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Proliferative Activity of Corneal Epithelium and Specific Features of Morphogenesis in Postmetamorphic *Rana arvalis* Nilss. in Urbanized Areas

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Abstract—The mitotic activity and sizes of corneal epithelial cells in postmetamorphic *Rana arvalis* were studied in the populations exposed to different degrees of anthropogenic impact in the city of Yekaterinburg. The studies were conducted at the initial stages of frogs' terrestrial life in three consecutive years. Mitotic activity exhibited a slight positive correlation with the liver index, which indirectly reflects the general metabolic rate in young frogs. The combined estimation of proliferative activity, epithelial cell size, and relative liver weight had the highest information value. There was a relationship between the mitotic index and liver index in young frogs from habitats with the highest level of anthropogenic transformation. This indicates that morphogenesis is well-balanced and is likely to decrease the probability of morphological abnormalities in frogs developing in the unstable environment. This physiological property allows the populations to exist and reproduce in urbanized areas.

Key words: amphibians, *Rana arvalis*, proliferative activity, morphophysiology, morphogenesis.

The biological characteristics of amphibians, which develop in water (outside the mother's organism) since fertilization, determine their considerable dependence on environmental conditions. Animals with various morphological abnormalities are common in amphibian populations (Borkin and Pikulik, 1986; Hebard and Brunson, 1963; Talvi, 1993). The abnormalities are often induced by byproducts of human activities, such as various pollutants. All abnormalities may be divided into two main groups: hereditary and acquired. The latter develop in the course of regeneration or ontogeny. The abnormalities resulting from developmental disturbances and atypical regeneration are largely determined by pollutant-induced inhibition or activation of the thyroid function. This leads to the suppression of proliferation and morphogenesis during larval development and organ regeneration (Syuzyumova, 1985) and affects the metabolic rate (Tokar' *et al.*, 1991).

Cytological parameters reflect the physiological state of the organism and result from its interaction with the environment. Tissue mitotic activity is an indicator of the organism's general activity and physiological state (Sokolov and Kuznetsov, 1978). The species and geographic specificity of mitotic activity in amphibians is known (Gatiyatullina, 1978). Effects of hormones, especially thyroid hormones, on mitotic activity have also been studied (Dournon and Chibon, 1974).

Amphibian development, including metamorphosis, is an ordered sequence of events. This sequence is partly determined by a gradual increase in the activity of the thyroid and sensitivity of different systems to the hormones secreted by it. Morphogenesis and regeneration are mainly governed by the pituitary–thyroid axis (Fischman, 1996; Menon *et al.*, 1996). Thyroid hormones are mainly catabolized in the liver and kidneys. In homoiotherms, thyroxin has a calorigenic effect related to an increase in oxygen consumption by most tissues. Poikilotherms are insensitive to the calorigenic effect of thyroid hormones (Gorbman and Bern, 1962). Administration of thyroid hormones to tadpoles of various amphibians (before metamorphosis) does not affect oxygen consumption. However, thyroid hormones substantially increase oxygen consumption in adult *Rana pipiens* (Warren, 1940); if the animals are kept at a temperature of 13°C or higher, the their body weight decreases.

Metabolic rate may be estimated directly, using physiological methods (based on oxygen consumption), or indirectly, using either morphophysiological methods (by determining the liver index) or cytological methods (by analyzing tissue mitotic activity).

We attempted to apply estimation of tissue proliferative activity, along with some other parameters, to ecological studies on the specificity of population processes in the recent biota. This makes it possible to estimate the significance of changes at the cell level in amphibians from populations exposed to different types of anthropogenic impact.

