Zoodiversity, 59(4):349–356, 2025

DOI 10.15407/zoo2025.04.349
UDC 597.851:504.064

SPECIES CHARACTERISTICS OF ANTHROPOGENIC POLLUTION EFFECT IN THE *PELOPHYLAX ESCULENTUS* WATER FROG COMPLEX (ANURA, RANIDAE). CHANGES IN THE HISTOSTRUCTURE OF THE LIVER

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urn:lsid:zoobank.org:pub:AA74D522-CB17-478C-B3FA-49601F89D488

Species Characteristics of Anthropogenic Pollution Effect in the *Pelophylax esculentus* Water Frog Complex (Anura, Ranidae). Changes in the histostructure of the liver. Akulenko, N. M. — The paper examined the specific features of hepatocyte damage, parenchymal repair processes and pigment accumulation in the livers of water frogs (*Pelophylax esculentus* complex) from a moderately polluted agrocenosis. It was demonstrated that hybrid individuals exhibit significant differences from their parental species with regard to the peculiarities of pigment metabolism in the liver. All hybrid frogs have a low pigment content in their livers. Additionally, the hybrid frog sample has a significantly higher proportion of animals with degraded connective tissue. The parental species do not differ in these parameters. The features of genetic material transfer that cause hybrids to differ reliably from parental species in terms of the presence of histological changes in the liver are discussed.

Key words: *Pelophylax esculentus* water frog complex, pollution, liver pigments, species differences.

Introduction

The water frogs (*Pelophylax esculentus* complex) represents a very interesting evolutionary group formed through intricate genetic and ecological processes, making it an exceptional subject for study among amphibians. In particular, among the am-

phibians of Ukraine, this complex stands out for its high resistance to anthropogenic influences. How extent genetic heterogeneity is related to ecological resilience is an understudied question. It has been shown that with increasing anthropogenic influence, the genetic composition of the population usually changes (Nekrasova, 2002, Nekrasova, Mezhzherin, Morozov-Leonov, 2004, Mezhzherin et al., 2023). In the case of weak pollution, Pool Frog (*Pelophylax lessonae*) and Marsh Frog (*Pelophylax ridibundus*) form mixed populations with a share of hybrid individuals (populations of the R–E–L type). Populations consisting of one of the parental species and hybrids (populations of R–E and L–E types) are often found. The latter is possible because hybrid individuals, as a rule, form gametes with a haploid set of chromosomes belonging to one of the parental species. The second set is thrown out during the formation of gametes; during such gametogenesis, crossing over, i. e., the exchange of chromosome sections, usually does not occur. So, hemiclonal heredity takes place, i. e. the haploid set of chromosomes obtained from the hybrid parent is transmitted to the offspring in an unchanged form, without further splitting of traits (Plenet et al., 2005, Shabanov et al., 2006; Dubey et al., 2019; Mezhzherin et al., 2023). In particular, the study of Kyiv and its surroundings showed that on the outskirts of Kyiv, among a large number of agrocoenoses, populations of the R–E–L type prevail. Populations of the R–E type predominate in the forest park zone of Kyiv. Only populations of Marsh Frogs (R–type) are found directly among urban buildings in Kyiv. This distribution is not random, but reflects the differentiated resistance of water frog to anthropogenic pollution. Hybrids mostly replace a share of Pool Frogs and are themselves subsequently replaced by populations consisting of Marsh Frogs (Nekrasova, 2002.)

Whether there is a connection between the complex genetic structure of water frog populations and their resistance to anthropogenic influences is an unanswered question. Its solution requires a complex approach and the study of various aspects. There is a point of view that the emergence of hybrid systems is a destructive process that should be fought (Christiansen et al., 2005; Quilodrán, Montoya-Burgos, Currat, 2015). However, taking into account the stability of hybrid populations in difficult environmental conditions, it is worth assuming in populations with a complex structure the presence of certain adaptive capabilities. Some evidence suggests that hybrids may be more resistant to certain parasites (Pascolini et al., 2003) and certain contaminants (Bucci et al., 2000; Prokić et al., 2016). We want to test the possibility that the hemiclonal mode of inheritance provides new opportunities for the survival of the species in conditions of anthropogenic pollution. This report examines the frequency of histological changes in the liver of water frog from a moderately polluted biocenosis depending on their species.

