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NOTE

Swim bladder as a primary site of mycobacterial infection in *Nothobranchius* 'belly sliders'

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ABSTRACT: The swim bladder inflates early after fish hatching via its interconnection with the digestive tract (ductus pneumaticus). This interconnection may serve as a portal to foreign particles, including bacteria, causing deficiencies in primary swim bladder inflation. We histologically examined 134 African annual killifish (genus *Nothobranchius*) with secondary loss of swim bladder function ('belly sliders'). We demonstrate that these fish lost the ability of air regulation in their swim bladders likely due to *Mycobacterium* spp. infection at an individual-specific age. Nearly all examined belly sliders had thickened swim bladder walls, and their swim bladder was filled with material containing mycobacteria, cell debris, young monocytic cells and phagocyting macrophages. Mycobacterial infection was restricted to the swim bladder in juveniles, where mycobacteria likely enter the host through the ductus pneumaticus. Infection in adults was systemic and mycobacteria were present in all examined organs. Presence of mycobacteria in the epithelial lining and submucosal layers of the digestive tract of adults suggests that it may also serve as the entrance site of infection. We suspect 2 sources of *Mycobacterium* contamination: dietary (with bloodworms) and/or contaminated hatching substrate. These sources of contamination may be eliminated by use of laboratory dry feed and egg disinfection prior to hatching.

KEY WORDS: Abnormal swimming · Model organism · Laboratory-reared killifish · Mycobacterial infection

1. INTRODUCTION

The impairment of fish behaviour within the water column has repeatedly been related to swim bladder inflation problems and altered swim bladder function (Woolley & Qin 2010). Non-inflation of the swim bladder associated with serious mortalities was recognized as a major obstacle in culture of larvae in species important for the aquaculture industry, farming and repopulation of water bodies (Marty et al. 1995, Woolley & Qin 2010). This is well documented in various aquarium-reared fishes including laboratory fish such as zebrafish *Danio rerio* (Goolish et al. 1999, Robertson et al. 2007) and annual killifishes (family Nothobranchidae: Wourms 1967; family Rivulidae: Podrabsky 1999; da Fonseca et al. 2018).

The general cause of non- or insufficient inflation of the swim bladder has been linked to suboptimal environmental conditions (Woolley & Qin 2010, Blecha et al. 2019). Foreign particles (e.g. food detritus) entering swim bladders via the ductus pneumaticus were considered the source of swim bladder

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inflation problems in various fish species (Marty et al. 1995). Information on bacteria associated with swim bladder inflation problems are rare in general, and in annual killifishes in particular (Marty et al. 1995, Gómez 2008). The scarcity of these data is in stark contrast to the intense scientific interest in mycobacterial infections caused by *Mycobacterium* spp. common in cultured, wild and laboratory-reared fish (Aubry et al. 2017, Davidovich et al. 2020). The zoonotic potential recognized in some *Mycobacterium* spp. associated with fishes and aquatic environments has further enhanced this interest (Aubry et al. 2017).

In annual killifish, the inability of sufficient swim bladder inflation was related to inappropriate egg incubation (Wourms 1967, Blažek et al. 2013) and suboptimal water chemistry during hatching (Podrabsky 1999). Killifish with swim bladder inflation problems reside on the bottom of the aquarium, move in a conspicuous way characterized as 'jumping' or 'bouncing' and have acquired the colloquial name 'belly sliders' (Genade et al. 2005, da Fonseca et al. 2018), which we use henceforth.

Prior to this study we made tentative diagnoses of mycobacterial infections based on transmission electron microscopy of 10 'belly slider' spleen samples obtained from the young adults of *Nothobranchius furzeri* (GRZ population, see Fig. 1A). A sudden increase in the occurrence of 'belly sliders' in *Nothobranchius* fishes in our breeding facility prompted us to focus on the aetiology of swim bladder problems in individuals which rapidly lost their previous non-problematic neutral buoyancy.

