



Distribution of *Alaria* spp. mesocercariae in waterfrogs

Antonia Christine Voelkel¹ · Sandra Dolle¹ · Martin Koethe¹ · Jenny Haas¹ · Gregor Makrutzki² · Stefan Birka¹ · Ernst Lücker¹ · Ahmad Hamedy³

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Abstract

The distribution of *Alaria*-spp.-mesocercariae within the host is relevant for the examination via *Alaria* spp. mesocercariae migration technique (AMT) regarding predilection sites and may indicate an interaction between parasite and host. Naturally *Alaria*-exposed frogs of *Pelophylax* species ($n = 13$) were examined for systemic distribution and localization-specific parasite density of *Alaria* spp. mesocercariae. The frogs were necropsied and their body was divided into the following localizations: inner organs, head, torso, forelimbs, and hind limbs. The localizations were analyzed individually and in toto using *Alaria* spp. mesocercariae migration technique. Our results showed neither statistical differences concerning the number of mesocercariae in the different localizations nor in respect of the rate of positive localizations. Therefore, an accumulation in a particular predilection site seems unlikely. Further research on a representative sample is necessary before final conclusions can be drawn.

Keywords Distomum musculorum suis · Ranidae · Pelophylax · Predilection site · Parasite host relationship

Introduction

Alaria spp. are trematodes with a three-host life cycle, which was reviewed by Möhl et al. (2009). It comprises carnivores (*Canidae*, *Felidae*, *Mustelidae*) as definitive hosts, snails (*Planorbis*, *Heliosoma*, *Lymnea*, and *Anisus*) as first intermediate hosts, and amphibians (especially tadpoles and adult frogs) as second intermediate hosts. The life cycle is significantly extended by paratenic hosts, including representatives of almost every vertebrate animal class, such as *Sus scrofa*, *Procyon lotor*, *Mus musculus*, and *Rattus rattus*. The distribution of mesocercariae within the host is relevant for the investigation via *Alaria* spp. mesocercariae migration technique (AMT) described by Riehn et al. (2010) with respect to predilection sites and may indicate an interaction between

parasite and host. Patelle et al. (2015) investigated the distribution and localization-specific parasite density of *Alaria alata* mesocercariae on two out of 59 positive specimens of the frog genus *Pelophylax* (species not stated). They used a modified Baermann technique, similar to the AMT. The authors examined head, periorbital region, visceral cavity, vertebral body, hind limb, and forelimb. They observed the highest parasite densities of mesocercariae in the tissues around the eyes.

In the present study, systemic distribution and localization-specific parasite density of *Alaria* spp. mesocercariae were examined on a larger sample size of *Pelophylax* species frogs. Due to legal constrictions regarding extraction of vertebrates from nature, by now, 13 specimens were examined to provide further data on the distribution of *Alaria* spp. mesocercariae in waterfrogs.

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✉ Antonia Christine Voelkel
antonia.voelkel@vetmed.uni-leipzig.de

¹ Institute of Food Hygiene, Faculty of Veterinary Medicine, Leipzig University, An den Tierkliniken 1, 04103 Leipzig, Germany

² Landkreis Leipzig, Landratsamt, Lebensmittelüberwachungs- und Veterinärämter, 04550 Borna, Germany

³ Hochschule Anhalt, Gebäude 20, Strenzfelder Allee 28, 06406 Bernburg, Germany

Material and methods

The systemic distribution and localization-specific parasite density of *Alaria* spp. mesocercariae in specimen of *Pelophylax* was examined as part of a study on the prevalence of *Alaria* spp. in frogs in Germany. For the present study, 15 frogs were sampled from waterbodies in the vicinity of Leipzig, Germany, known for the presence of the

mesocercariae (unpublished). Sampling was performed using a landing net and frogs were transported to the Institute of Food Hygiene, Faculty of Veterinary Medicine, Leipzig University, in water-filled containers. The frogs were euthanized by a cranial concussion followed by decapitation. Special permission for the collection and euthanasia of the amphibians for scientific purpose was granted by the competent authorities (AZ 364.620/17/10/4 and T 27/14; AZ 364.620/33/6/2 and T 45/16) in accordance with the German animal protection act (TierSchG 2014). Handling, transport, keeping, and euthanasia of the animals were performed in accordance with national legal regulations (TierSchG 2014; TierSchVerV 2013) and international guidelines (*Guide for the care and use of laboratory animals*; European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes).