Material and Methods

The research was conducted on a sample of water frogs (*Pelophylax esculentus* complex) from a moderately polluted agrocenosis. The sample included Marsh Frogs (*Pelophylax ridibundus*) — 10 specimens; Pool Frogs (*Pelophylax lessonae*) — 8; and hybrid forms — 9 (Akulenko, Zhalai & Nekrasova, 2012). The level of anthropogen-

ic pollution of a particular area depends on the combined effect of many pollutants. Therefore, we determined it taking into account the physiological effect on frogs. All specimens showed pathological changes in the liver. However, the most reliable criterion for the severity of liver damage is the altered content of pigment inclusions (Akulenko 2015, 2021). In this case, the average content in the general group is close to normal. There are several animals with hyperpigmentation, which is a compensatory, not a pathological change. The population is numerous, it has many large (i. e. old) animals. Pollution that causes changes in the liver of all sexually mature animals, but does not affect the population size and lifespan, can be defined as moderate. A sample of 9 Marsh Frogs specimens, caught in an ecologically unpolluted zone, served as a control. All individuals were sexually mature and close to the maximum possible size (9–10 cm body length, for *Pelophylax ridibundus* and hybrids and 6–8 cm body length for *Pelophylax lessonae*.) The number of pigment inclusions depends on the season (Akulenko, 2012), therefore, animals caught in August-September, when animals recovered after participating in reproduction, were taken. The studies were carried out on sections stained with hematoxylin and eosin. The area of pigment inclusions was calculated using a glass mesh and the data were statistically processed using standard methods (Akulenko, 2016). The presence of changes in the liver histostructure was determined by the presence-absence criterion and the data were statistically processed using standard methods for alternative features. Statistical processing was performed using a method suitable for qualitative characteristics. Comparison of histological changes in the samples was made according to the presence-absence criterion, which is used to analyze qualitative characteristics (Akulenko 2015). To analyze the results of each sample, average errors, reliability of differences with the control and between samples were calculated. Calculations were carried out in Microsoft Excel according to a previously developed methods (Akulenko, 2015, 2016).

Results

Analysis of the results shows significant species differences in the response of cellular and non-cellular elements of the liver to anthropogenic pollution (Tables 1, 2). A significant difference between Marsh Frogs and others is the presence of pronounced foci of fatty degeneration of hepatocytes. This seems surprising because Marsh Frogs are more resistant to urban pollution, which consists mainly of fat-soluble substances. Meanwhile, the presence of foci of fatty degeneration of hepatocytes is a marker for this type of contamination (Akulenko, 2015). However, in Marsh Frogs areas of fatty degeneration largely retain a clear cellular structure (Fig. 1), while in Pool Frogs and hybrid frogs they turn into foci of necrosis in which hepatocytes are destroyed (Fig. 2). Such vacuolated areas must be considered as foci of necrosis. The preservation of large areas of intact hepatocytes with fat vacuoles may, on the contrary, indicate greater Marsh Frog resistance to necrosis. It can be noted that the number of individuals with necrosis is reduced in Marsh Frogs. There are no other significant differences between Pool Frog and Marsh Frog. In the control group, individuals with foci of inflammation in the liver parenchyma were found, which may be caused

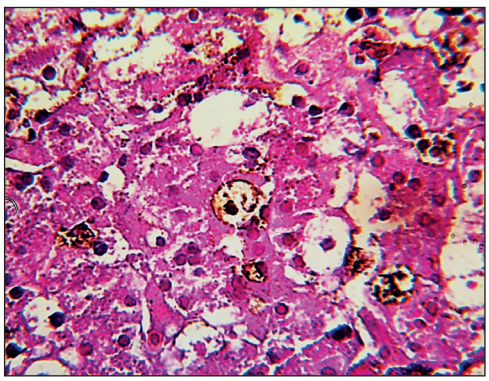

Fig. 1. Area of fatty degeneration in the liver of a Marsh Frog. Fat vacuoles are visible. Hematoxylin and eosin staining. Magnification 200

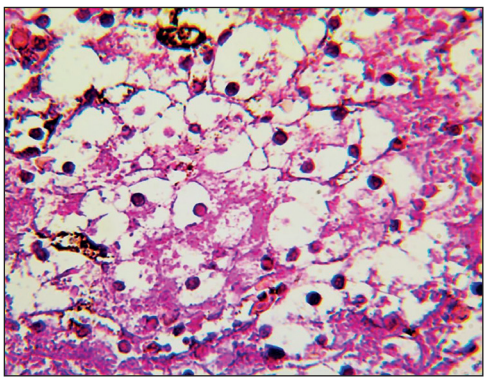

Fig. 2. Area of fatty degeneration of hepatocytes, turning into necrosis in the liver of a Pool Frog. Hematoxylin and eosin staining. Magnification 200