2. MATERIALS AND METHODS

2.1. Husbandry

Hatching, housing and maintenance of fish in communal tanks followed the protocol by Polačik et al. (2016). In short, a mixture of peat (spawning substrate) and eggs was removed from parental aquaria to wet paper and left to dry for 24 h. Eggs were incubated in sealed plastic bags with peat for several months in an incubator at 19°C. Hatching was stimulated by pouring water (15–17°C) over peat with fully developed eggs in glass hatching tanks. After 1–4 d, hatched fish were placed to larger tanks with a water temperature of 24–28°C. Fish were fed *Artemia* nauplii for the first 10–14 dph (d post hatching) and later weaned on bloodworms (frozen *Chironomus* larvae).

2.2. Histology

Only individuals that exhibited behavioural abnormalities suggestive of swim bladder inflation problems following fully functional swimming performance at a younger age were histologically examined. A total of 134 juvenile and adult *Nothobranchius* fishes of 2 species and 4 populations reared in laboratory conditions were used in this study (Table 1). Fish were fixed with 10% neutral buffered formalin and processed for histology using the standard paraffin technique. Mid-sagittal and parasagittal sections through the entire fish bodies were stained with hematoxylin and eosin (H&E) supplemented with Ziehl-Neelsen (ZN) staining for acid-fast bacteria. Given the age-cohort specific nature of our findings, the results are presented separately for juvenile and adult fish. Fish were considered adult once all males in a specific cohort had fully developed nuptial coloration.

2.3. Bacterial species determination

The bacterial species affecting the GRZ cohort investigated herein were subjected to molecular identification after completion of the histopathological study (i.e. at older age), by full length 16S rRNA analysis of 4 frozen kidney tissues, in accordance with Dyková et al. (2021). Bacterial DNA was extracted using ZymoBIOMICS DNA Miniprep Kit (Zymo Research); 16S metagenomic libraries were prepared by LoopSeq™ 16S Microbiome SSC 24-Plex Kit (Loop Genomics) and sequenced on NextSeq (v2.5 reagents, 300 cycles). Sequence results were processed through the SILVA138.1 Small Subunit rRNA Database and Global Catalogue of Microorganisms (Type Strains Genome Database). 16S rRNA hypervariable regions V3/V4 were analysed according to Klindworth et al. (2013).

3. RESULTS

Approximately 90% of 134 fish examined had mycobacterial infection (Table 2) as shown with ZN staining (Figs. 1 & 2). Damage caused by mycobacteria in organs other than the swim bladder differed between juvenile and adult fish. Molecular findings included *M. marinum* CCUG 20998 and DSM 44344 in 1 GRZ sample and *M. chelonae* subsp. *gwanakae* (MOTT36W, the type strain) and *M. saopaulense* (ID EPM 10906 and CCUG 66554) in 3 GRZ individuals.