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MATERIALS AND METHODS

The material was collected during studies on the natural populations of amphibians living within the city limits. We distinguished four zones differing in the anthropogenic impact on amphibian populations. The subdivision into zones was based on the extent of housing development (average number of stories, density, etc.), general development of the area, and pollution level. The city area was divided as follows: zone I, the city center with multistory buildings, massive asphalt pavement, water bodies polluted by industrial wastes, and small rivers or creeks enclosed in tubes; zone II, areas with multistory buildings and areas under development adjacent to the city center; zone III, areas with low buildings, mainly private houses with garden plots, city outskirts, vacant grounds, and parks; often the biotopes of this zone bordered on forest parks; zone IV, the park-forest belt of the city. A population living in a forest 23 km away from Yekaterinburg served as the control group. Results of hydrochemical analysis of the main spawning grounds in the habitats studied confirmed the adequacy of this subdivision. Several amphibian populations lived in each zone. The object of this study was *Rana arvalis* Nilss., which is abundant in Yekaterinburg and suburbs. The material for cytological studies was collected within the city limits from 1995 to 1997. In 1997, we also estimated mitotic activity in postmetamorphic frogs grown in the laboratory from eggs collected in the same populations.

The eye cornea was used for cytogenetic analysis. The corneal epithelium is convenient for cytological analysis because it contains no blood vessels, and metabolites are supplied to the cells uniformly, through the basement membrane. This tissue fulfills a protective function and is in direct contact with environmental pollutants. The protoplasmic growth in such tissues may be measured by counting dividing cells per unit area in a population or a sample of animals (Ebert, 1968).

Carnoy's liquid was used for fixation (Kal'yuste, 1968; Lillie, 1969). Total preparations were made using the standard method (Epifanova, 1965) and stained with hematoxylin according to Bemer (Roskin and Levinson, 1957). To estimate the cell density when studying tissue growth, we counted cells within the microscopic field limited by a rectangular diaphragm (3025 μm^2) and calculated the mean size of an epithelial cell (in μm). The mitotic activity was determined in the same preparations. The number of mitoses was counted in 50 microscopic fields (3025 μm^2 each) at the total magnification of 1350×. The preparations were examined under a Biolam D.13 microscope with a 90× immersion objective lens and a 10× eyepiece, along two mutually perpendicular diameters. The mitotic index (MI) was expressed as the number of mitoses per thousand cells.

In addition to cytological parameters, we used the heart and liver morphophysiological indices (Shvarts *et al.*, 1968). Regression relationships between MI and these parameters were analyzed.

RESULTS AND DISCUSSION

The data obtained demonstrated considerable interannual and interpopulation differences, which were determined by the specific conditions of each year and the characteristics of the habitats. In 1995, the corneal epithelium of postmetamorphic *R. arvalis* from the park-forest zone exhibited the highest mitotic activity ($F = 11.84$, $p \ll 0.01$). The populations from the zone of multistory buildings and the control area did not differ significantly with respect to this parameter. In 1996, the MI increased with an increase in urbanization level of the habitats (6.7, 7.3, and 10.1‰). In 1997, the MI was higher in young frogs from the city than in control animals (Table 1), which might be explained by higher monthly average temperatures in the urban habitats (Vershinin, 1997).

It is known (Odum, 1975) that the average air temperature in the centers of large cities is higher than at the outskirts by 1–2°C. The water temperatures in water bodies of Yekaterinburg exhibit the same trend (Vershinin, 1983, 1997). The average water temperatures in frog spawning grounds in May were 3°C higher in zones II and III than in zone IV and outside the city ($p < 0.0001$); the respective minimum temperatures differed by 0.5–1°C (Table 2). The mitotic activity of corneal epithelium is known to depend on temperature, larval population density, and some other factors (Li, 1963; Dournon and Chibon, 1974; Gatiyatullina, 1975, 1978; Syuzyumova, 1985). Decrease in temperature may cause a 1.5-fold decrease in the MI in young *R. arvalis*, whereas larval development at a high population density may cause a 1.9-fold increase in this parameter.

The MI of the corneal epithelium of young frogs that developed at a constant temperature of 20°C in the laboratory (in 1997) differed from MI estimated in natural populations in the same year. In all laboratory groups, the MI was lower than in the corresponding field groups; it was the lowest in the laboratory frogs originating from the zone of multistory buildings (Table 1).

