

RESEARCH ARTICLE

Strigea robusta (Digenea: Strigeidae) infection effects on the gonadal structure and limb malformation in toad early development

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Funding information

Russian Science Foundation, Grant/Award Number: 21-74-00079

Abstract

The anomaly P is a mass morphological anomaly reported in some water frog populations across Europe. It was found that polydactyly is only a mild attenuated form of heavy cases of the anomaly P syndrome, which have strong deformations of the hindlimbs and, partly, forelimbs. It was shown that the anomaly P is caused by the trematode *Strigea robusta* and this syndrome can be considered as a special case of strigeosis in amphibians. The anomaly P for a long time considered to be specific for water frogs of the genus *Pelophylax*. Herein, we describe polydactyly and heavy forms of the anomaly P syndrome in toads of the genera *Bufo* and *Bufo*tes, as a result of exposure to *S. robusta* cercariae. A total of 150 tadpoles of *Bufo bufo*, 60 tadpoles of *Bufo*tes *viridis*, and 60 tadpoles of *Bufo*tes *baturae* were divided into five experimental and four control groups (30 tadpoles in each group). All anomalies in the toads were similar to those observed in water frogs. The survival of tadpoles in the experimental groups was 76%. The anomaly P was observed in 57.9% of toad tadpoles (51.8% of mild forms and 6.1% of heavy forms). The occurrence of the anomaly P varied among groups from 19% to 78%. Heavy forms of the anomaly P were found in all experimental groups. We described rare asymmetrical cases of the anomaly P. According to severe modification of limb morphology, we supposed changes of gonadal morphology (any modifications of the germ and somatic cells). The gonadal development of infected tadpoles was however the same as in uninfected toad tadpoles, and heterochromatin distribution within gonocytes had no differences as well. It seems like the parasite doesn't have any effect on the gonadal development of the toads. The lack of heavy forms in natural populations of toads, as well as a development of gonads were discussed.

KEYWORDS

anomaly P, *Bufo*, *Bufo*tes, host-parasite interactions, limb deformities, morphology, palaeartic toads, *Strigea robusta*, trematode, trematode-induced malformations

1 | INTRODUCTION

The manipulation of hosts by parasites is a widespread phenomenon, which is not completely understood. Trophically transmitted parasites can manipulate their intermediate hosts via changes in their host activity (increased or decreased) and microhabitat choice (Lafferty & Shaw, 2013; Moore, 2002). The altered activity may be caused by affection of internal and external morphology that make intermediate hosts more susceptible to predation by definitive hosts (Lafferty & Kuris, 2012; Lafferty & Shaw, 2013). Among amphibians, several variants for changing host morphology are known to be induced by parasites and decrease their activity. One of the most investigated and famous case is deformations of tadpoles caused by infection of the trematode *Ribeiroia ondatrae* occurring in many freshwater communities across United States (Blaustein et al., 2011; Haas et al., 2018; Johnson et al., 1999, 2013; Johnson & Haight, 2001; Johnson & Ritchie, 2001; Johnson & Sutherland, 2003). *R. ondatrae* induces additional limb growth (polymely) in various places of the body of amphibians (depend on cysts localization) and some other types of anomalies (Johnson et al., 1999, 2011; Johnson & Haight, 2001). Another variant of limb anomaly is amely (missing limbs) caused by the trematode *Acanthostomum burminis* from Sri Lanka (Jayawardena et al., 2013, 2016; Rajakaruna et al., 2007, 2008). Infection of the parasitic copepod *Lernaea* can lead to amely in frog tadpoles as well as in rare cases to polydactyly (Kupferberg et al., 2009; Leong, 2001).

An intriguing case of morphological anomaly (known as the "anomaly P") was observed in European water frogs of the genus *Pelophylax* Fitzinger, 1843 (Anura: Ranidae) after infection of the trematode *Strigea robusta* (Szidat, 1928) Heneberg et Sitko, 2018 (Digenea: Strigeidae) (Svinin et al., 2020). *S. robusta* (Digenea: Strigeidae) has a complex three-host lifecycle: planorbid snails serve as the first intermediate hosts, amphibians serve as the second intermediate hosts, and the anatid birds are usually definitive hosts (Heneberg et al., 2018; Odening, 1965; Sudarikov, 1984). The anomaly P includes mild and severe forms (Dubois, 1979, 2017; Rostand, 1958, 1971). Benign (mild) forms consist of symmetrical cases of polydactyly, while severe forms are represented by strong limb transformations that includes shortening parts of hindlimbs (brachymely in femur and tibia), taumely (flexions of hindlimbs also known as bony triangles), an increase in the number of digits on hindlimbs (polydactyly that looking like a "brush"), and sometimes, forelimbs, presence of bony spikes, outgrowths, and excrescences especially in the inguinal region (Dubois, 2017; Rostand, 1958, 1971). Recently, we found that the anomaly P in European water frogs is induced by metacercariae of the trematode *S. robusta* (Svinin et al., 2020). Nevertheless, it remains unknown how this trematode affects other amphibian species.

Polydactyly in palaeartic toads of the genera *Bufo* Garsault, 1764, *Bufo* Rafinesque, 1815, and *Epidalea* Cope, 1864 is often recorded in natural populations (see Table S1; Henle et al., 2017, 2017a, 2017b). It was found in France (Dubois, 1974; Rostand, 1947, 1949a,b, 1951a,c), Ukraine (Borkin & Flyaks, 2004;

Marushchak & Muravynets, 2018), Estonia (Talvi, 1992), Belarus (Borkin & Pikulik, 1986), and Russia (Fayzulín et al., 2018; Henle et al., 2017b; Lada, 1999; Vershinin, 2010; Zamaletdinov, 2003, 2014). Thus, the first aim of our study was to check the trematode *S. robusta* effects on the limb development in toads. To test this, two West Palaeartic (*Bufo* *viridis* [Laurenti, 1768] and *Bufo* *bufo* [Linnaeus, 1758]) and one Central Asian (*Bufo* *baturae* Stoeck, Schmid, Steinlein et Grosse, 1999) species of toads were used in our experiments.

