REPTILES & AMPHIBIANS

Anomalies in Marsh Frogs (*Pelophylax ridibundus*) and Hybrid Waterfrogs (*P. esculentus*) (Anura: Ranidae) from Two Ponds in the Kharkiv Region of Ukraine

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Anomalies in amphibians are present in natural populations in eastern Europe and have been documented for many years (e.g., Dubois 1979, 2017; Borkin and Pikulik 1986; Vershinin 1989, 2015; Borkin et al. 2012; Borkin 2014; Henle et al. 2017; Svinin et al. 2019). Anomalies can be caused by both genetic and environmental factors, with the latter including extreme and unstable conditions such as temperature, pH of water, occupying the margin of the species' range, high density of tadpoles, hormonal imbalances, water pollution, parasitic trematodes, and fungal and other infections (Vershinin 1989; Bezman-Moseyko et al. 2014). Most amphibians are extremely susceptible to environmental conditions because they develop outside of the mother in eggs lacking stiff envelopes (Neustroeva 2012). Vershinin (2015) presented a classification of anomalies based on principal causes: genetic anomalies, ontogenetic anomalies (disruption of development caused by environmental influences), regenerative anomalies (appear in anurans before metamorphosis due to faulty regeneration), and deformities caused by parasites, predators, or injuries.

Various amphibian anomalies (e.g., malformed limbs, vertebral column, and head, bone and skin outgrowths, absence or disruption of pigmentation, ocular deformation) have been documented in Ukraine (Vershinin 1989, 2015; Fayzulin 2011; Nekrasova 2012; Mikitinets 2012, 2014; Svinin 2014; Nekrasova and Kuibida 2018; Marushchak and Muravynets 2018). In the Kharkiv Region, Katrushenko (2020) examined specimens in the collection of the Kharkiv National Museum of Nature and compared the occurrence of anomalies with levels of pollution.

The Siverskyi Donets River Basin in the Kharkiv Region is a center of waterfrog diversity that has been described

Table 1. Waterfrogs (Marsh Frogs, *Pelohylax ridibundus*, and hybrids, *P. esculentus*) sampled over a three-year period from Dobrytskyi and Koriakiv Ponds in or adjacent to the National Nature Park "Homilshanski Lisy," Chuhuiv District, Kharkiv Region, Ukraine, respectively. Question marks (?) indicate sex unknown.

		Species, Sex, Ploidy									
		Marsh Frogs (*P. ridibundus*)			Hybrids (*P. esculentus*)						
Year	**Pond**	♂	♀	?	♂		♀		?		**Total**
			2n		2n	3n	2n	3n	2n	3n	
2019	Dobrytskyi	6	3	3	46	3	12	2	2	0	77
	Koriakiv	0	1	0	105	7	7	2	4	1	127
2020	Dobrytskyi	1	3	1	26	3	4	8	0	0	46
	Koriakiv	0	1	0	19	1	7	1	11	0	40
2021	Dobrytskyi	1	14	1	45	2	3	1	0	0	67
	Koriakiv	1	0	0	85	12	24	2	0	0	124
Total	**Dobrytskyi**	**8**	**20**	**5**	**123**	**8**	**30**	**11**	**2**	**0**	**190**
	Koriakiv	**1**	**2**	**0**	**229**	**20**	**43**	**5**	**15**	**1**	**291**

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by Shabanov et al. (2009, 2020). The hybrid waterfrog *Pelophylax esculentus* is represented by both diploid and triploid forms of both sexes and by one of its parental species, the Marsh Frog (*P. ridibundus*). The other parental species, the Pool Frog (*P. lessonae*), is absent. Genetic peculiarities of triploids can contribute to developmental anomalies (Spirina 2009) and these waterfrogs are consequently one of the most vulnerable groups of amphibians and have the highest levels of abnormalities (Borkin 2014; Katrushenko 2020). Herein we present the results of an investigation of waterfrog anomalies conducted during the summers of 2019–2021 as a part of the annual monitoring of population systems inhabiting two ponds in the Siverskyi Donets River Basin.

