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BRIEF REPORT

Effects of minimum *Strigea robusta* (Digenea: Strigeidae) cercariae doses and localization of cysts on the anomaly P manifestation in *Pelophylax lessonae* (Anura: Ranidae) tadpoles

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Abstract

The anomaly P is a mass morphological anomaly found in some populations of anuran amphibians (water frogs of the genus *Pelophylax* and toads of the genera *Bufo* and *Bufotes*) caused by the parasitic flatworm *Strigea robusta*. Minimum dose of cercariae for the appearance of the anomaly P remains unknown. However, it is important information for understanding of host population dynamics after invasion and the effects of the parasite on the second intermediate hosts. Herein, the invasion properties of *S. robusta* in *Pelophylax lessonae* tadpoles (Anura: Ranidae) and minimum dose for appearance of mild and severe forms of the anomaly P syndrome were described after direct experiments with certain numbers of cercariae exposure. Experimental groups of tadpoles have been exposed to eight doses of cercariae (2, 4, 6, 8, 10, 12, 14 and 16). A total of 63.8% tadpoles survived to the end of this experiment. It was revealed that a mild form of the anomaly P (polydactyly) can appear after infection by two cercariae, while the severe form traits appear after infection by four cercariae. The mean number of detected encysted metacercariae was reached to 53.5%. Differences in infection rates can be explained by the presence of an individual immune response in tadpoles or by the presence of different genetic lineages of the parasite infecting the same snail, which have different infectious potential. Low doses of infection leading to the induction of anomalies characterize *S. robusta* as a highly pathogenic species for amphibian species that are susceptible to infection and show an abnormal phenotype.

Keywords: *Strigea robusta* · Anomaly P · Water frogs · Amphibian morphological anomalies

Introduction

The strigeiosis is a trematode infectious disease caused by over 50 species of the genus *Strigea* Abildgaard, 1790 (Digenea: Strigeidae) (Sudarikov 1984; Heneberg et al. 2018; Sitko and Heneberg 2021). Species of this genus have a complex life cycle including several stages and three hosts (Sudarikov 1984). Birds serve as the final hosts (Lunaschi and Drago 2012, 2013; Heneberg et al. 2018; Sitko and Heneberg 2021). Planorbid snails (Gastropoda: Planorbidae) are known as first intermediate hosts (Sudarikov 1984; Faltýnková et al. 2007, 2008; Cichy et al. 2011). Some groups of vertebrates (amphibians, reptiles, mammals, and birds) serve as the second intermediate hosts as well as reservoir, paratenic and accidental hosts (Vojtek, 1972; Sudarikov 1984; Patrelle et al. 2015; Zhigileva and Kirina 2015; Sinsch et al. 2018, 2019; Hamann et al. 2019; Kuzmin et al. 2020). At least one

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species of the genus has a teratogenic effect on the second intermediate host at the metacercariae stage: *Strigea robusta* (Szidat, 1928) Heneberg et Sitko, 2018 causes polydactyly and severe morphological anomalies in water frog tadpoles (Svinin et al. 2020) known in herpetological literature as the "anomaly P" (Rostand 1971; Ouellet 2000; Dubois 2017). The life cycle of the parasite *S. robusta* includes transmission from molluscs (genera *Anisus* Studer, 1820, *Gyraulus* Charpentier, 1837, *Segmentina* Fleming, 1818, *Bathyomphalus* Charpentier, 1837, *Planorbis* Müller, 1774, *Planorbarius* Duméril, 1806) to amphibian larvae (genera *Lissotriton* Bell, 1839, *Bombina* Oken, 1816, *Bufo* Garsault, 1764, *Bufotes* Rafinesque, 1815, *Rana* Linnaeus, 1758, *Pelophylax* Fitzinger, 1843). In some amphibian larvae (genera *Bufo*, *Bufotes*, *Pelophylax*), parasites change their morphology, and they become more accessible prey for birds, most often ducks *Anas platyrhynchos* Linnaeus, 1758 and *Aythya fuligula* (Linnaeus, 1758) (Heneberg et al. 2018), serving the parasite as the final hosts. Developing severe anomalies prevents the active movement of frogs, which makes them easier prey for waterfowl.