Many parasites have an intensity-dependent impact on reproductive success of the first intermediate host leading to castration (Baudoin, 1975; Lafferty & Kuris, 2009; Moore, 2002). However, only few studies investigated the effect of trematodes on reproductive success of the second intermediate host (Fredensborg & Poulin, 2006; Zelmer & Esch, 1998). For example, it was noted that the trematode *Codonocephalus urnigerus* (Rudolphi, 1819) lead to castration of the second intermediate hosts, marsh frogs (*Pelophylax ridibundus* (Pallas, 1771)), inhabiting water bodies at Eastern Europe (Chikhlyayev & Ruchin, 2021; Ivanov et al., 2012; Litvinchuk, 2018). Individuals with the anomaly P syndrome had no changes in general morphology of gonads (Rostand, 1971), but there is no information about internal morphological structure of gonads. Therefore, the second aim of present paper was to find out effects of *S. robusta* metacercariae on development of tadpole gonads at early stages of development.

2 | MATERIALS AND METHODS

2.1 | Toad species used for breeding

Three toad species were chosen for experiments (Table 1): *B. bufo*, *B. viridis*, and *B. baturae*. *B. baturae* were bred during the winter of 2019–2020 in the laboratory of the K. A. Timiryazev Agricultural Academy. A line of laboratory breeding *B. baturae* originated from Sasyk-Bulak thermal spring (72.889°E, 37.731°N, Tajikistan). One pair of *B. viridis* were caught in the pond in the city of Yoshkar-Ola (56.638°N, 47.823°E, Mari El, Russia), and one pair of *B. bufo* was caught in the pond near the Gusevo settlement (56.973°N, 47.751°E, Mari El, Russia). Pairs of *B. baturae* were injected with 400 µl of surfagon (synthetic analog of luteinizing hormone) at 5 µg/ml, and females started to spawn egg strings after 12–48 h (Dedukh et al., 2017). Wild-caught toads bred without injections. Eggs were placed in 40 L aquariums filled of dechlorinate tap water. Wild animals were released back into field sites after spawning. Experimental design and procedures were approved by the Mari State University Ethics Committee (Yoshkar-Ola, Russia), protocol #2/22.10.2021.

2.2 | Mollusks and trematode cercariae used in experiments

A freshwater snail *Planorbis cornutus* (Linnaeus, 1758) infected by the trematode *S. robusta* was caught from oxbow in the

TABLE 1 Design of experiments with exposure to low dose of the trematode *Strigea robusta* cercariae: number of tadpoles, doses of cercariae, localities for toads, and mollusks used for cercariae emerging

Species	Dose ^a	Locality for toads	Cercariae from mollusk host	Locality for mollusk host	Number of tadpoles ^b	
					N	n
A <i>Bufo bufo</i>	8	Gusevo village (56.973°N, 47.751°E)	<i>Planorbis planorbis</i> 1	Medvedevo settlement (56.642°N, 47.752°E)	30	25
B <i>Bufo bufo</i>	8	Gusevo village (56.973°N, 47.751°E)	<i>Planorbis planorbis</i> 2	Medvedevo settlement (56.642°N, 47.752°E)	30	21
C <i>Bufo bufo</i>	8	Gusevo village (56.973°N, 47.751°E)	<i>Planorbis planorbis</i> 3	Medvedevo settlement (56.642°N, 47.752°E)	30	26
D <i>Bufo viridis</i>	8	Yoshkar-Ola (56.638°N, 47.823°E)	<i>Planorbis planorbis</i> 3	Medvedevo settlement (56.642°N, 47.752°E)	30	15
E <i>Bufo baturae</i>	8	Laboratory animals originated from Sasyk-Bulak thermal spring (37.731°N, 72.889°E)	<i>Planorbis planorbis</i> 1	Privolzhskaya Lesosteppe (52.815°N, 44.459°E)	30	27
C1 <i>Bufo bufo</i>	0	Gusevo village (56.973°N, 47.751°E)	-	-	30	29
C2 <i>Bufo bufo</i>	0	Gusevo village (56.973°N, 47.751°E)	-	-	30	30
C3 <i>Bufo viridis</i>	0	Yoshkar-Ola (56.638°N, 47.823°E)	-	-	30	27
C4 <i>Bufo baturae</i>	0	Laboratory animals originated from Sasyk-Bulak thermal spring (37.731°N, 72.889°E)	-	-	30	30

^aDose is number of cercariae used for infection (where the 0 cercariae is a control group without infection).

^bN is number of tadpoles used in the beginning of experiment; n is number of surviving metamorphs used for calculation of abnormality rates.

Ostrovtsovskaya Lesosteppe part of Privolzhskaya Lesostep Nature Reserve (52.815 N, 44.459°E) in August of 2019. Initially, this snail was used to infect water frogs (Svinin et al., 2020), and afterwards we used it in experiments with *B. batourae* (Table 1). The species of trematode was determined by both morphological (Faltýnková et al., 2008) and molecular (Tkach et al., 2000) diagnostics.

Three freshwater snails *Planorbis planorbis* (Linnaeus, 1758) were collected from the pond near Medvedevo settlement (56.642 N, 47.752°E) in June of 2020. *S. robusta* from these snails was determined by the sequencing of part of COI gene. Cercariae from these snails were used in experiments with *B. viridis* and *B. bufo* (Table 1).

The emergence of cercariae was stimulated by heating containers with a lamp of 45 W for 1–2 h; the distance between the lamp and container was 15–20 cm.

2.3 | Molecular diagnostics of *S. robusta* cercariae

The molecular analysis was performed according to our previous work (Svinin et al., 2020). The DNA was extracted from fixed in 70% ethanol cercariae using the standard salt-extraction method (Aljanabi & Martinez, 1997).