We sampled frogs from Lower Dobrytskyi Pond (hereafter "Dobrytskyi Pond"; 49°37'40"N; 36°16'58"E) and Koriakiv Pond (49°36'57.3"N; 36°18'45.2"E), which are in or adjacent to the National Nature Park "Homilshanski Lisy," Chuhuiv District, Kharkiv Region, Ukraine, respectively. During the reproductive seasons (June–July) in 2019–2021, we captured frogs (Table 1) at night, measured snout-vent length (SVL) using a caliper, and determined sex and species using morphological features (Shabanov 2015). For *P. esculentus* we also identified ploidy by measuring erythrocyte length (Ogielska-Nowak 1978; Bondarieva et al. 2012). Those individuals for whom ploidy identification by erythrocyte length was not possible were examined using karyoanalysis (Biriuk et al. 2016). We described anomalies according to the classification proposed by Nekrasova (2008) supplemented with anomalies identified by Vershinin (2015) and Katrushenko (2020).

Waterfrogs in this study are not listed in the Red Data Book of Ukraine and *P. ridibundus* is listed as being of Least Concern (LC) on the IUCN Red List (Kuzmin et al. 2009). All procedures involving animals were conducted according to the Guidelines for Use of Live Amphibians and Reptiles in Field and Laboratory Research (ASIH 2004) and The Ukrainian Law on the Protection of Animals from Cruelty, and were approved by the Committee on Bioethics of V.N. Karazin Kharkiv National University.

We identified 12 types of anomalies of limbs, digits, color, and muscles in 30 individuals from Dobrytskyi Pond and seven individuals from Koriakiv Pond (Table 2). The most frequent anomalies were brachydactyly (Fig. 1B) – 13 cases in Dobrytskyi Pond, schizodactyly (Fig. 1C) – one case in Dobrytskyi Pond, and four cases in Koriakiv Pond, and color abnormalities (curved lines, black/gray dots, etc.) (Fig. 1J) – four cases in Dobrytskyi Pond and one case in Koriakiv Pond. Of the 37 waterfrogs with anomalies, three males from Dobrytskyi Pond had more than one anomaly (with brachydactyly as a common abnormality for three of them).

We also found a vocalization anomaly in a male *P. esculentus* from Dobrytskyi Pond. It did not produce any sound while being subjected to gentle compression of the flanks to imitate amplexus. It made all the movements necessary for calling (expelling air from the lungs through the larynx to the vocal sacs while nostrils were closed and subsequently

Table 2. Anomalies detected in waterfrogs (Marsh Frogs, *Pelohylax ridibundus*, and hybrids, *P. esculentus*) sampled over a three-year period from Dobrytskyi and Koriakiv Ponds in or adjacent to the National Nature Park "Homilshanski Lisy," Chuhuiv District, Kharkiv Region, Ukraine, respectively. Asterisks (*) indicate individuals with two or more anomalies. Brachydactyly = digits reduced in length; schizodactyly = missing middle digits; syndactyly = conjoined digits; oligodactyly = missing or reduced digits; ectrodactyly = cleft manus or pes with missing digits; ectromelia (hemimelia) = long bones in limbs missing or underdeveloped; taumelia = long bones in limbs severely bent.

	Lower Dobrytskyi Pond				Koriakiv Pond			
Anomaly	**2019**	**2020**	**2021**	**Total**	**2019**	**2020**	**2021**	**Total**
Brachydactyly	6*	4*	3*	**13**	0	0	0	**0**
Schizodactyly	1	0	0	**1**	0	0	4*	**4**
Syndactyly	1	0	1	**2**	0	0	0	**0**
Oligodactyly	0	0	3*	**3**	1	0	0	**1**
Ectrodactyly	3	0	1	**4**	0	0	0	**0**
Ectromelia (hemimelia)	1	0	0	**1**	1	0	0	**1**
Taumelia	1	0	0	**1**	0	0	0	**0**
Dilated pupil	1*	0	1	**2**	0	0	1*	**1**
Color/pattern	0	3	0	**3**	0	0	1	**1**
Muscular dystrophy	3*	0	0	**3**	0	0	0	**0**
Vocalization (see text)	0	1*	0	**1**	0	0	0	**0**
Total	**16**	**7**	**8**	**34**	**2**	**0**	**5**	**8***