The anomaly P symptoms provoked by *S. robusta* go from a mild deformation (symmetrical polydactyly) to significant deviations from normal development such as symmetrical shortening of the hind limbs (brachymely), flexion of the hind limbs (taumely), development of outgrowths and tumour-like formations, as well as additional distal parts of limbs in the inguinal region (Rostand 1971; Dubois 1979, 2017; Svinin et al. 2019). It is important to note polydactylies are considered mild due to their insignificant effect on froglet locomotion activity and not affecting the survival of individuals, because polydactylies are massively found in adults (Svinin et al. 2019). Severe forms have never been noted in adult individuals (as well as in individuals after wintering), which indicates that these individuals do not survive in the natural habitats. Data about the laboratory keeping of individuals with severe forms show that some individuals are able to live up to 1.5 years (Rostand 1971). Probably, the death of such individuals in nature is due to the fact they more often become the prey of predators.

Cysts of *S. robusta* are located in various parts of the body of tadpoles (Svinin et al. 2020): some of them were found in the head, back and flanks, tail, fore- and hind limb regions. However, special studies on how the localization site affects the development of the limbs have not been conducted.

It was shown that all doses used in our experiments (16, 32 and 48 doses of *S. robusta* cercariae) led to the appearance of severe deformations (e.g., in the case of 16 dose, they were 20%) in *Pelophylax lessonae* (Camerano, 1882) tadpoles on early stages of limb bud development (Svinin et al. 2020). As the anomaly P syndrome can have a great impact on tadpole morphology, studying the minimum infection dose of *S. robusta* cercariae is crucial to understand the development of the syndrome from mild (polydactyly) to severe symptoms. Such data may be useful in the context of the host-parasite interactions between *S. robusta* and water frog species (*Pelophylax* spp.; Anura: Ranidae), since the anomaly P syndrome can significantly reduce survival (~up to 80% of tadpoles, Dubois, 2017) or even extinct host populations (*Lissotriton vulgaris*, Sinsch et al. 2018).

Materials and methods

Collection, maintenance of molluscs and cercariae emergence

Molluscs *Planorbarius corneus* naturally infected with *S. robusta* were collected from an oxbow in Privolzhskaya Lesostep' Nature Reserve (52.8183° N, 44.4545° E). Maintenance and screening of the molluscs was carried out in the field. The captured molluscs were kept in 10-l aquariums (30 individuals) until they were placed in small containers for screening procedures. To obtain cercariae, molluscs were placed individually into small plastic containers (200 ml) and placed 40 cm away from 60-W incandescent lamp (Faltýnková et al. 2008). The initial identification of cercariae was carried out by morphology (Combes 1980; Faltýnková et al. 2008). Cercariae morphologically identified as *S. robusta* were selected for molecular analysis. The snails producing *S. robusta* cercariae were transported to the laboratory for further infection experiments. In the laboratory, these snails were kept in 60-l aquarium with settled dechlorinated tap water. A third of the water in the containers was changed twice a week. The snails were fed with plant-based Tetra fish food (TetraMin Inc., Melle, Germany). The experiment began a one week after the molluscs were transported.

Freshly emerged *S. robusta* cercariae (i.e. 1–2 h old) from only one infected mollusc were used for infecting the tadpoles. Cercariae of *S. robusta* were calculated with the use of a dissection microscope and transferred into containers with tadpoles.

Tadpoles for experiments were obtained by a laboratory breeding of a pair of pool frogs, *Pelophylax lessonae* (Anura: Ranidae), catching in June 2020 from a pond near Krasniy Most settlement in Mari El Republic, Russia (56.593° N, 47.075° E). The frogs were placed in 90-l aquariums (filled by 60 l of water) with pieces of polystyrene. Dechlorinated tap water was used for keeping the frogs. Reproduction was induced by injection of 400 µl of Surfagon (synthetic analogue of luteinizing hormone) at 5 µg/ml (Dedukh et al. 2017). Reproduction began two days after the frogs were transported to the laboratory and injected with surfagon. In order to avoid intraspecific variability, we took a clutch of eggs from one pair of spawners. A clutch of eggs was transported to a 60-l aquarium with dechlorinated tap water. The water in containers was changed twice a week and the tadpoles were fed daily with fish food (TetraMin Inc., Melle, Germany). The starting dose of food for tadpoles was 0.05 g, and the feed dosage increased as the tadpoles grew (Matushkina et al. 2017). At the start of the experiment, the tadpoles were kept together; as they grew, tadpoles were seated in several aquariums with a density of 30 tadpoles per 60 l. Then tadpoles on stage 25 (Gosner 1960) were then randomly selected for experiment.