We used two nuclear (ITS2 and 28S rRNA) and one mitochondrial (COI) markers. The primers are described in Table S2 from Supporting Information. The polymerase chain reaction (PCR) reaction mixture (25 µl) contained 50–100 ng of DNA, 0.5 µM of each primer, 0.2 mM dNTPs, 1.5 mM MgCl₂, 2.5 µl 10× PCR bufer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), and 2 U of Taq polymerase (Termo Fisher Scientific, Oyster Point). The thermocycling profile for primers were taken from previously published studies (Bowles et al., 1995; Hugall et al., 1999; Miura et al., 2005; Snyder & Tkach, 2001; Tkach et al., 1999, 2000).

The PCR fragments were prepared for sequencing by elution with a high-salt solution from a 6% polyacrylamide gel. Sequencing was performed on an ABI 3500 automatic sequencer (Applied Biosystems) using the BigDye[®] Terminator 3.1 kit (Applied Biosystems). The same primers were used for amplification. Screening of the primary sequences was made using the BLAST algorithm (Altschul et al., 1990).

Sequences of COI were deposited in GenBank under numbers OM943856–OM943858. Sequences of ITS2 and 28S rRNA were deposited in GenBank earlier (Svinin et al., 2020) under numbers MT075803 and MT075841.

2.4 | Design of the experiment

After hatching, larvae of the trematode *S. robusta* were deposited into experimental containers. For experiments, we used tadpoles at the 25 stage according to Gosner (1960). We divided tadpoles into nine experimental groups (Table 1): (1) *Bufo bufo* I; 30 tadpoles; dose: 8 cercariae; (2) *Bufo bufo* II; 30 tadpoles; dose: 8 cercariae; (3) *Bufo bufo* III; 30 tadpoles; dose: 8 cercariae; (4) *Bufo bufo* (C1); 30 tadpoles; dose: 0 cercariae (control); (5) *Bufo bufo* (C2); 30 tadpoles; dose: 0

cercariae (control); (6) *B. viridis*; 30 tadpoles; dose: 8 cercariae; (7) *B. viridis* (C3); 30 tadpoles; dose: 0 cercariae (control); (8) *B. batourae*; 30 tadpoles; dose: 8 cercariae; (9) *B. batourae* (C4); 30 tadpoles; dose: 0 cercariae (control).

Thus, each group contained 30 tadpoles in the beginning of the experiments. A total of 270 tadpoles were used: 150 tadpoles of *Bufo bufo*, 60 tadpoles of *B. viridis*, and 60 tadpoles of *B. batourae*. All tadpoles in these experimental groups were exposed to an 8 cercariae dose, because in water frogs of the genus *Pelophylax* such dose allowed to obtain both mild and some severe forms of the anomaly P (our unpublished data).

To find out whether the trematode acts on toad tadpoles (if eight cercariae do not cause any effect), we undertook an experiment on hyperinvasion, previously done with water frog tadpoles (Svinin et al., 2020). Although the number of cercariae that infect tadpoles has not been determined, such infestation gives an indication of the most severe forms, in the case of water frogs it was comparable to those observed in nature. In this additional experiment (hyperinvasion), 20 tadpoles of *B. batourae* were kept together with *P. cornutus* snail (localities for mollusk host see in Table 2) infected with *S. robusta* in a 45 L aquarium for 20 days.

2.5 | Morphological examination of anomalies

Following Rostand (1971), we considered cases of polydactyly to be a mild form, when the limbs are not strongly affected, and the individual can move freely. Other changes of limbs morphology we attributed to heavy cases, which included strong changes in the hind and forelimbs and their girdles, in which individuals were not able to move freely. Such changes expressed in a trait syndrome included limb inversion (taumely), limb shortening (brachymely), bony spines, tumor-like outgrowths, and numerous additional digits (polydactyly), and sometimes mirror-image polydactyly. Our classification of anomalies corresponded to that proposed by Henle et al. (2017a). Anomalies were examined using a stereomicroscope Zeiss Discovery V.8 SteREO (Zeiss).

2.6 | Gonadal morphology: Whole mount immunostaining

To compare gonadal morphology between infected and uninfected tadpoles, we examined gonads of 13 infected and 15 uninfected individuals under a laser scanning confocal microscope. Tadpole gonads consisted of somatic cells (follicular and prefollicular in ovaries) and primary germ cells (oogonia and oocytes). Antibodies against Vasa protein allowed precise identification of germ cells. Additionally, we analyzed morphology of cysts as well as potential effect of metacercariae invasion on tadpole gonadal morphology. Gonads were dissected from infected and uninfected tadpoles and fixed in 2% paraformaldehyde for 2 h, and then placed to 1× phosphate-buffered saline (PBS) with 0.02% NaN₃ for long-term storage. Whole bodies of infected tadpoles were fixed in 2% PFA for

TABLE 2 Survival (%) and abnormality rates (%) of mild and heavy forms of the anomaly P in tadpoles of three toad species in experiments with exposure to low dose of the trematode *Strigea robusta* cercariae

Experimental groups	Species	Survival (%)	Anomaly P (%)		Normal tadpoles (%)
			Mild form	Heavy cases	
A	<i>Bufo bufo</i>	83	52	8	40
B	<i>Bufo bufo</i>	70	14	5	81
C	<i>Bufo bufo</i>	87	69	8	23
D	<i>Bufo viridis</i>	50	60	7	33
E	<i>Bufo baturae</i>	90	59	4	37
C1	<i>Bufo bufo</i>	97	0	0	100
C2	<i>Bufo bufo</i>	100	0	0	100
C3	<i>Bufo viridis</i>	90	0	0	100
C4	<i>Bufo baturae</i>	100	0	0	100

Note: The water in the aquariums was changed three to four times per week. Tadpoles were fed fish food (TetraMin Inc.) ad libitum.