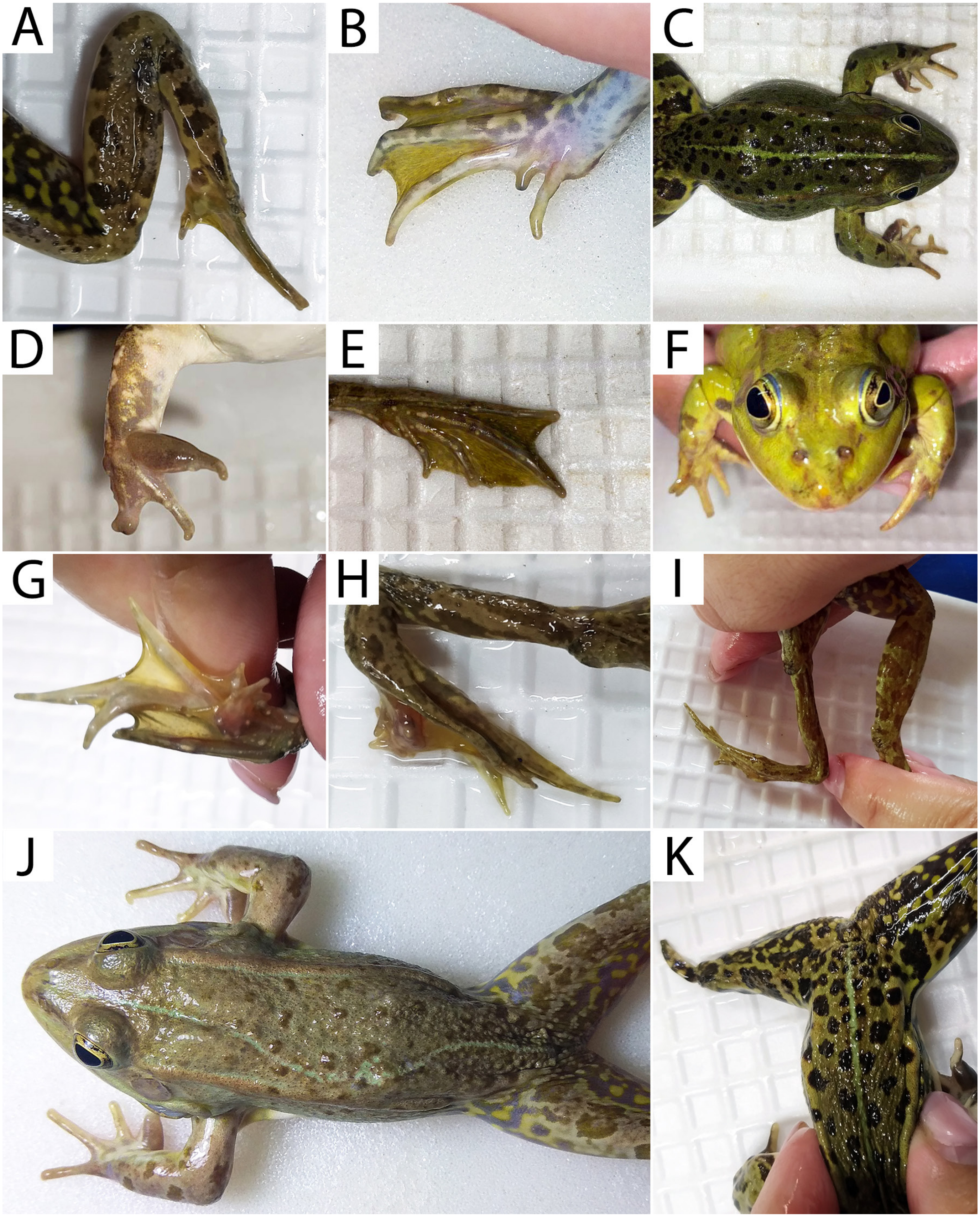

Fig. 1. Anomalies detected in waterfrogs (Marsh Frogs, *Pelophylax ridibundus*, and hybrids, *P. esculentus*) sampled over a three-year period from Dobrytskyi and Koriakiv Ponds in or adjacent to the National Nature Park "Homilshanski Lisy," Chuhuiv District, Kharkiv Region, Ukraine, respectively: ectrodactyly (A); brachydactyly (B); schizodactyly (C, F); syndactyly (D); oligodactyly (E); dilated pupil (F); taumelia (G, H); muscular dystrophy (G, H, I); color/pattern anomaly (twisted middorsal line) (J); hemimelia (K). Photographs by Anna Fedorova, Eleonora Pustovalova, Polina Verchoturova, Ksenia Pereslavska, and Hostkina Taisia.

Fig. 2. Koriakiv (left) and Lower Dobrytskyi (right) Ponds adjacent to or in the National Nature Park "Homilshanski Lisy," Chuhuiv District, Kharkiv Region, Ukraine, respectively. Photographs by Anna Fedorova.

opened) (Gans 1973) but remained absolutely mute. All of the other frogs in the sample produced typical release calls.

We observed significant differences in the portion of individuals with anomalies ($p < 0.0001$), frequency of anomalies during each year of the study ($\chi^2 = 6.97$, $p = 0.0306$), and types of anomalies ($\chi^2 = 24.05$, $p = 0.0125$) between the ponds. However, we found no significant differences in the frequency of anomalies between *P. esculentus* and *P. ridibundus* in both ponds (p = 0.1063 for Dobrytskyi Pond and p = 0.7848 for Koriakiv Pond) or between diploid and triploid hybrids (p = 0.3295 for Dobrytskyi Pond and p = 0.0903 for Koriakiv Pond).

We found a variety of bone deformations (fractures, dislocations, bone fragility, malunion, etc.) in frogs from Dobrytskiy Pond. Moreover, many frogs from that pond had noticeable problems with blood coagulation; most bled copiously and required additional attention and care after we took a blood sample. We found no bones deformations or problems with coagulation in frogs from Koriakiv Pond, suggesting that frogs in Dobrytskyi Pond might suffer nutritional deficiencies leading to disruptions of calcium metabolism and bone fragility (Densmore and Green 2007).