Experimental design

A total of ninety tadpoles were divided into nine groups of ten individuals. To determine the minimum dose of *S. robusta* cercariae for the occurrence of the anomaly P, each group was exposed to a *S. robusta* cercariae-dose (*n* cercariae/tadpole infection): 0 (control), 2, 4, 6, 8, 10, 12, 14, and 16. Tadpoles and cercariae of certain dose were placed in 200-ml cup for individual infection, and then tadpoles were then kept in the cups for 24 h. Two months after infection, the tadpoles were fixed in ethanol 70% and evaluated under a stereodissecting microscope Zeiss Discovery V.8 (Carl Zeiss AG, Oberkochen, Germany), to determine metacercarial success and morphological anomalies rate by scoring the side-effects using standard classification of amphibian anomalies (Nekrasova 2008; Henle et al. 2017a, b). We have identified several areas on the body of the tadpole (Fig. 1): the tail region (from the distal part of the tail to the proximal one, at the junction with the body); the back region (includes all cysts located near the spinal column under the skin); the flanks region (the region is delimited by the gill slit in front, and the border between body and tail in the back); the region of the fore- and hind limbs is located around the bases of limbs (we have never observed cysts directly in the limbs); the region of the head was delimited by the gill slit and the base of the skull in the region of the back. The position of the cyst was also taken into account: on the right side, on the left or medially (some cysts in back and tail regions). Some tadpoles were at non-diagnostic stages (is not possible to determine whether anomalies are mild, severe, and the tadpole abnormal or not) and were assigned to the group of long-developing tadpoles.

Rates of survival analysed using logistic regression with Firth's correction in R with use of *logistf* package to obtain penalized maximum likelihood estimates. Alive or dead tadpoles were the response variables; cercariae dose was a predictor. Comparison of frequencies was carried out using the Chi-square test in the native stats package in R. The package *dplyr* was used for comparison of mean ranks among groups by Kruskal-Wallis H test. All calculations were performed using RStudio v. 2021.09.0 + 351 "Ghost Orchid" Release.

Results and discussion

Experimental infections of tadpoles with *S. robusta* cercariae showed a 63.8% survival rate (51/80 infected tadpoles) while the uninfected ones showed a 100% (10/10 tadpoles control). Among the experimental groups of tadpoles, statistically significant differences in the survival rates were found (logistic regression with Firth's correction: Wald test = 13.9, p = 0.0002). Twenty tadpoles out of 51 (39.2%) were long-developing tadpoles and not reach the diagnostic stages (for the anomaly P) by the end of the experiment.

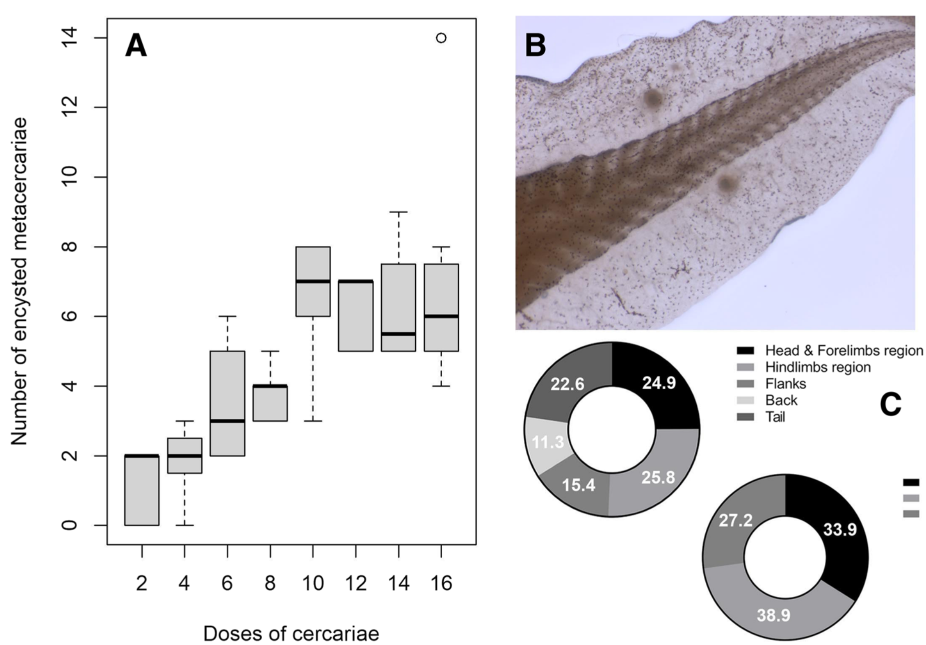

Fig. 1 Dependence between the dose of cercariae and the number of encysted metacercariae (**A**), cysts in the tail of the *Pelophylax lessonae* tadpole (**B**), and localization of cysts on different sides and regions of the tadpole body (**C**)