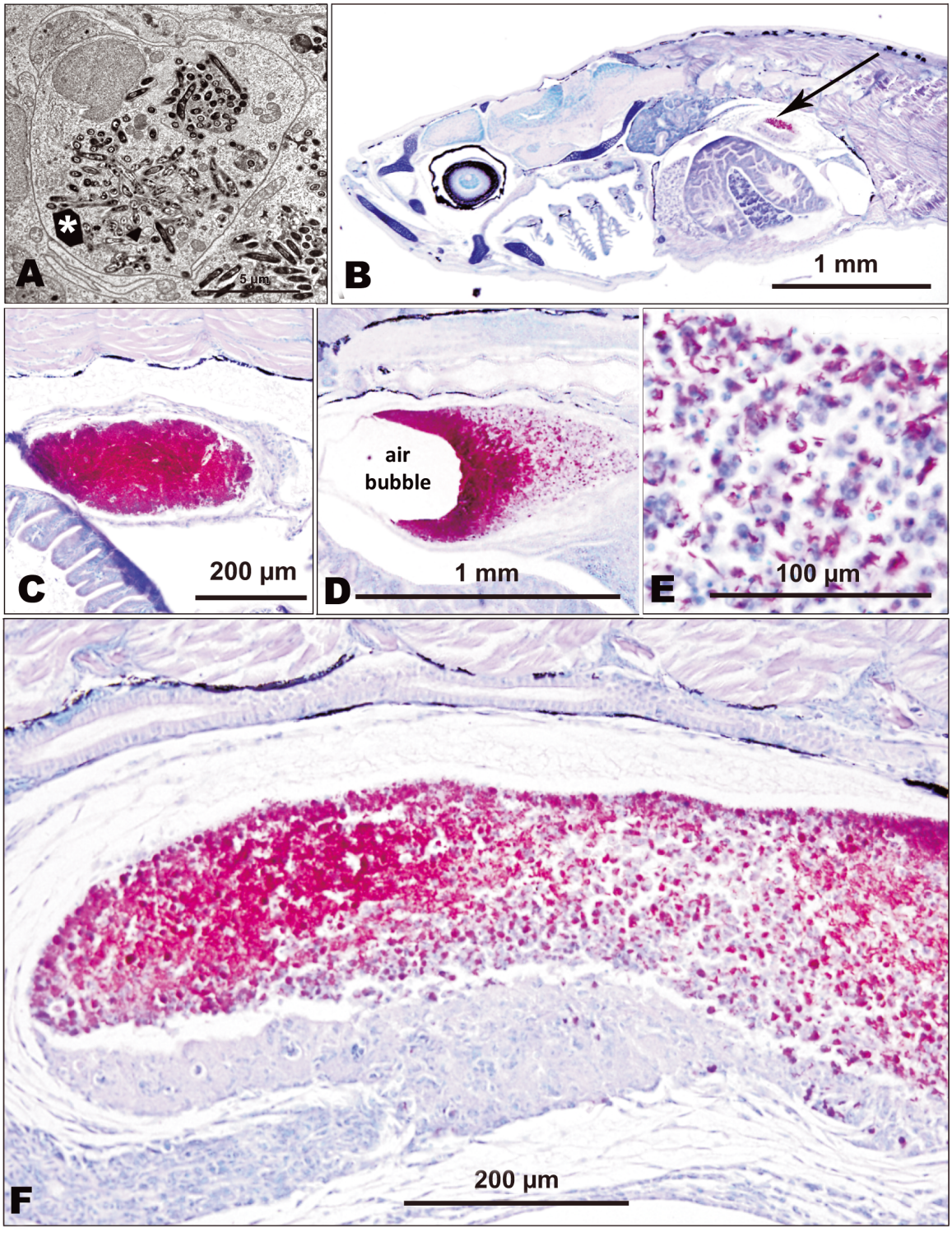

Fig. 1. Mycobacterial infections in juvenile *Nothobranchius furzeri* swim bladders shown in (A) electron and (B–F) light micrographs. (A) Spleen macrophage from *N. furzeri* examined 32 d post hatching (pilot study, not part of cohort study). Cytoplasm of the cell contains large numbers of suspected mycobacteria together with electron-dense crystals (*). (B) Parasagittal section through anterior part of 9 d old fish. Arrow: infected swim bladder. Ziehl-Neelsen staining visualizes mycobacteria in red. (C) Densely packed mycobacteria completely fill swim bladder cavity in 10 d old fish. (D) Mycobacteria crowded around air bubble in 12 d old fish. (E) Intra- and extracellularly localized mycobacteria together with macrophages and cell debris characterize moderate infection. (F) Extensive infection in 12 d old fish

Table 1. Histologically examined *Nothobranchius* 'belly sliders'. dph: d post hatching; N_{invest}: no. of individuals with at least 1 examined organ; N_{swb}: no. of investigated swim bladders. Some tissues were lost or contained artefacts which prevented proper diagnosis; thus, number of investigated fish and investigated tissues may differ. Exact number of investigated tissues given in Table 2

Nothobranchius sp. (population)	Age (dph)	Age cohort	N_{invest}/N_{swb}
N. furzeri (NF 121)	8–14	Juvenile	46/44
N. furzeri (GRZ)	32–40	Adult	70/57
N. furzeri (NF 222)[a]	48	Adult	4/4
N. furzeri (NF 222)[a]	67	Adult	5/5
N. guentheri[b]	53	Adult	11/11

[a]Different cohorts; [b]obtained from a hobbyist

Table 2. Proportions of *Mycobacterium* infected organs in *Nothobranchius* fish according to age cohort. Pos: positive: neg: negative; na: not assessed

