INTRODUCTION

Teleost fishes with short lifespans have a special position among the fish species which are used in laboratory research. They were introduced as model organisms for ageing studies by Markofsky (1979), Markofsky and Milstoc (1979) and Cooper et al. (1983). Since then, the importance of annual killifish, especially *Nothobranchius furzeri* (the shortest-lived vertebrate currently cultured in captivity), for ageing studies has been highlighted by a series of key articles (Baumgart et al., 2015; Blažek et al., 2017; Cellerino et al., 2016; Di Cicco et al., 2011; Harel et al., 2015; Hu & Brunet, 2018; Terzibasi et al., 2007; Valdesalici & Cellerino, 2003; Wendler et al., 2015).

Studies on spontaneous lesions and the cause of death form an important part of the research on short-lived fish and annual killifish in particular. Di Cicco et al. (2011) performed an analysis of spontaneous lesions in organs of *N. furzeri* and found histopathological changes in the liver (90% of individuals), kidney (75%), heart (70%) and gonads (40%). A high proportion of these changes were considered to be neoplasias classified by these authors as hepatic and...
renal carcinomas, respectively. The diagnosis was supported by immunohistochemical and genetic markers but morphological features were not well described, being referred to as nodules of neoplastic tissue and documented in low magnification images of organ sections. The same images (Di Cicco et al., 2011) were reproduced in a key review by Cellarino et al. (2016) who, in accordance with Di Cicco et al. (2011), presented Nothobranchius fishes as organisms particularly suited for the investigation of biological and molecular aspects of ageing and ageing-associated dysfunctions. An identical classification of lesions in the livers of Nothobranchius furzeri and N. korthausae was published by Baumgart et al. (2015) who also stressed the age-related onset of hepatomas and hepatocarcinomas observed in the same organ sample. Similar opinions on liver tumours and tumour-associated kidney swellings were incorporated into a study on the intraspecific divergence in life span and ageing of African annual fishes by Blažek et al. (2017).

A different opinion on the nature of histopathological changes in the kidney and liver of ageing N. guentheri was published in an earlier, well-documented paper for its time, by Cooper et al. (1983). These authors confirmed previous observations by Markofsky (1979) and Markofsky and Milstoc (1979) and classified histologically similar cell aggregates found in the kidney and liver as a nodular type of histiocytic lymphoma.

Studying these problems, one should not ignore the potential role of mycobacteria, which are ubiquitously distributed microorganisms and a known problem also in zebrafish research facilities and cyprinodont fish used for toxicological and carcinogenicity assays (Kent et al., 2004; Watral & Kent, 2007; Whipp et al., 2012). In terms of our objectives, one of the most relevant studies is that by Broussard et al. (2009) who experimentally tested cancer risk associated with chronic Mycobacterium marinum infections in Japanese medaka Oryzias latipes (Temminck & Schlegel, 1846). Results of these experiments did not prove a direct correlation; however, it revealed mycobacteria in liver lesions similar to those classified in the above-mentioned Nothobranchius studies as hepatic carcinomas.

Despite intensive exploitation of Nothobranchius fishes as emerging model organisms for research on ageing (Hu & Brunet, 2018; Platzer & Englert, 2016), significant gaps in the knowledge of cellular and tissue manifestations of Nothobranchius disease conditions are apparent. In order to supplement such data, we studied spontaneous histopathological lesions in laboratory-reared Nothobranchius fishes with the aim of obtaining a general overview of spontaneous and proliferative lesions specifically and compiling a histopathological classification of these changes.

2 MATERIAL AND METHODS

2.1 Fish

The principal research subjects of the study were two strains (MZM 0410 studied in 2019 trial and GRZ studied in 2020 trial) of the turquoise killifish Nothobranchius furzeri (N = 247) bred according to the protocol by Polačik et al. (2016). Adult fish were fed once daily ad libitum. A pelleted diet (Biomar Inicio) was fed to one-third of the fish in the GRZ trial, one-third fed Grygera bloodworms and the remaining third Petra-Aqua bloodworms. The MZM trial was fed Grygera bloodworms. The diet did not have a significant effect on our findings (Table S1) and thus was not considered in our final conclusions. Comparative material (N = 80) was obtained from Nothobranchius fishes bred in 2013, the housing details of which are presented in Blažek et al. (2017). Complementary material contained Nothobranchius fishes (N = 84) examined in diagnostic services rendered by us to local fish breeders (housing not known) in 2000–2010 (Table 1).

2.2 Material examined

Histopathological examination included samples of the kidney, liver, spleen and, if indicated, swim bladder of Nothobranchius furzeri MZM 0410 strain (MZM hereafter) collected in six samplings from individuals aged 6-21 weeks (N = 78) and of N. furzeri GRZ strain sampled at the terminal age of 12-19 weeks (N = 169). This recently collected material from 247 individuals was supplemented by re-examination of previously sectioned material on organ lesions of a total 411 Nothobranchius fishes (Table 1).

