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# A MOLLUSK *Planorbarius corneus* IS AN INTERMEDIATE HOST OF THE INFECTIOUS AGENT OF ROSTAND'S "ANOMALY P" IN GREEN FROGS

**A. O. Svinin,[1] I. V. Bashinskiy,[2] S. N. Litvinchuk,[3] L. A. Neymark,[2] A. Yu. Ivanov,[4] O. A. Ermakov,[4] A. A. Vedernikov,[1] and A. Dubois[5]**

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Here we present the first new data about the mysterious "anomaly P" of green frogs (genus *Pelophylax*) in about 50 years. We established that the gastropod *Planorbarius corneus* could be an intermediate host (or vector) of the infectious agent of the anomaly P. Symmetrical cases of polydactyly, the anomaly "cross" and heavy cases of the anomaly P, which were previously found in natural populations in the European part of Russia and recently obtained in laboratory, can be caused by this infectious agent. As the most probable cause, we assume a species of trematodes, for which the first intermediate host is *P. corneus*, from which they infest tadpoles of green frogs.

**Keywords:** Amphibia; Anura; *Pelophylax*; anomaly P; mollusks; *Planorbarius corneus*; trematode invasion.

## INTRODUCTION

In 1952, the famous French biologist Jean Rostand (1894 – 1977) found mass anomalies in green frogs of the genus *Pelophylax* Fitzinger, 1843 (Rostand, 1952a, 1959, 1971; Dubois, 2017). He called them "anomaly P". It consists in a polymorphic syndrome, which includes symmetrical cases of polydactyly, polyphalangy, brachymely, flexion of the hindlimbs and various tumours and excrescences (Rostand, 1952a, 1959; Dubois, 2017). In search of causes, Rostand experienced a large number of factors, and only after 20 years of research he managed to make a significant discovery: the anomaly P was obtained experimentally by feeding tadpoles of green frogs with contents of the intestines of two fish species (Rostand and Darré, 1969; Rostand, 1971; Dubois, 2017). Rostand suggested that the anomaly P could be caused by some infectious agent transmitted by fish, which he hypothesized to be a teratogenic virus with a temporary effect on limb development (Rostand, 1952; Dubois, 2017).

Recently, we found Rostand's anomalies at a huge distance from previously known localities in Europe and Africa (France, Morocco, and Netherlands). In 2016, green frogs with the anomaly P were found in the "Privolzhskaya Lesostep" Nature Reserve located in the European part of Russia (Svinin et al., 2018). In this paper, we describe the cases of the anomaly P, which were obtained in the laboratory after rearing tadpoles of *Pelophylax esculentus* (Linnaeus, 1758) together with the snail *Planorbarius corneus* (Linnaeus, 1758).

## MATERIAL AND METHODS

In September 2017, a female of *Pelophylax lessonae* (Camerano, 1882) was caught in a pure population of this species in waterbodies near Novotroitsk settlement, and a male of *Pelophylax ridibundus* (Pallas, 1771) was caught in a pure population of that species in the forest park "Sosnovaya Rozsha" near Yoshkar-Ola city (Mari El Republic, Russia). In none of these two populations the anomaly P had so far been observed in natural conditions. The frogs were placed in plastic containers in a refrigerator

[1] Mari State University, Lenina sq., 1, Yoshkar-Ola, 424000, Russia.

[2] A. N. Severtsov Institute of Ecology and Evolution, Russian Academy of Sciences, Leninskiy pr. 33, Moscow, 119071, Russia.

[3] Institute of Cytology, Russian Academy of Sciences, Tikhoretsky pr. 4, St. Petersburg, 194064, Russia.

[4] Penza State University, Krasnaya str. 40, 440026, Penza, Russia.

[5] Muséum National d'Histoire Naturelle, Institut Systématique, Evolution, Biodiversité, 25 rue Cuvier, CP 30, Paris, 75005 France.

for artificial hibernation at 4 – 6°C for two months. They were taken from the hibernation place at the end of October 2017. Surfagon (Mosagrogen©, synthetic analog of luteinizing hormone-releasing hormone) was injected into the lymphatic sacs of both specimens. After female oviposition of some unfertilized eggs in the container water, we obtained some eggs from the female by pressure of the flanks. The eggs were fertilized with a suspension of spermatozoa obtained from testes of the male according to the method of artificial fertilization suggested by Berger et al. (1994). From all fertilized eggs, only 13 individuals survived till Gosner's (1960) stage 18, whereas at the time of hatching (stage 19) only eight tadpoles were alive. These 8 specimens were diploid *P. esculentus*, with one *ridibundus* and one *lessonae* genome. Parental specimens and offspring were identified using PCR in the Laboratory of Molecular Ecology and Animal Systematics at the Department of Zoology and Ecology of the Penza State University, using standard techniques (Ermakov et al., 2013). For identification of green frog species, we used a nuclear DNA marker (SAI-1), which has been used in previous studies of green frogs (Akin et al., 2010).