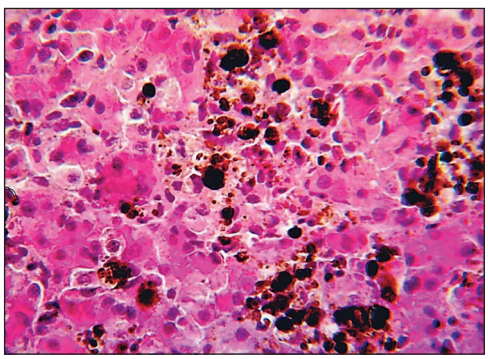

Fig. 3. Large melanomacrophage accumulations with significant loss of pigment inclusions in the liver of a Marsh Frog. Dark melanin inclusions are visible. Some cells are filled with melanin. In others, small inclusions are observed, that is, remnants of melanin. Hematoxylin and eosin staining. Magnification 200

by infections and invasions. Other pathological changes in liver of the frogs from control group are absent (Table 1).

Hybrid individuals exhibit significant differences from both parental species (Tables 1, 2). The content of pigments in the liver of individuals with different genetic backgrounds deserves special attention. Individuals with liver hyperpigmentation were found in Pool and Marsh frog samples; according to our assumptions, the increase in the area of pigment inclusions in the liver (hyperpigmentation) is an adaptive response to pollution. When the compensatory capabilities of the system are exhausted, the next phase begins — hypopigmentation (Akulenko, 2015, 2016.) Among the parent species there are individuals with hypo- and hyperpigmentation (Table 1). Large melanomacrophage accumulations with pigment residues are also found (Fig. 3). Hypopigmentation is found in all hybrid individuals from this population. Thus, it was previously possible to assume that hybrid individuals are more difficult to tolerate toxic effects in anthropogenically altered biocenosis. However, this assumption contradicts the known facts. In polluted biocenoses, Pool Frogs are replaced by Marsh Frogs, but the hybrid part of the population is usually preserved (Nekrasova, 2002, Akulenko; Zhalai, Nekrasova, 2012, Mezhzherin et al., 2023). Thanks to hemiclonal inheritance, hybrid individuals can reproduce from generation to generation without one of the parental species (Shabanov et al., 2006), therefore, for anthropogenically polluted biocenoses, populations consisting of Marsh Frogs and hybrids are quite typical. In this population, the hybrid individuals we captured were very large. Consequently, they tolerated this level of pollution well for several years. Thus, the preliminary conclusion needed at least a thorough check.

Table 1. **Presence of histological changes in the liver of *Pelophylax esculentus* water frog complex from agrocenosis and the area of pigment inclusions, %**

Indicator	Control	Pollution (combined group)	Hybrid	Pool Frog	Marsh Frog
Number of animals	9	27	9	8	10
Thrombosis	0	29.63	33.33	42.86	18.18
Inflammation	25	37.04	22.22	57.14	36.36
Necrosis	0	81.48	88.89	85.71	72.72
Degradation of connective tissue	0	59.26	88.89	42.86	45.45
Anisocytosis	0	25.93	11.11	28.57	36.36
Presence of cords	0	18.52	11.11	14.29	27.27
Fatty degeneration of hepatocytes	0	11.11	0	0	27.27
Hypopigmentation	0	55.56	100	28.57	36.36
Hyperpigmentation	0	18.52	0	42.86	18.18
Area of pigment inclusions	1.58 + 0.49	2.01 + 0.48	0.61 + 0.07	3.08 + 0.99	2.37 + 0.6

Table 2. **Reliability of differences in the frequencies of histological changes in the liver of *Pelophylax esculentus* water frog complex from an agrocenosis**

Indicator	Control — combined group	Hybrids — Pool Frog	Hybrids — Marsh Frog	Pool Frog — Marsh Frog
Tthrombosis	$p < 0.05$	–	–	–
Inflammation	–	–	–	–
Necrosis	$p < 0.001$	–	–	–
Degradation of connective tissue	$p < 0.01$	$p < 0.05$	$p < 0.05$	–
Anisocytosis	$p < 0.05$	–	–	–
Presence of cords	$p < 0.05$	–	–	–
Fatty degeneration of hepatocytes	$p < 0.1$	–	$p < 0.1$	$p < 0.1$
Hypopigmentation	$p < 0.01$	$p < 0.01$	$p < 0.01$	–
Hyperpigmentation	$p < 0.05$	$p < 0.05$	–	–
Area of pigment inclusions	–	$p < 0.05$	$p < 0.05$	–