Organ	Juvenile (%) (pos/neg)	Adult (%) (pos/neg)
Swim bladder	93 (41/3)	90 (69/8)
Kidney	0 (0/44)	71 (60/25)
Liver	0 (0/44)	47 (41/47)
Spleen	na (0/0)	71 (20/8)

3.1. Histopathology of juvenile *Nothobranchius* belly sliders

All 44 juvenile individuals (aged 8 to 14 dph) had altered swim bladders but no lesions in other body organs (Table 2). Their swim bladders contained mycobacteria in variable densities, intermixed with cell debris, young monocytic cells and phagocyting macrophages (Fig. 1). Mycobacteria in dense masses were present in 93% of individuals (N = 41) whereas absence of mycobacteria was rare (N = 3). Extremely dense ZN-positive masses were found to surround a residual free space of swim bladders with a corresponding limited volume of air (Fig. 1D).

3.2. Histopathology of adult *Nothobranchius* belly sliders

In all 90 adults (aged 32 to 67 dph) swim bladder lesions were of the same obstructive nature as in the juveniles. The composition of swim bladder contents differed among individuals in terms of the density of mycobacteria and in proportion, type and condition of infiltrated host cells. Host inflammatory response was dominated by macrophages. Together with mononuclear precursors (young phagocytes), macrophages were extremely abundant in swim bladders and participated in the formation of necrotic masses. The swim bladder wall was usually thickened due to connective tissue response and proliferation of secreting/resorbing epithelial cells.

In adults, mycobacterial infection had a systemic nature with a pronounced affinity for the kidney and liver (Table 2). Mycobacteria replicated in the kidney interstitium in a diffuse manner, caused proliferation of young mononuclear phagocytes and massive accumulation of macrophages which slightly reduced excretory structures of the kidney. Severe mycobacterial infections were also detected in 71% of the histologically examined spleen samples (only severely enlarged spleens were sectioned, N = 28; Table 2).

In the liver, mycobacteria densities were lower than in kidney and concentrated mostly in macrophage centres. In both these organs there were foci of inflammatory reaction with the most pronounced alterative component of this process, i.e. necrosis. There were no signs of a granulomatous organization of inflammatory lesions which were previously seen in livers of zebrafish infected by *M. haematophilus* (Whipps et al. 2007).

The image of systemic infection of adult belly sliders was completed by additional rare findings in the submucosal layers of the pharynx and intestine (Fig. 2A) and in the epithelial lining of the intestine (Fig. 2B), which implies that the digestive tract could also play the role as a portal of infection entry. The visualisation of mycobacteria in the central nervous system (Fig. 2C), connective tissue of intrahepatic islet of exocrine pancreas (Fig. 2D), capillary networks (rete mirabile) of the eye, gas gland, and pseudobranchia (Fig. 2E) underlined the role of high-level bacteraemia and haematogenous spread of infection.

4. DISCUSSION

Our histopathological examination of juvenile and adult *Nothobranchius* 'belly sliders' yielded mycobacterial infection as the key finding. The positive ZN staining results obtained in our study narrows the spectrum of candidate agents to acid fast and partially acid fast rod-shaped actinobacteria (Whipps et al. 2007, Gauthier & Rhodes 2009). The molecular analysis performed on subsample of 4 fish narrowed this to *Mycobacterium marinum*, *M. chelonae* and *M.*