10–16 h followed by transferring them in 1× PBS with 0.02% NaN₃. Before immunofluorescence staining, gonads and cyst were carefully dissected from tissues of infected tadpoles and transferred in 1% solution of Triton ×100 in 1× PBS for 4–5 h at room temperature. After washing in 1× PBS, tissues were incubated for 1–2 h in a 1% blocking solution (Roche) prepared with 1× PBS. Following primary antibodies were used: rabbit polyclonal antibodies DDX4 ([C1C3]; GeneTex) against Vasa protein specific only for germ cells. To check the difference in epigenetic modification, we visualized H3K9Me3 heterochromatin modification within the germ cells in infected and uninfected individuals by application of rabbit polyclonal antibodies against H3K9Me3 (Abcam) heterochromatin mark (ab8898; Abcam). In addition, detection of alfa-tubulin using mouse monoclonal antibodies (ab7291; Abcam) allows to visualize cytoskeleton of germ cells as well as accuracy of cell divisions in the gonads. Incubation with primary antibodies was carried out at room temperature overnight. After washing in 1× PBS with 0.01% Tween (ICN Biomedical Inc.), tissues were incubated in secondary antibodies conjugated with Alexa-594-conjugated goat anti-rabbit IgG (H + L) (Thermo Fisher Scientific) and Alexa-488-conjugated goat anti-mouse IgG (H + L) (Molecular Probes) for 12 h at room temperature. Tissues were then washed in 1× PBS with 0.01% Tween (ICN Biomedical Inc.). Additionally, visualization for actin filaments was performed by phalloidin conjugated with Alexa-488 (A12379; Thermo Fisher Scientific). Finally, tissues were counterstained with DAPI (1 µg/µl) (Sigma) in 1× PBS at room temperature for overnight.

2.7 | Gonadal morphology: Confocal laser scanning microscopy

Tissues were placed in a drop of DAPI with Vectashield (1.5 mg/ml) (Vector), solution and mounted with cover slides. Analysis was

performed under Leica TCS SP5 confocal microscope based on the inverted microscope Leica DMI 6000 CS (Leica Microsystems). Specimens were analyzed using HC PL APO x20, x40, and x63 objective. Diode, argon, and helium-neon lasers were used to excite the fluorescent dyes DAPI and fluorochromes Alexa-488 and Alexa-594, respectively. The images were captured using LAS, AF, and processed in LAS AF Light software (Leica Microsystems).

2.8 | Statistical analysis

We used logistic regression for comparison of survival and abnormality rates in experimental groups, and the χ^2 test with Yates's correction for 2 × 2 tables for comparison of frequencies. Calculations were performed using *stats* and *ggplot2* packages in R version 4.2.0 (R Core Team, 2014).

3 | RESULTS

3.1 | Anomaly P manifestation in toads

A total of 114 (76%) tadpoles in experimental groups under treatment survived until metamorphosis: 70%–87% of *B. bufo* tadpoles, 50% of *B. viridis* tadpoles, and 90% of *B. baturae* (Table 2). In control groups, the tadpole survival varied from 90% to 100%. The anomaly P manifested in all three toad species studied (Figure 1). The dose of eight cercariae induced the anomaly P manifestation in 57.9% of tadpoles (51.8% of mild forms and 6.1% of heavy forms). In tadpoles of all three toad species, the occurrence of anomaly P (predominantly polydactyly) varied from 19% to 78% (Table 2 and Figure 2). Individuals with severe cases of the anomaly P were found in all test groups (3.7%–8.0%).