After hatching from eggs, the tadpoles were raised in a 60-liter container with 31 large specimens (average shell length 26.7 ± 0.592; range 19 – 30 mm) of the water snail *Planorbarius corneus* (Linnaeus, 1758) (Mollusca, Gastropoda), caught in the forest oxbows in "Ostrovtsovskaya Lesostep'" (a part of the "Privolzhskaya Lesostep'" Nature Reserve; see Svinin et al., 2018), where heavy forms of the anomaly P have been observed. The mollusks were transferred twice to new clean water after capture and before experiment. The water in the aquarium was taken in Yoshkar-Ola, Mari El, and was not changed during the first month of the experiment. Then all surviving mollusks and tadpoles showing the anomaly P were displaced and the water was completely changed. The tadpoles were fed with prepared aquarium fish feed. The temperature in the container was 21 – 23°C. The duration of the experiment was 98 days (from November 3, 2017, to February 9, 2018, when the last tadpole passed metamorphosis).

As a control, in the autumn of 2016 and the summer of 2017, tadpoles of the three green frogs species *P. lessonae* ($n = 130$), *P. ridibundus* ($n = 32$), and *P. esculentus* ($n = 121$) from some localities in Mari El Republic (*P. ridibundus* forest park in Yoshkar-Ola city "Sosnovaya Roshcha," *P. esculentus* and *P. lessonae* from Kuguvan settlement, Pin'zhedur village and Krasnooktyabrskiy settlements), in which we have never seen the anomaly P, were reared under identical conditions but without mollusks. They developed normally and did not show a single case of the anomaly P.

We performed the amputation of a hindlimb in a tadpole as follows: the tadpole on stage 35 which had the first features of the anomaly P (enlarged limb buds) was taken out of water and cut the left limb (Fig. 1).

## RESULTS

On day 7 after fertilization, and after hatching, mollusks were introduced in the aquarium with the frog larvae at stage 19. On day 31 of development, the hindlimbs' buds of tadpoles looked swollen. On the 38$^{th}$ day of development, it was possible to observe the presence of developing deformations in two of three of the fastest developing tadpoles. The hindlimbs of the third tadpole looked normal. However, later, symmetrical polydactyly was found in this tadpole. It was therefore possible to notice the initial manifestation of severe forms of the anomaly P since stage 27.

We amputated a newly formed hindlimb in one of the tadpoles at stage 35. Isolated from mollusks at this stage up to metamorphosis, it had the anomaly P, expressed in development of outgrows ("spurs") and a small paw on the distal part of the limb. However, the limb did not regenerate, except for the proximal part of the femur.

All eight tadpoles obtained had deformed hindlimbs. However, three of them (37.5%) showed mild forms of the anomaly P: symmetric and asymmetric cases of polydactyly. The other five had heavy forms of the anomaly P. All tadpoles that passed metamorphosis had a specific character, which was called by us a "cross" and was met previously by us in natural populations (Svinin et al., 2018: Fig. 2*d*). It is interesting to note that the "cross" was observed in several individuals of green frogs from natural populations in which no other abnormalities of limbs were recorded.

## DISCUSSION AND CONCLUSION

The previous works on the anomaly P, mostly by Rostand (1971), reviewed by Dubois (1979, 1984, 2018), allowed establishing a rather good "portrait" of this syndrome, including in particular the following six pieces of information:

[I1] This syndrome has so far been established with certainty only in species belonging to the genus *Pelophylax*, but in this genus in at least 5 different species (Dubois, 2017: 53) living in different parts of the Palearctic region. The other amphibian species living in sympatry in the same localities never show any symptom of this syndrome.