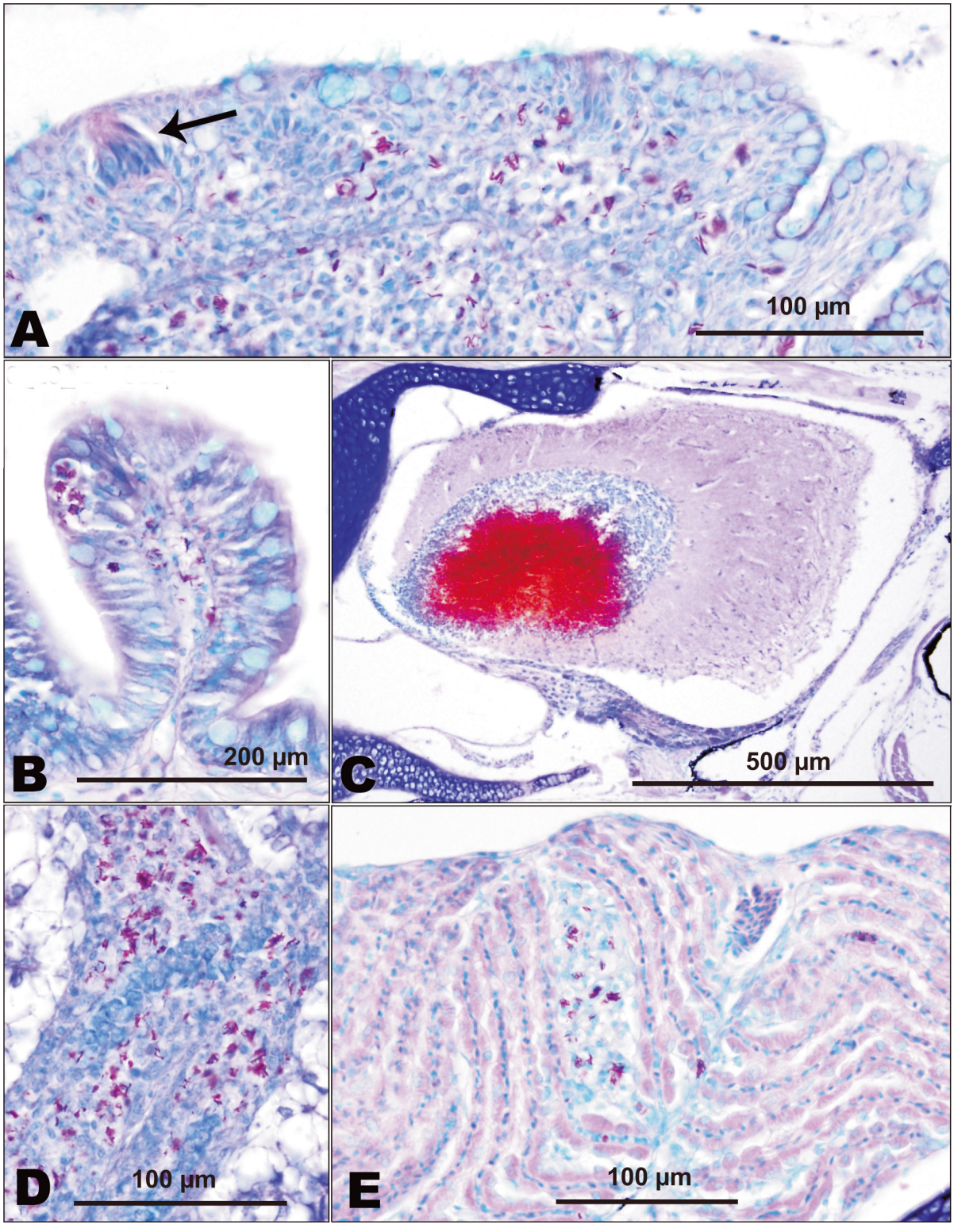

Fig. 2. Mycobacterial infections of adult *Nothobranchius furzeri* (GRZ) seen in organs other than swim bladder. Ziehl-Neelsen staining visualizes mycobacteria in red. (A) Mycobacteria individually scattered in subepithelial connective tissue of digestive tract, between pharynx and esophagus. Arrow: taste bud. (B) Mycobacteria localized in epithelial lining of intestine. (C) Severe alteration of brain optic lobe. (D) Mycobacterial clusters seen in connective tissue around intrahepatic islet of exocrine pancreas. (E) Mycobacteria scattered between pseudobranch lamellae

saopaulense. The finding of mixed infection by multiple *Mycobacterium* species in some fish complicates the assignment of our histopathological findings to the single pathogen. Mycobacteria are widely distributed in the environment, strictly aerobic and have a growth optimum in temperatures close to the water temperature in killifish breeding facilities (24–28°C; Beran et al. 2006, Polačik et al. 2016, Aubry et al. 2017). We speculate that the substantial difference in affected organs between juvenile and adult cohorts in the present study may be attributed to progress of the infection as fish age, to different sources of infection for juvenile vs. adult killifish or to age-specific defence mechanisms. Thus, possible causes and the pathogenesis of swim bladder alterations associated with function impairments in all age cohorts deserve more attention.