Based on conditions in the two ponds, we expected that frogs in Koriakiv Pond would have been subjected to more factors that could disrupt ontogenesis than those in Dobrytskyi Pond. Koriakiv Pond is located between the protected area of National Nature Park "Homilshansky Lisy" and agricultural land. A field and a road are located above the pond and fertilizers, pesticides, and other contaminants could drain into the pond. In fact, the water is completely covered by floating aquatic plants (Fig. 2), mostly duckweed in the genus *Lemna* and Common Duckweed (*Spirodella polyrhiza*), presumably attributable to fertilizers draining into the pond. On the other hand, Dobrytskyi Pond, which is clear of floating vegetation (Fig. 2), is in the core area of the park and surrounding land in the catchment area is occupied by oak (*Quercus* sp.) groves. These conditions suggest that Koriakiv Pond is more conducive for the development of parasitic fauna and mycobiota, which can increase the frequency of anomalies (e.g., Sessions and Ruth 1990; Johnson et al. 2001, 2002). Because we found more anomalies in Dobrytsky Pond than in Koriakiv Pond, additional factors affecting the development of frogs in the former remain to be identified.

Causes of any observed anomaly are difficult to determine, and most anomalies should be considered products of synergistic effects in a particular environment (Vershinin 2015; Marushchak et al. 2021). Consequently, reports of amphibian anomalies should be sufficiently informative to identify common potentially causative factors (Ohler and Dubois 2018). Also, additional data are needed to investigate the frequency of anomalies in other species of sympatric amphibians to discern any species-specific anomalies in the *P. esculentus* complex (Ohler and Dubois 2018; Vershinin 2018). Further investigations in the Kharkiv Region also are important, particularly since the presence of Rostand's anomaly or "anomaly P" (Rostand 1971) has been confirmed in waterfrogs in some parts of Ukraine (Kurtyak and Krulko 2007; Marushchak and Muravynets 2018).

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