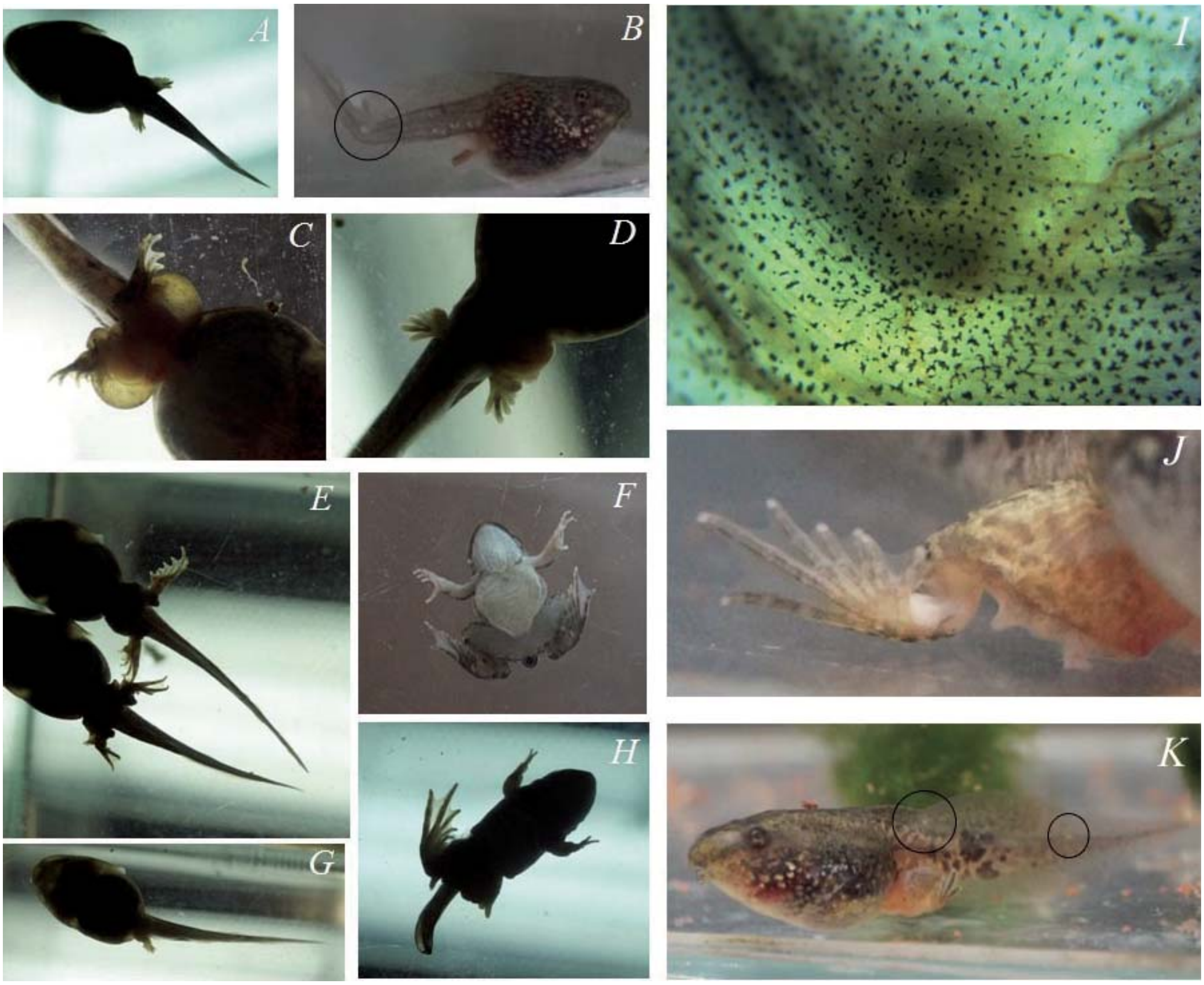


**Fig. 1.** Tadpoles of *Pelophylax esculentus* with the anomaly P: *A*, *B*, *C*, *E*, *G*, *H*, individuals with severe forms of the anomaly P; *F*, a specimen with mild form of the anomaly P (polydactyly); *G* and *H*, experiment with hindlimb amputation; *B*, metacercaria of trematode in skin of tadpole (in the course of developing a severe form of the anomaly P) that led to tail deformation; *I*, cysts from tadpole under the letter *B* (×400 magnification); *J*, hindlimb with polydactyly, additional mini-limb and spur; *K*, tadpole with two cysts (trematode metacercariae) in tail.

[I2] Where it occurs, the anomaly usually concerns a large proportion of the larvae, but the larvae showing the most severe symptoms usually do not survive to metamorphosis, so that if only the adults of a population are surveyed only the mild forms of the anomaly (mostly polydactyly) are observed.

[I3] The anomaly P syndrome covers a variety of anomalies that concern mainly the limbs (polydactyly, hyperphalangy, brachymely, polymely, bony excrescences, etc.), as well as, in the severe forms, the tissues of the inguinal region (tumours). Among the morphological characters of the anomaly (Dubois, 2017: 50), the following are diagnostic of the latter: [a] not only the hindlimbs, but also the forelimbs, can show polydactyly; [b] the structure of the polydactylous hindlimbs is particular: the most axial additional toe is usually longer than the first "normal" one, giving the foot a very special aspect (see Dubois, 2017: Fig. 2); [c] a good, although not always perfect, bilateral symmetry is always observed, the number of additional digits being always roughly the same on both sides, with sometimes one more toe or finger on one side, but never an excess of several digits on one side (e.g., 5 – 6, 6 – 7, 7 – 7 or 7 – 8 toes, but never 5 – 7 or 6 – 8); and [d] the anomaly shows gradients of severity, including a postero-anterior one (the forelimbs being affected by polydactyly only if the hindlimbs show more than 6 toes) and the fact that the most severe forms of the syndrome (such as brachydactly, polymely, bony excrescences and tumours) are present only in frogs already affected by postero-anterior polydactyly.