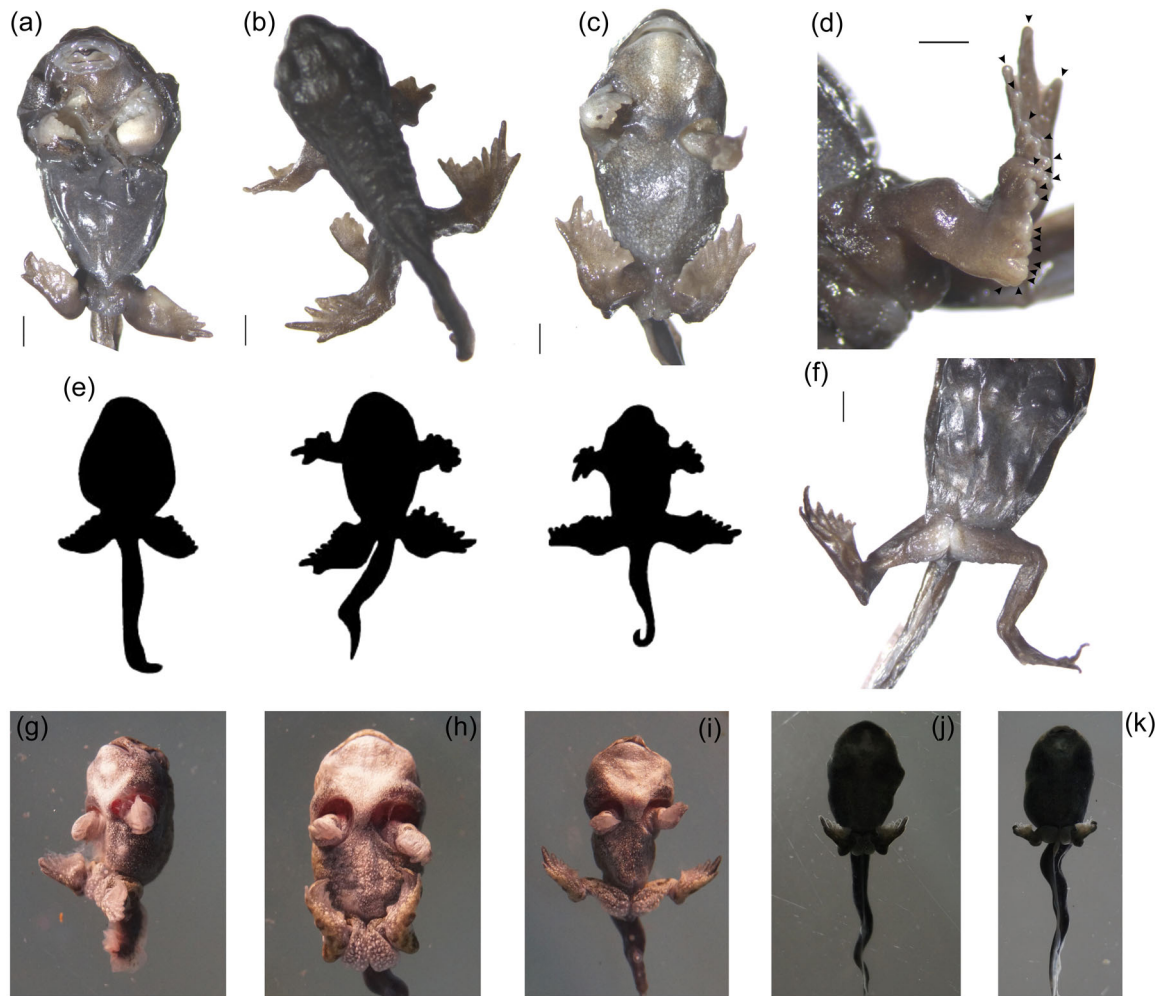


FIGURE 1 Severe cases of the anomaly P in toad tadpoles: (a) A tadpole from experimental group *Bufo bufo* III with brachymely (shortened leg), polydactyly on fore- and hindlimbs, flexion of hindlimbs; (b) A tadpole from experimental group *B. bufo* I with mirror-image polydactyly on the right hindlimb and polymely (additional leg) on the left hindlimb; (c) A tadpole from experimental group *B. bufo* III with brachymely, polydactyly on fore- and hindlimbs, limb flexion, and taumely; (d) A tadpole from experimental group *B. bufo* II with more than 20 digits on the hindlimbs, brachymely, and limb inversion (flexion); (e) First and unique report of heavy cases of the anomaly P in *B. bufo* tadpoles from natural populations (Yakovlev, 1984; with modifications); (f) A tadpole from experimental group *B. bufo* I with asymmetrical heavy case that includes limb flexion and polydactyly on the right hindlimb; (g–k) *Bufo baturae* tadpoles on various stages of development after exposure to *Strigea robusta* cercariae hyperinvasion. Scale bar in a, b, c, f = 1000 μm ; scale bar in d = 00 μm .

Despite high mortality rate (50% in the group of *B. viridis*; Table 2), the frequency of anomalous individuals in *B. viridis* was similar to *B. baturae* and *B. bufo* I + III (Figure 2). In *B. bufo* II, a smaller number of anomalous tadpoles was observed (19%; Figure 2).

Under exposure to low doses of cercariae, standard combination (fingers/toes formula = 4/5) of digits was relatively numerous (42.1%; Figure 3). The next most frequent combination (36.8%) was with a normal number of fingers and an increased number of toes (4/6). Polydactyly on the forelimbs was less frequent (20.2%) than on the hindlimbs (51.8%), and these differences in the frequency of occurrence were statistically significant (Yates corrected $\chi^2 = 10.91$; $df = 1$; $p = 0.001$). Polydactyly on the forelimbs without polydactyly on the hindlimbs were not observed. The combination with seven toes and five fingers was also found (9.7%). Six fingers were observed only in individuals with six or

seven toes (Figure 3). Polydactyly on the hindlimbs (51.8%) was a little more frequent than the normal combination (42.1%), but differences were not statistically significant (Yates corrected $\chi^2 = 0.58$; $df = 1$; $p = 0.447$; Figure 3).

The average number of digits on the forelimbs varies among studied groups (Figure 4). The average number of digits on the forelimbs in *B. viridis* was four, while in *B. baturae* it was closer to group of *B. bufo* I tadpoles (average 4.2). It seems that *B. viridis* toads are less susceptible to development of polydactyly on the forelimbs.

In the additional (hyperinvasion) experiment with tadpoles of *B. baturae*, we found that exposure to high doses of cercariae after coexistence of mollusk and tadpoles leads to severe cases of the anomaly P, in which the hind and forelimbs are significantly modified. Such tadpoles had serious problems with movements during and after metamorphosis (Figure 1g–k).

FIGURE 2 Frequency of tadpoles without anomalies, mild (polydactyly) and severe forms of the anomaly P in tadpoles from experimental groups of three Palearctic toad species (*Bufo bufo*, *Bufo viridis*, and *Bufo baturae*) after exposure to eight *Strigea robusta* cercariae on early stages of limb development