Thirty-one tadpoles out of 51 survived (60.7%), had the anomaly P (52.9%) or healthy phenotype (7.8%). Ten specimens (19.6%) had severe cases of the anomaly P, while 17 specimens (33.3% out of 51 tadpoles) had one to four additional digits on hindlimbs.

A mild form of the anomaly P (polydactyly) can develop after infection by two cercariae. The number of digits in such individuals did not exceed six on the hindlimbs, and polydactyly was not observed on the forelimbs.

A severe form can develop after infection by four cercariae. Groups of tadpoles infected by 4 cercariae had polydactyly, inversion (flexions or taumely) of the limbs, outgrowths and brachymely. The number of severe forms, as well as the number of digits on the forelimbs (up to 5), increased slightly with an increase in the doses of cercariae.

Normal individuals were found in experimental groups under exposure to 2 and 10 cercariae.

The mean number of detected encysted metacercariae was reached to 53.5% (Fig. 2; Table 1). There are no differences in cyst localization between left, right sides of the body and in middle position ($\chi^2 = 4.62$; $df = 2$; $p = 0.099$). Cysts are most often localized in the region of the hind limbs (25.8%), the head region (24.9%), and in the tail (22.6%) (Fig. 1). The frequency of cysts in the head, in the region of the hind limbs, on the flanks, back and tail was the same in polydactyl individuals and individuals with severe form of the anomaly P ($\chi^2 = 10.13$; $df = 5$; $p = 0.071$). No differences were found among cyst number in long-developing tadpoles, tadpoles with polydactyly, severe forms and without anomalies (Fig. 2; Kruskal-Wallis test: $H = 4.27$; $p = 0.234$).