[I4] Crosses carried out in controlled laboratory conditions among frogs affected by the anomaly P or between them and "normal" specimens never resulted in transmission of the anomaly to the offspring, thus allowing to dismiss the possibility of a genetic basis of the anomaly.

[I5] Experimental unilateral amputation of hindlimb buds in tadpoles affected by the anomaly P resulted in the regeneration of normal limbs, while the other limb developed as polydactylous, thus showing that the teratogenic agent responsible for the anomaly was active only in the first stages of development.

[I6] Just hatched tadpoles fed exclusively at the beginning of development with feces from fish captured in a lake where the anomaly P was present developed very severe forms of the anomaly P, thus showing that the infectious teratogenic agent in question, whatever it is, was present in these feces. Although Rostand (1971) sticked to the hypothesis of M. Caullery (in Rostand, 1952) that this infectious agent might be a virus, no data supported this hypothesis.

In the meanwhile, new data were obtained, especially in North America (see review in Henle et al., 2018). Some trematodes are now known to be liable to cause limb anomalies in amphibians (Johnson et al., 1999). For example, Session and Ruth (1990) showed that high incidence of polymely in the frog *Hyliola regilla* (Baird and Girard, 1852) and in the salamander *Ambystoma macrodactylum* Baird, 1850 was associated with high concentration of metacercariae at the proximal part of additional limbs. Johnson et al. (1999) showed that the trematode *Ribeiroia ondatrae* Looss, 1907 may lead to the development of additional limb development and other limb anomalies. Metacercariae of trematodes were later shown to cause development of additional or abnormal limbs in about 60 amphibian species from 46 states in the USA (Johnson and Sutherland, 2003). Rediae of *Ribeiroia ondatrae* develop in freshwater mollusks as their intermediate hosts, in which they reproduce asexually. Free-swimming larvae (cercariae) of the worm are formed after penetrating tadpoles through the skin and becoming encysted (metacercariae). These metacercariae produce retinoid acid, which acts as a morphogen in the development of hindlimbs and may induce development of additional or abnormal hindlimbs in amphibian larvae (Johnson and Sutherland, 2003). Rohr et al. (2009) suggested that agrochemicals may play an indirect role on frogs' development through immunodepression, making tadpoles more sensitive to the action of trematodes.

So far, the possibility that the anomaly P could be caused also by trematodes does not seem to have been explored seriously, apparently for two main reasons. First, the anomaly P is very specific in its action, as it affects only frogs of the genus *Pelophylax*, all other amphibian species in the same habitats being unaffected, whereas in North America *Ribeiroia ondatrae* and other trematodes have been shown to have teratogenic effects on limb development in a vast array of species from various genera in different taxonomic groups. Second, the morphological diagnostic characters of the anomaly P reminded above under [I3] are morphologically very different from the (varied) limb anomalies described in various North American amphibian species for limb anomalies shown, or thought, to be caused by trematode infections.

It is striking that our data complete, and do not contradict, those of Rostand (1971) regarding the different topics listed above. Our data confirm the items [I1] to [I3]. We have no data on [I4] and [I5], as we did not perform crosses between anomalous specimens and as our unique amputation experiment was unsuccessful. Finally, our observations provide an explanation for Rostand's results mentioned above in [I6], which had suggested that the anomaly P was caused by a teratogenic agent present in fish feces but which was unknown. Although not explored then, the hypothesis that it could be linked to infection by trematodes present in these feces is quite appealing.

In conclusion, the idea that trematodes could be the cause for the anomaly P is supported by our new data, which therefore provide the first important breakthrough regarding this mysterious anomaly in almost 50 years. It is now necessary to search for species of trematodes, the life cycle of which include *Planorbarius corneus* as an intermediate host, and fish and frogs as reservoir hosts for meso- or metacercariae. Further direct experiments will allow to determine the infectious agent of the anomaly P and to prove experimentally its teratogenic effect on limb development. Many stimulating questions remain open, which we plan to address in the coming years, a few of which can already be listed. How does this factor interfere with limb development? Why is this factor active only in the genus *Pelophylax*? Why are the symptoms observed in *Pelophylax* so different from those observed in North American species? What could be the effects of this agent in other groups of vertebrates?

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