The frogs were examined in toto after dissection into five localizations (inner organs, head, torso, forelimbs, and hind limbs). For dissection, the torsos were opened with scissors in supine position in order to extract the inner organs. Subsequently, the bodies were divided into the respective localizations. The five localizations were analyzed individually using AMT described by Riehn et al. (2010). In brief, the body tissue was cut into cubic pieces of 0.5 cm edge length and transferred to a plastic sieve. The sieve was placed in a funnel with a rubber hose, which was closed by a clamp. The funnel was filled with warm water (46–48 °C) to cover the tissue samples. After 45 min, a 20-ml liquid sample was transferred into modified counting basins (Trichoview, Makrutzki et al. 2014), a 50-ml acrylic cylinder with a basal plate featuring a grid. The sample fluid was examined using an inverse microscope (Zeiss Primo Vert, Jena, Germany) at 25- to 40-fold magnification.

The identification of the frog specimens as members of the waterfrog sensu lato group (*Pelophylax ridibundus* and *Pelophylax* kl. *esculentus*) was based on morphological criteria according to Nöllert and Nöllert (1992) and the nomenclature of Frost et al. (2006). The identification of *Alaria* spp. mesocercariae was based on morphological characteristics such as shape, size, and movement pattern as reviewed by Möhl et al. (2009). Additionally, five isolated mesocercariae were selected randomly. Their DNA was extracted individually using the DNeasy Blood & Tissue Kit (Qiagen). Subsequently, their identity was confirmed by a conventional *Alaria* spp.-specific PCR assay. The PCR protocol was based on the protocol published by Riehn et al. (2011) with slight modifications. Each PCR reaction consisted of 20 µl 1, 1× ReddyMix PCR Master Mix (containing 0.625 U Taq polymerase, 75 mM Tris-HCl, 20 mM (NH₄)₂SO₄, 2.5 mM MgCl₂, 0.01% Tween 20, 0.2 mM each dNTP; Thermo Fisher Scientific), 5 pmol forward primer (5'-CTTA GCTGCGGGTTCCTGCT-3'), 5 pmol reverse primer (5'-CGGCACATAAGCAAATACCTCG-3'), and 1 µl template

DNA. Two *Alaria* spp. specimens previously identified by the German Federal Institute of Risk Assessment served as positive control while PCR grade water was included as a negative control. Amplification products were size-separated by 2% agarose gel electrophoresis.

The numbers of mesocercariae isolated from different localizations were tested for statistically significant differences by ANOVA since data followed Gaussian distribution as demonstrated by the Kolmogorov-Smirnov test. To compare the observed frequencies of mesocercariae detection of the different localizations, a chi-squared test was applied. All analyses were performed using Prism statistical software (version 4.00, GraphPad Software, Inc.).

Data availability All data analyzed and generated during this study are included in the present article.

Results and discussion

Alaria spp. mesocercariae were detected in 13 out of 15 examined frogs. Table 1 shows the distribution of mesocercariae in the five localizations. A total of 96 mesocercariae was detected with a parasite burden of two to 20 mesocercariae per frog. In every positive frog, mesocercariae were isolated from one to four out of the five localizations. By far, most mesocercariae were isolated from torso (38/96) and hind limbs (35/96) while in the remaining localizations only eight (forelimbs and head) or seven (inner organs) mesocercariae were found. Figure 1 illustrates the sum of positive localizations and the range of mesocercariae isolated from positive frogs. For every localization, at least four frogs displayed positive findings. Hind limbs and torsos were positive in most frogs (9/13 and 8/13, respectively) followed by forelimbs (6/13), head (6/13), and inner organs (4/13). Interestingly, although heads of six frogs were positive, only one single mesocercaria each was isolated from five out of them, while three mesocercariae were isolated from the sixth frog. In contrast, by far, the highest number of mesocercariae was detected in the torso of one frog (18 mesocercariae) and the hind limbs of another one (16 mesocercariae). There were neither statistically significant differences in the number of mesocercariae (ANOVA, $p = 0.09$) nor in the frequency of positivity (chi-squared test, $p = 0.322$) between the different localizations.

The findings of the present study are in line with previous reports insofar as *Alaria* spp. mesocercariae were detected in all localizations as described by Bugge (1942b), Andreas (2006), and Patrelle et al. (2015) in frogs. Most mesocercariae were found in the hind limbs and torsos, which is consistent with the results of Shoop and Corkum (1981) in amphibians. Similarly, Freeman et al. (1976) described a high number of the parasite in the hind limbs of a big bullfrog. Bugge (1942b)