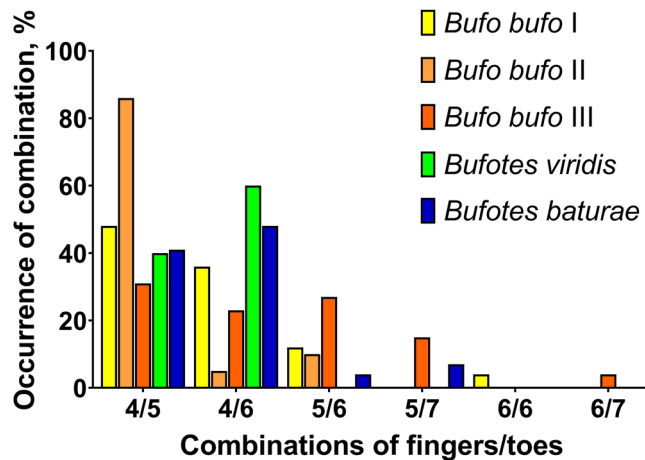
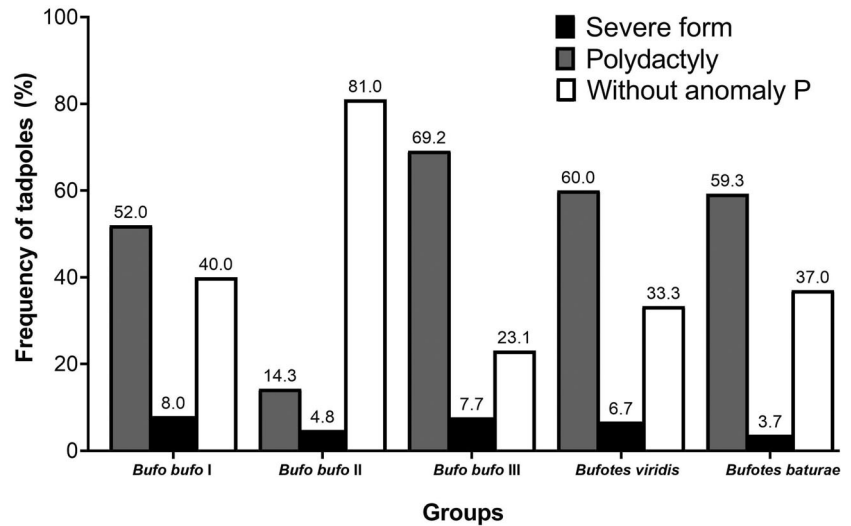


FIGURE 3 Occurrence of digits combinations on the fore- and hindlimbs in experimental groups of three Palearctic toad species (*Bufo bufo*, *Bufo viridis*, and *Bufo baturae*) after exposure to eight *Strigea robusta* cercariae on early stages of limb development (100% is a sum of specimens within experimental groups)

Observed deformations were very close to manifestations of the anomaly P in water frogs of the genus *Pelophylax*. They had severe or minor malformations of hind and forelimbs. Some polydactylous individuals had six to seven toes and five fingers (Figures 3, 4). In severe cases of the anomaly P, toads had flexions of hindlimbs (taumely), tumor-like formations, polydactyly, and brachymely.

Asymmetric cases of the anomaly P were observed in three polydactylous individuals: one in *B. bufo* (experiment A; only right hindlimb strongly deformed) and two in *B. viridis* (only right hindlimbs).

3.2 | Gonadal development in infected and uninfected tadpoles

The somatic cells tightly adhered to the germ cells and had pyramidal nuclei with clearly visible heterochromatin patterns, whereas the

germ cells had bigger spherical nuclei (15–25 μm in diameter) and less intensive DAPI staining. We clearly identified diplotene oocytes in proximal part of the gonad while distal part was presented mostly by gonocytes.

In both infected and uninfected tadpoles, we did not find differences in stages of gonadal development. Infected tadpoles exhibited similar to uninfected tadpoles distribution of diplotene oocytes and gonocytes throughout the ovary.

Moreover, we did not detect any difference in distribution of heterochromatin epigenetic modifications within germ cells and somatic cells in gonads of infected and uninfected tadpoles. In both groups, immunostaining against H3K9me3 showed accumulation on the periphery of cell nuclei as well as inside the nucleus on the periphery of nucleoli.

Cysts are usually surrounded with the two to three layers of somatic cells (Figure 5). Nuclei of such cells exhibit prolonged morphology and intensive DAPI staining. Immunostaining of such cells with antibodies against H3K9me3 mark showed heterochromatin accumulation in the nuclei of such cells (Figure 5). Surrounding cells are characterized by more intensive staining of actin filaments. Cells of the parasite within cyst have intense DAPI staining. Actine filaments were accumulated in peripheral cells. An accumulation of heterochromatin epigenetic modifications didn't detected due to hampered penetration of antibodies within the cyst.

4 | DISCUSSION

4.1 | Anomaly P manifestation in toads

Despite two different genera of toads used in our experiments, all three species had similar manifestations of the anomaly (symmetrical cases of the hindlimbs flexions, brachymely, and polydactyly). Observed manifestations of the infection in toads were similar to the anomaly P in water frogs of the genus *Pelophylax* (Dubois, 2017; Rostand, 1958, 1971). Low abnormality rate (19%) registered in one