The problem of swim bladder non-inflation is not constrained to early life stages. We previously documented adenocarcinoma as a cause of 'belly sliding' in senescent adults of African annual killifish (Dyková et al. 2020). A detailed study on histopathology of post-hatching swim bladder non-inflation associated with bacteria was published for *Stizostedion vitreum* larvae (Marty et al. 1995). In agreement with our preliminary *Nothobranchius* study (Fig. 1A), Marty et al. (1995) described the bacteria as rods but could not apply the specific ZN staining for mycobacteria in resin embedded material. The histopathological study of the swim bladder *Austrolebias nigrofasciatus* 'belly sliders' (da Fonseca et al. 2018) also did not include the specific staining for mycobacteria. In contrast to those 2 works, which investigated fish with swim bladder non-inflation early in life, we demonstrate that fish, which successfully inflated their swim bladder early in life, later lost the ability of air regulation in their swim bladder due to *Mycobacterium* spp. infection at an individual-specific age.

The symptoms in *Nothobranchius* spp. appear as an increased frequency of pectoral fin movements and an upward head position as the infected individual struggles to maintain neutral buoyancy in the water column. This initial phase lasts hours in juveniles to days in adults. Juvenile belly sliders usually die within a few days after displaying first symptoms, whereas adult belly sliders may survive several days to weeks.

Among possible sources of mycobacterial infection in laboratory-reared fishes, contamination of the aquarium environment is considered essential (Beran et al. 2006). In annual killifishes, the consumption of contaminated food poses a possible risk of infection. Aquatic invertebrates may serve as the primary or concurrent agents of infections (Chang et al. 2019, Davidovich et al. 2020). In view of this, bloodworms used as a sole food source for laboratory annual killifish, although treated by freezing, pose a serious risk for distribution of mycobacteriosis. The control of mycobacteria in chironomids by ZN staining faces a detection problem because the principal compound (fenolized fuchsin) binds to some chironomid structures. In order to reduce risk of mycobacterium introduction and to improve research replicability (Barnard et al. 2009), the introduction of a formulated diet is required.

Hatching of annual killifish from parent-contaminated peat can be another source of mycobacterium infection. Annual killifish embryos need at least a brief non-aquatic period, and various humid peats are used as incubation substrata (Polačik et al. 2016, Dodzian et al. 2018). It is likely that humid peat allows the survival of *Mycobacterium*. This explains our observations where fish were already infected by *Mycobacterium* at the age preceding introduction of bloodworms to their diet (<10–12 d). Previous protocol developed by Polačik et al. (2016) did not recognized the risk of *Mycobacterium* as a pathogen and neglected the possibility of contamination from spawning substrate. Hence, we recommend following a step introduced by Dodzian et al. (2018), which is the careful use of sterile egg incubation media to prevent brood contamination. We recommend egg sterilization prior to fish hatching (prior to or during the diapause DII stage) using bleach (Polačik et al. 2016) or peracetic acid (Zusková et al. 2011) when following the protocol of Polačik et al. (2016) for hatching *Nothobranchius* fishes.

5. CONCLUSIONS

Histopathological lesions observed in *Nothobranchius* juveniles imply that (1) contact with mycobacteria happens at the initial attempts of fish to inflate their swim bladder; (2) mycobacteria extensively proliferate in the swim bladder; (3) swim bladder lesions can be severe enough to cause 'belly sliding' and mortalities of juvenile and adult individuals; (4) the systemic nature of mycobacteriosis in adult fish is attributed to hematogenous spread of infection, in which both the swim bladder and digestive tract can be the primary sites of infection; and (5) spontaneous mycobacterial infections are valuable for comparison with host defence reactions to intraperitoneally inoculated mycobacteria. Our results point to the importance of continuous screening for

the presence of mycobacterial infection in breeding facilities, and, as part of a screening strategy, the test of adult belly sliders for mycobacterial load is suggested. The careful sterilization of embryos prior to annual killifish hatching, replacement of live food by formulated food and discarding old mycobacterium susceptible fish may serve to prevent the spread of diseases (Mason et al. 2016).

Ethics. All procedures involving laboratory animals were conducted according to all institutional and national legal regulations of the Czech Republic.

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