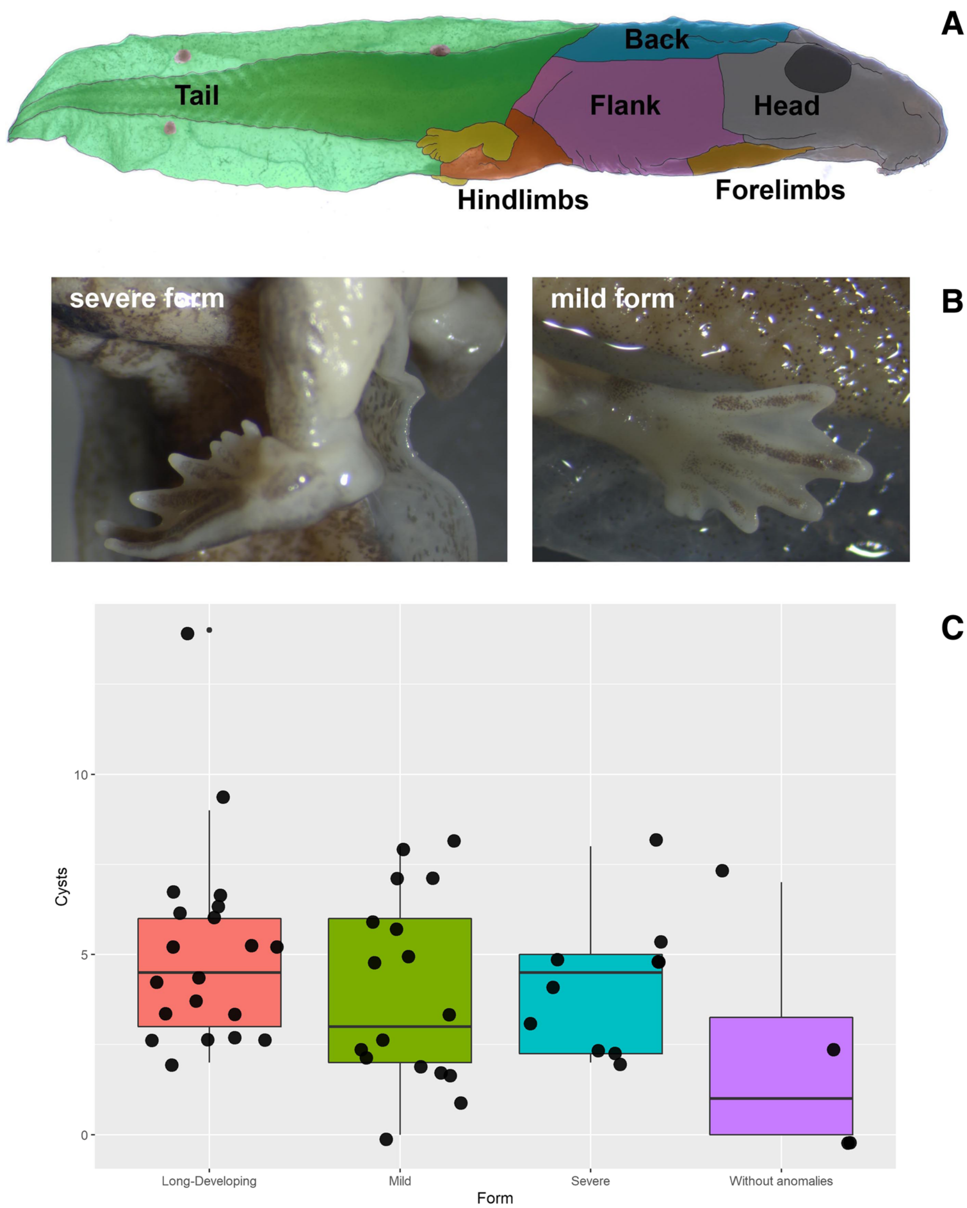

Fig. 2 Body areas of tadpole (**A**), severe and mild forms of the anomaly P syndrome (**B**) and cyst number in long-developing tadpoles, tadpoles with severe and mild form of anomaly P and without anomalies (**C**)

Table 1 Percentage of encysted metacercariae (%) out of cercariae dose in experimental groups of *Pelophylax lessonae* tadpoles calculated for survived tadpoles.

Dose (D)	Number of survived tadpoles (N_1)	Number of cercariae per survived tadpoles ($N_2 = D \times N_1$)	Number of cysts in tadpoles (N_3)	% of encysted metacercariae ($P = N_3/N_2 \times 100$)
2	6	12	8	66.7
4	7	28	13	46.4
6	10	60	37	61.7
8	7	56	22	39.3
10	5	50	32	64.0
12	5	60	31	51.7
14	4	56	25	44.6
16	7	112	49	43.8

The largest number of cysts (9 and 14) detected in our study was found in long-developing tadpoles. Probably, the presence of a large number of cysts, which triggered a cascade of biochemical processes, caused the developmental delay in these tadpoles by hormonal imbalance (perhaps due to the group effect; for example, Bellakhal et al. 2014). The opposite variant may also be possible: individuals prone to long-term development are more susceptible to infection by the parasites.

Thus, according to our results, an important role in the manifestation of anomalies is played not only by the dose of exposure, but also by other factors. Localization of cysts is the most likely factor (Johnson et al. 1999; Johnson et al. 2011); however, it was not highly significant in our study. It was logical to assume that cysts can cause either mild or severe forms of the anomaly or generally lead to a normal phenotype of individuals depending on proximity to the developing limb buds. However, in our study, the location of cysts in severe forms does not differ from mild forms or normal individuals. Severity of anomalies may also be due to the resistance of some tadpoles to the effect of cercariae, i.e. to an individual immune response. It can be explained by the presence of different genetic lineages of the parasite-infected snail, which have different infectious potential. Low doses of infection that lead to the anomaly appearance could characterize *S. robusta* as a highly pathogenic species for amphibians that can lead to decline of populations and can be a new detected threat for global amphibian communities.

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Author contributions A.S., O.E. and S.L. designed the study; A S. provided data curation and made an original draft preparation; O.E. and S.L. reviewed and edited the manuscript. All the authors have read and agreed to the published version of the manuscript.

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Data availability All data used are available upon request from the corresponding author.

Declarations

Ethics approval The Mari State University Ethics Committee (Yoshkar-Ola, Russia) (#2/22.10.2021) approved experimental design and procedures.

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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