Also in hybrids, the proportion of individuals with pronounced degradation of connective tissue was significantly increased (Tables 1, 2). There is a mention in the literature that pigment-containing cells in the liver are associated with the utilization of collagen (Gutierre et al., 2018), but in this case the differences rather relate to the characteristics of its synthesis. According to our data, macrophages and pigment cells of the liver take an active part in the processes of liver repair after toxic lesions

(Akulenko 2021). However, among hybrid individuals, the proportion of animals with hepatocyte damage is not increased, which indicates normal reparation. Theoretically, the antioxidant role of melanin can be performed by intermediate, less polymerized products of its synthesis. Soluble compounds can also be a source of iron for enzyme synthesis. Thus, it can be assumed that the reduced amount of pigments in the liver of hybrid frogs is caused not by depletion, but by reduced activity of their synthesis. In particular, in hybrid individuals, depleted melanomacrophage clusters are not detected. In any case, the presence of reliable differences in the amount of pigments and collagen indicates the existence of peculiarities in the metabolic processes inherent in hybrids.

Discussion

Currently, the issues related to the decline in the number of tailless amphibians are still relevant and mostly do not have ready solutions (Fasola et al., 2015; Bernabò et al., 2017). Nowadays, the problems of environmental pollution have been added to the problems of climate change, which also threaten the existence of many amphibian populations (Di Rosa et al., 2006). Measures to protect amphibians are complicated by insufficient study of the physiology of amphibians, in particular, their differences from mammals. In amphibians, there are mechanisms of "emergency regeneration" of hepatocytes, which allow replacing large foci of necrosis not with connective tissue, but with young, functionally active cells (Akulenko, 2015). The functions of large pigment inclusions in the liver also require further study (Gutierre et al., 2018). The genetic heterogeneity of the synanthropic complex of *Pelophylax esculentus* complex species can be important, determining the physiological plasticity of individual populations. In particular, the absence of crossover in hybrids can ensure the presence of a complex of co-adapted genes that are not split in the process of meiosis in hybrid individuals (Akulenko, Zhalai, Nekrasova, 2012). Studies of the genetic characteristics of *Pelophylax esculentus* water frog complex populations in various biotopes are quite numerous (Plenet et. al., 2005; Bove, Milazzo, Barbuti, 2014; Mikulíček et al., 2014, Dubey et. al., 2019; Mezhzherin et al., 2023, etc.) Of particular interest are the results of Bove, Milazzo, Barbuti (2014), showing that genetic variations found in hybrids in the homozygous state are lethal. The authors justify the accumulation of mutations by statistical regularities, but we do not find this convincing. Variations in alleles that are lethal in the homozygous state may be a case of incomplete dominance and in the heterozygous state be responsible for altered metabolic features. The data of Bucci et al. (2000) indicate greater resistance of hybrids to anthropogenic pollution. However, the relationship between the genetic characteristics of animals and their physiological characteristics has hardly been studied. The data we present show that hybrid individuals from the population we examined have pronounced changes in metabolism.

Recent research (Plenet et. al., 2005; Mikulíček et al., 2014; Dubey et. al., 2019; Mezhzherin et al., 2023) has discovered self-sustaining populations consisting entirely of hybrid individuals. Unfortunately, in these works there is no analysis of the ecological situation in which fully hybrid populations exist. However, our results show the potential for hybrid individuals to transmit complex genetic combinations without

segregation. This method of transferring genetic material allows certain metabolic features to spread very quickly in the population. In the case of chemical pollution, this possibility can be important for the survival of the population. It is unclear whether the emergence of unusual forms of inheritance is an evolutionary dead end or carries some potential for development (Plenet et al., 2005; Mikulíček et al., 2014).

Conclusions

The presence of reliable differences in the metabolic features of hybrid green frogs suggests that mechanisms of hemiclonal inheritance are involved in adaptive physiological changes. These mechanisms may be particularly crucial for the survival of synanthropic species because anthropogenic transformation processes occur rapidly. The emergence of species complexes with hemiclonal inheritance allows adaptive changes to spread rapidly to a significant proportion of the population. However, disturbances in the processes of gametogenesis in hybrid individuals mean that we cannot definitively conclude that this path is an evolutionary dead end.

Acknowledgments. The author thanks E. I. Zhalai for the provided research material and O. D. Nekrasova for consultations regarding the composition of the *Pelophylax esculentus* water frog complex in various biocenoses.

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Received 12 December 2024
Accepted 21 August 2025