We found a weak significant regression relationship between the MI and the liver index. The regression coefficients (R) for this relationship in the field material from zone II were 0.258 ($p = 0.012$), 0.317 ($p = 0.0127$), and 0.295 ($p = 0.046$) in 1995, 1996, and 1997, respectively. In the laboratory material, this relationship was found for the groups originating from zone III ($R = 0.73$, $p = 0.011$) and from the control population ($R = 0.67$, $p = 0.0022$).

The MI demonstrated a significant relationship with the liver weight only in the laboratory groups (in 1997)

Table 1. Mitotic activity in the corneal epithelium of young frogs (1995–1997)

1995	Zone	*N*	*L*, mm	MA	MI, ‰	*S*, mm^2
	II	43	17.5 ± 0.27	21.7 ± 0.96	7.61 ± 0.5	74.29 ± 0.79
	III	No data				
	IV	22	15.9 ± 0.36	27.5 ± 2.68	9.96 ± 0.7	73.95 ± 1.87
	Control	28	15.0 ± 0.23	22.3 ± 1.54	7.82 ± 0.6	74.3 ± 1.34
1996	II	11	16.6 ± 0.18	35.7 ± 2.3	10.1 ± 0.68	59.3 ± 0.66
	III	No data				
	IV	30	16.4 ± 0.16	24.9 ± 1.36	7.27 ± 0.41	61.6 ± 0.35
	Control	21	14.3 ± 0.15	21.2 ± 1.49	6.55 ± 0.43	65.6 ± 0.46
1997 (field)	II	46	15.8 ± 0.28	22.2 ± 2.3	6.8 ± 0.71	65.0 ± 1.29
	III	23	14.1 ± 0.42	16.5 ± 1.54	5.47 ± 0.52	69.9 ± 1.0
	IV	63	15.0 ± 0.22	24.9 ± 1.54	7.99 ± 0.51	67.5 ± 0.73
	Control	18	15.3 ± 0.45	15.4 ± 1.9	4.97 ± 0.57	69.5 ± 1.25
1997 (laboratory)	II	28	13.6 ± 0.24	8.1 ± 0.82	3.06 ± 0.31	79.3 ± 0.58
	III	11	13.5 ± 0.32	12.36 ± 2.1	4.58 ± 0.8	78.0 ± 0.78
	IV	13	12.8 ± 0.59	12.4 ± 1.56	4.52 ± 0.54	77.0 ± 0.83
	Control	18	13.8 ± 0.37	11.2 ± 1.8	4.15 ± 0.68	78.4 ± 1.7

Note: *S* is the cell area; MA is the number of mitoses in 50 microscopic fields.

of young frogs originating from the control population ($R = 0.68$, $p = 0.00186$) and from zone II ($R = 0.369$, $p = 0.05$).

When we analyzed pooled data for all years, the MI and liver index in frogs from the zone of multistory buildings exhibited a weak but highly significant relationship ($R = 0.31$, $p = 0.0014$), whereas there was no significant relationship between the MI and liver weight ($R = 0.191$, $p = 0.0057$). In the field population of zone II and the laboratory population originating from this zone, the values of MI in 1997 differed from those for other zones ($p = 0.05$ and $p = 0.0087$, respectively). In general, the patterns of zonal changes in MI were similar, except for the animals from the zone of multistory buildings (field samples), where this parameter was higher than in the zone of smaller houses and in the control area. This is most probably related to selective elimination of animals with low metabolic rate and, hence, low MI from natural populations (Pyastolova and Vershinin, 1999).