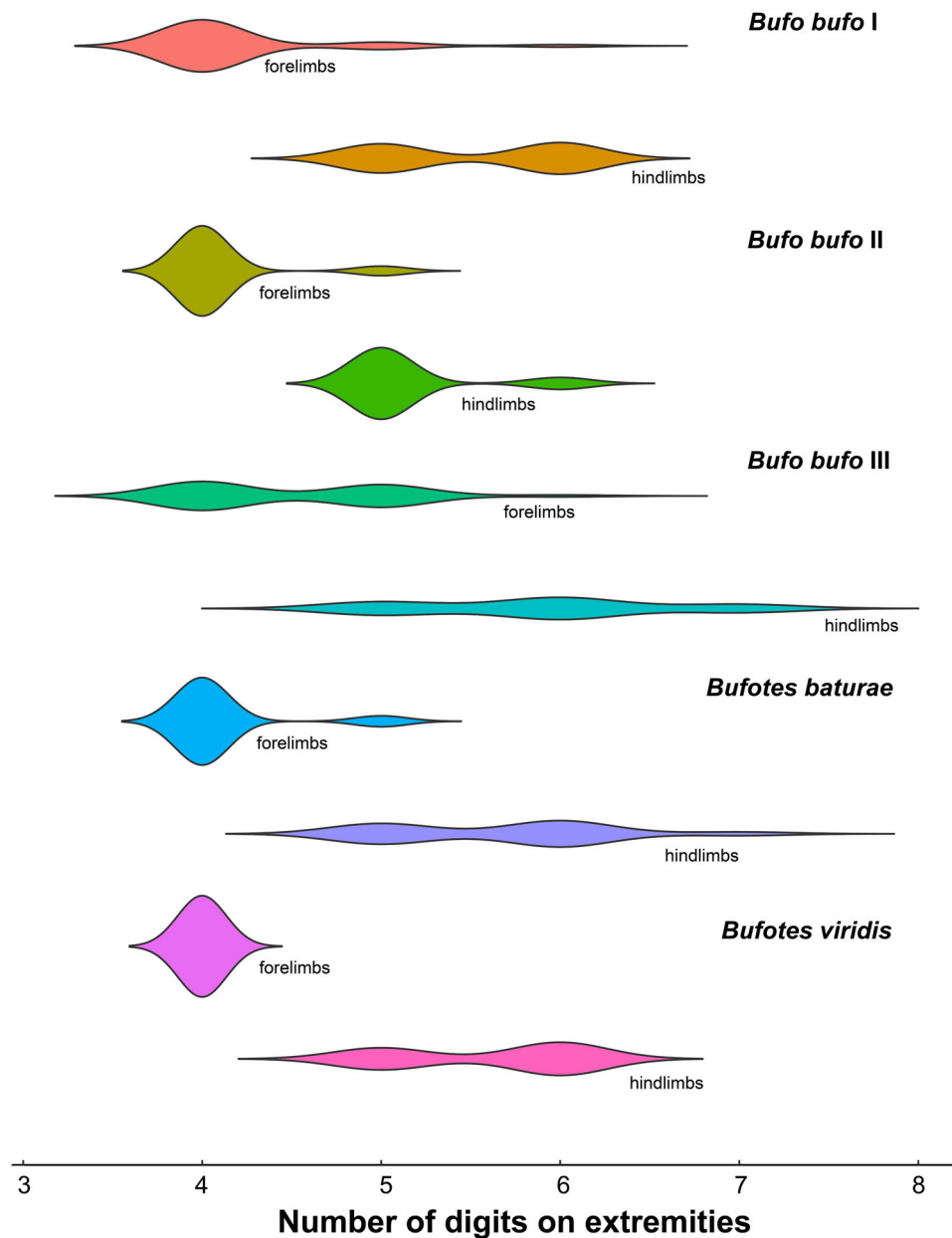


FIGURE 4 Number of digits (violin plots) on fore- and hindlimbs in experimental tadpoles (including individuals with and without anomalies)

experimental group of *B. bufo* tadpoles can be associated with the individual resistance to infection (Table 1). High variability in survival and morphological traits (such as the number of digits) can indicate the presence of individual resistance to parasite action.

For the first time, we observed three strongly asymmetric cases of the anomaly P in toads such as severe cases developed on the right side of the body. In field sites from Ostrovtsovskaya Lesosteppe, we also observed three asymmetric cases of the anomaly P in *P. ridibundus*, which had additional structures on the right side of body (our unpublished data). Such unexplained right-sided asymmetry is a characteristic trait for another type of morphological anomalies in amphibians—the Woitkewitch's anomaly (Woitkewitch, 1961), that includes additional supernumerary legs predominantly on the right side

of the body. If the Woitkewitch's anomaly is also caused by a trematode, such trend of trematodes to form right-sided asymmetric morphological supernumerary structures seems to be a very intriguing phenomenon.

4.2 | Gonadal development and heterochromatin distribution do not differ between infected and uninfected tadpoles

We did not find any alterations in gonadal development of infected tadpoles suggesting that the parasite does not have any effect on the gonads. We also did not detect differences in heterochromatin

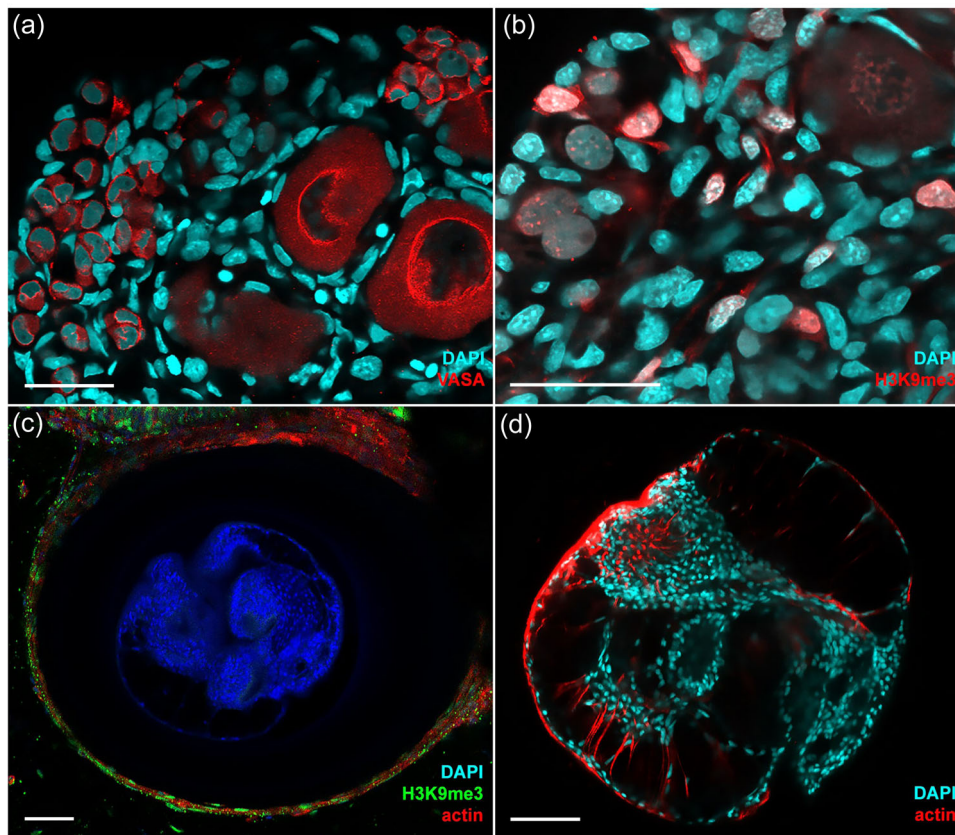


FIGURE 5 Microanatomy of gonads (a,b) from infected tadpoles and the *Strigea robusta* cyst (c,d). Whole-mount immunofluorescent staining with antibodies against Vasa protein (red) identifying germ cells (a) and heterochromatin modification H3K9me3 (red) (b). (c) Epigenetic modification against heterochromatin (green) and actin filaments (red) accumulates in cells nuclei and cytoplasm correspondingly. They show intensive signals in those cells, which surround the cyst. (d) Detailed microanatomy of cercariae with detection of actin filaments accumulating in its peripheral parts and inner components. DAPI is visualizing chromatin (blue in a, b, c, cyan in d). Scale bar = 50 μ m