Table 1 Distribution of *Alaria* spp. mesocercariae in 13 water frogs

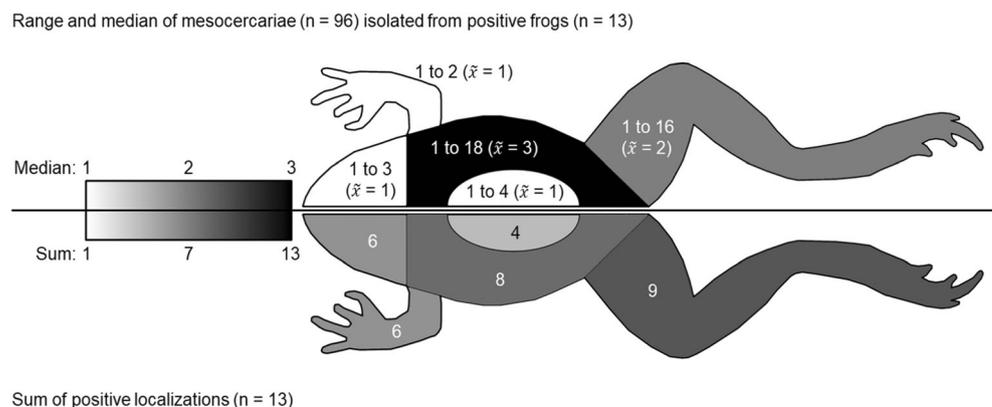
Localization	Detected mesocercariae per frog													Sum of detected mesocercariae
	Frog													
	1	2	3	4	5	6	7	8	9	10	11	12	13	
Hind limbs	0	0	3	6	1	16	1	4	0	0	1	1	2	35
Torso	0	18	0	3	3	0	2	3	1	2	0	6	0	38
Forelimbs	1	1	0	0	2	0	1	2	0	0	0	1	0	8
Head	3	1	0	1	1	1	0	0	0	0	1	0	0	8
Inner organs	0	0	0	4	0	0	0	1	1	0	0	1	0	7
Sum of detected mesocercariae	4	20	3	14	7	17	4	10	2	2	2	9	2	96

not only detected mesocercariae in the forelimbs and hind limbs of frogs but also in the abdominal and thoracic cavity, in the abdominal muscles, chest, and back, which corresponds to our findings in the torso of frogs. Furthermore, the results match the assumed preference of mesocercariae for fat and muscle tissue as described by Bugge (1942a) in pigs, Odening (1961) in a rhesus monkey, Shoop and Corkum (1981) in raccoons and opossums, and Riehn et al. (2010) in wild boars.

Overall, the distribution of *Alaria* spp. mesocercariae showed no significant accumulation of the mesocercariae in a particular localization. Although most mesocercariae were found in the hind limbs and torsos of the frogs, they did not obviously show any preferential localization, as there were no statistically significant differences regarding the number of mesocercariae in the different localizations and the rate of positive localizations.

These findings contradict the results of Patrelle et al. (2015), who observed the highest parasite densities of mesocercariae in the tissues around the eyes of two specimens. They suspected that the mesocercariae may have an impact on frogs' or tadpoles' vision which possibly reduces its ability to avoid predators. In the present study, only one single mesocercaria each was isolated from five out of six positive heads, while no mesocercariae were detected in the heads of

the further seven positive frogs. The highest mesocercariae number was observed in the torso of one frog and the hind limbs of another one. The discrepancy does not result from divergent study designs as a direct comparison of the presented data to the data of Patrelle et al. (2015) is possible: head and periorbital region jointly correspond to the localization "head" in the present study, the visceral cavity corresponds to the "inner organs," and the vertebral body corresponds to the "torso". Hind limbs and forelimbs were examined equally in both studies. Furthermore, the conflicting results compared to the findings of Patrelle et al. (2015) cannot be attributed to the frog species or methodology, as in both studies frogs of the genus *Pelophylax* were examined by means of AMT. A possible explanation for the discrepancy could be the strongly varying parasitic burden. While Patrelle et al. (2015) detected 164 and 314 mesocercariae in two specimens, the 13 frogs in the present study were infected with two to 20 mesocercariae. There could be a correlation between the parasitic burden and the distribution of *Alaria* spp. mesocercariae in frogs. However, Patrelle et al. (2015) analyzed only one froglet and one sub-adult frog for distribution of mesocercariae in different localizations. Therefore, the increased occurrence of mesocercariae in the frogs' heads the authors described could have been a random finding.

Fig. 1 Range and median (\bar{x}) of *Alaria* spp. mesocercariae isolated from positive water frogs and sum of positive localizations

Conclusion

By now, based on varying study results no final conclusions can be drawn regarding the distribution of *Alaria* spp. mesocercariae in frogs. In contrast to the findings of Patrelle et al. (2015) on two specimens, mesocercariae showed no statistically significant preference for a certain localization in the present study on 13 frogs with a well-comparable study design. Therefore, an accumulation of the mesocercariae in the head with a specific impairment of the host seems unlikely. For the final assessment, a study should be carried out on a representative sample.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international and national guidelines for the care and use of animals were followed.

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