Table 2. Monthly average temperatures at the initial stages of amphibian development in zones with different levels of urbanization (pooled data for 1980–1997)

Zone	Temperature, °C		*N*
	average	limits of variation	
II	15.33 ± 0.39	4.5–28.5	155
III	14.05 ± 0.47	5.0–27.0	96
V	11.09 ± 0.38	4.0–28.0	180
Control	12.39 ± 0.52	4.0–28.0	91

Thus, young frogs from zone II exhibited a slight but statistically significant relationship between the MI and liver index (p fell within the interval 0.0012–0.046).

We revealed no zonal differences in the sizes of corneal epithelial cells in the *R. arvalis* that underwent metamorphosis in 1995. The differences might be smoothed due to the considerable decrease in the sizes of water bodies and increase in larval population densities by the time when metamorphosis was completed because of an unusually dry summer.

In 1996, corneal epithelial cells of young frogs from habitats with different levels of urbanization significantly differed in size ($F = 3.64$, $p = 0.015$) (Table 1). The smallest cells were found in *R. arvalis* populations exposed to the strongest anthropogenic impact: the average cell areas were 65.2, 61.5, and 59.6 μm^2 in the control population, the population from the forest-park zone, and the population from zone II, respectively. In 1997, the cells of young frogs from the natural population of zone II were also the smallest ($F = 3.55$, $p = 0.016$), whereas the cells of laboratory frogs were slightly larger than in other zones (Table 1). This was apparently related to the rapid growth of the frogs in the zone of multistory buildings, where the young of the year usually had the largest sizes (Vershinin, 1983). Young frogs from the urban populations were characterized by a larger body size and smaller corneal epithelial cells compared to the control population. Cell sizes in young *R. arvalis* from zones with different levels of

Table 3. Frequencies (%) of morphological abnormalities in young *R. arvalis* (1995–1997)

Zone	Field								Laboratory	
	1995	*N*	1996	*N*	1997	*N*	average	*N*	1997	*N*
II	23.4	158	18.2	11	4.44	90	16.6	259	0	60
III	No data				6.52	92	6.52	92	23.3	30
IV	3.03	33	7.5	40	7.14	252	6.7	325	6.67	30
Control	1.59	126	0	32	0	56	0.94	214	0	30

urbanization did not differ significantly from one another, except that they were smaller in zone II than in the control area. The dependence between body size and epithelial cell size has not been found earlier (Gatiyatullina, 1978).

The frequency of various morphological abnormalities is known to increase in the urban environment (Vershinin, 1982, 1989, 1995). It is the highest in the populations from the zones of multistory buildings, i.e., under the unstable conditions of anthropogenic landscapes, where the probability of disturbances in ontogeny or regeneration is relatively high. Table 3 shows the frequencies of morphological abnormalities in young frogs grown in the laboratory and sampled from different zones of the city.

As noted above, abnormalities of development and regeneration often result from the effects of pollutants on the activity of the thyroid, which is responsible for the control of cell division and morphogenesis. In other words, pollutants disturb the balance of cell growth, proliferation, metabolic rate, and the activity and catabolism of thyroid hormones. Probably, only the frogs with strictly balanced MI and liver index survived in the zone of multistory buildings, which determined the observed relationship between these parameters. This physiological property allows the frogs to live and reproduce under conditions of strong anthropogenic impacts. This suggestion is confirmed by the absence of abnormalities in the tadpoles and young frogs that developed in the laboratory (in clean water) from eggs collected in zone II.

Studies on the survival of *R. arvalis* eggs yielded similar results. Laboratory experiments demonstrated a high tolerance of *R. arvalis* embryos from the populations of zone II. When these eggs were placed into clean water, the embryonic survival rate increased to 93.6–96.7% ($p < 0.001$, $\chi^2 = 152.19$), i.e., to values considerably higher than those for the control population (32.4–78.8%) (Vershinin and Trubetskaya, 1992). This suggests that adaptive changes have occurred in city frog populations.

Thus, morphological and physiological specificity and the balance between it and cellular and tissue processes are characteristic features of populations living in anthropogenically transformed areas. Apparently, these characteristic features favor the survival of new generations in the polluted and urbanized environment.

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