distribution within gonocytes between infected and uninfected tadpoles. In contrast to ranid species, germ cell nuclei in *Bufo* species exhibit irregular morphology and are highly lobulated (Haczkiwicz et al., 2017; Ogielska, 2009; Ogielska & Kotusz, 2004). Heterochromatin epigenetic modifications within germ cells and somatic cells were analyzed in water frog hybrids previously. Toad tadpoles have similar heterochromatin epigenetic modifications as in frogs (Chmielewska et al., 2018). Normal gonadal development of tadpoles can be beneficial for survival of parasites and its perpetuation in toad populations via reproduction success of hosts. The fact is that a large percentage of occurrence of abnormal tadpoles in various localities (up to 80%; Dubois, 2017) can lead to a significant reduction of amphibian populations or their extinction. This species of trematode can cause a decline of a local population of the smooth newt *Lissotriton vulgaris* (Linnaeus, 1758), which can be the same in water frogs or toads. An additional effect on the gonads with a decrease in survival, can cause significant damage to population and final decline, which should not be beneficial to the parasite. The gonadal lesions described for *C. urnigerus* are probably adaptive. This species of trematode strongly changes behavior and reduced motor activity of frogs, which increases their chances of being eaten by bitterns, their final hosts (Chikhlyayev & Ruchin, 2021; Ivanov et al., 2012).

Nevertheless, it is still unknown how *S. robusta* affects the developing limbs of the tadpole and does not affect the development and differentiation of gonads.

4.3 | Host-parasite interactions

S. robusta is a widespread species and its range includes Germany, Czech Republic, Romania, Lithuania, Ukraine, Russia, Kazakhstan, Turkmenistan, and Kyrgyzstan (Heneberg et al., 2018; Sinsch et al., 2018, 2019; Sudarikov, 1984; Zhigileva & Kirina, 2015). Toads of the genera *Bufo* and *Bufo* are widely distributed in the Palearctic Realm and, accordingly, can easily be infected by *S. robusta* trematode, due to coincidence of their ranges. The genus *Bufo* is represented by about 13–16 species (Frost, 2021), and the genus *Bufo* includes about 15 species, which inhabits Europe, Asia, and North Africa (Dufresnes et al., 2019). Despite the records of polydactyly in toads in Europe and Asia, severe forms of the anomaly P were described in toads in some rare cases only. Asymmetrical phocomely in *B. bufo* from Sagra settlement in Russia observed by V. L. Vershinin (Henle et al., 2017a, fig. 36) have similarities with hindlimb inversion observed in some anomaly P severe cases.

Undoubtedly, records of abnormal tadpoles of *B. bufo* near Yailu from Lake Teletskoye is belonging to the records of severe cases of the anomaly P in toads (see Figure 1), and these findings are the first and unique report of heavy cases of the anomaly P in toads (Yakovlev, 1984).

Even though we have demonstrated the ability of tadpoles to become infected and to show the anomaly P phenotype, there is a deficit of anomaly P severe forms observations in natural populations of Palaearctic toads. It can be associated with phenological asynchrony in the time of larval development and the mass emergence of *S. robusta* cercariae from planorbid snails. We have previously shown that infection of water frog tadpoles with the *S. robusta* trematode depends on stages of larval development (Svinin et al., 2020). Probably, time of mass release of *S. robusta* cercariae does not coincide with the time of limb bud formation in toad tadpoles. However, the cercariae can affect late larval stages, in which such infection leads to polydactyly. The weather in certain years may be warmer than usual, resulting in record numbers of toads with polydactyly. Such observations were made, for example, in a *B. bufo* population in the vicinities of Tambov (Lada, 1999). Asynchrony in phenology of amphibians and helminths developments can be a determining factor in distribution of abnormality patterns in natural amphibian populations (McDevitt-Galles et al., 2020). Nevertheless, some other abiotic or biotic factors can affect the anomaly P manifestation as well. The study of interactions between phenology of spring migrations of birds, time of breeding of amphibians, and development of their larvae, lifespan features of mollusks, from the one side, and trematodes lifecycle features, from another, will help to answer on question about mosaic distribution of the anomaly P hotspots observed in nature.

ACKNOWLEDGMENTS

We are thankful to E. Simonov (Tyumen, Russia) and two anonymous reviewers for their constructive comments, which helped us to improve the manuscript. We are thankful to F. Letoutchaia for English correction (Michigan, USA), and our field team V. Osipov (Penza, Russia), L. Neymark (Moscow, Russia). Special thanks to A. Ivanov (Penza, Russia) for assistance molecular analysis of trematodes. The research was supported by the Russian Science Foundation grant No. 21-74-00079, <https://rscf.ru/en/project/21-74-00079/>

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

All data used in this study are available upon request from the corresponding author.

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SUPPORTING INFORMATION

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How to cite this article: Svinin, A. O., Matushkina, K. A., Dedukh, D. V., Bashinskiy, I. V., Ermakov, O. A., & Litvinchuk, S. N. (2022). *Strigea robusta* (Digenea: Strigeidae) infection effects on the gonadal structure and limb malformation in toad early development. *Journal of Experimental Zoology Part A: Ecological and Integrative Physiology*, 1–12. <https://doi.org/10.1002/jez.2599>