21 than on stages 32-43. The fact is that during the first period larvae do not feed and the water contains the odorants only, in the second period as the tadpoles begin to feed the chemical marker needs to be mixed with a foodstuff thus providing a scent mixture. One can also propose that frogs discriminate between water from native and strange ponds by a composition of odorants, rather than by single learnt components.

It is known that frogs and toads use olfaction for spatial orientation (Sinsch, 1992). Whether the frogs prefer or reject the native pond odour largely depends on the biology of a species. Common toad (Bufo bufo L.) leaves the native pond soon after metamorphosis rejecting the native pond odorants. On the contrary, froglets of the pool frog (R. lessonae) are strongly attached to the native pond and they reveal preference to native pond odorants (Bastakov, 1992). The present study shows that reaction of the pool frog to native pond odour changes gradually with age and these changes correspond with the strategy of behaviour of different age groups of this species. According to the results of our censuses (unpublished), after wintering, 8-9 months after metamorphosis, young frogs usually occupy strange ponds and constantly move from one pond to another. At this time we register indifferent or even negative reaction to the native pond odour. It is very likely that amphibians return for first breeding, at the age of 2-3 years, to their native pond (Breden, 1987). Adult pool frogs are also strongly attached to their breeding ponds (Sjögren, 1994). In our experiment we were able to register the preference for native pond odour in frogs nearly 2 years old. Thus it is very likely that frogs use native pond odour for spatial orientation in natural conditions.

Our data confirm the possibility of memorization and use of odorants in a pool frog. Frogs memorize different odorants during larval development. No visible reward is necessary for such learning. The periods of learning are rather short, but the reaction to

this odour is maintained for a long time after metamorphosis. All the facts indicate that olfactory imprinting which was discovered earlier in fish (Hasler and Scholz, 1978), reptiles (Grassman, 1993) and mammals (Hudson, 1999) also exists in amphibians.

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