2.3 Sample collections

The sampling methods for histological examinations applied in MZM and GRZ strains consisted of euthanasia with an overdose of clove oil, fast, careful and sterile removal of the left-side body wall followed by organ size scoring of the kidney, liver and spleen of each individual. Kidney size was scored on 5-point ordinal scale (0—normal size, 1—mild, 2—moderate, 3—severe and 4—extreme kidney enlargement). Liver and spleen size was scored binomially (natural size/enlarged organ). Colour changes of liver and spleen were recorded binomially as natural or altered colour. After organ scoring and extraction, organs were divided into two parts, one of which was frozen in dry ice and stored for future genetic studies (not part of this study) while the other was fixed for histopathological study.

2.4 Sample preparation for histology

MZM organ samples, divided into subsamples of suitable size, were fixed in 2% osmium tetroxide, dehydrated in acetone dilution series and embedded in Spurr resin for examination of semithin and ultrathin sections using light or electron microscope, respectively. GRZ organ samples were fixed in 10% neutral-buffered formalin (NBF) and processed using the standard paraffin technique. The same technique was applied to samples from 2013 and archived material from diagnostic history of N. furzeri, N. kadleci and N. orthotus (see Table 1) fixed in NBF or Davidson fixative. All paraffin
sections evaluated in the study were stained with haematoxylin and eosin (H&E). Ziehl–Neelsen (ZN) differential staining (Roberts, 1978) of parallel/semiserial sections was used for identification of acid-fast organisms such as members of the genus *Mycobacterium*. Bacterial presence in MZM was noted when rod-shaped bacteria were detected in ultrathin sections of MZM during slide examination. 

### 2.5 | Histological evaluation

The evaluation of histopathological changes was based on the generally accepted diagnostic criteria for degenerative, inflammatory, proliferative non-neoplastic and neoplastic lesions using the standardized nomenclature for proliferative and non-proliferative lesions in fish (Boorman et al., 1997; Goodman, 2007; Keenan et al., 2015; Spitsbergen et al., 2012; Thoolen et al., 2010; Wolf et al., 2015) and the specific criteria for distinguishing hyperplastic from neoplastic lesions (Fournie et al., 2005). Our stepwise diagnostic process followed the recommendation of Orazi (2007) and consisted of screening for the presence (“yes/no”) of proliferative lesions, decision on the proliferation type (neoplastic/non-neoplastic), detection of the reactive condition if present and classification of neoplastic proliferation using comparative data available in advanced studies of model tetrapod vertebrates, rats and mice (Thoolen et al., 2010).

### 2.6 | Species determination of mycobacteria

A partial determination was undertaken of *Mycobacterium* species in kidney tissue samples of 11 *N. furzeri* (4 GRZ and 7 MZM). For full-length 16S rRNA analysis, bacterial DNA was extracted from frozen tissues using ZymoBIOMICS DNA Miniprep Kit (Zymo Research) according to the manufacturer’s instructions. 16S metagenomic libraries were prepared using LoopSeq™ 16S Microbiome SSC 24-Plex Kit (Loop Genomics) and sequenced on NextSeq (v2.5 reagents, 300 cycles) to finally generate long-read sequences (~10,000 molecules per sample). Raw reads were processed following the standard Loop Genomics protocol for full-length 16S sequencing. 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).

For whole-genome metagenomic analysis, DNA was extracted from frozen tissues using DNeasy Blood & Tissue Kit (QIAGEN) according to the manufacturer’s instructions. Libraries were prepared using Truseq DNA PCR-free Low Throughput Library Prep Kit (Illumina) and sequenced using Illumina NovaSeq 6000 System (NovaSeq 6000 S2 Reagent Kit, 200 cycles). Raw sequencing data were mapped to the NFU reference NFZv2.0 (Bradshaw & Valenzano, 2020) using the BWA tool (Li, 2013). Unmapped reads were extracted using samtools (Li et al., 2009). An accurate strain-level microbial profiling was performed using the MetaPhlAn tool (Beghini et al., 2020) on extracted reads with the latest database of unique clade-specific marker genes (i.e., mpa_v30_CHOCOPhAn_201901). Based on the results, a new alignment was set using BWA on unmapped reads to the particular genomes.

### 2.7 | Statistical analysis

Statistical comparisons of gross necropsy findings and histological findings were done for cohorts of MZM and GRZ, for which appropriate sample sizes were available. In each case, backward selection of the most parsimonious model was performed based on removal of non-significant variables from the model. Overviews of full models, the most parsimonious model and their Akaike information criteria (AIC) are provided in Table S1.

Age dependency of kidney size in MZM was analysed by ordinal generalized additive model (GAM) with kidney size as the ordinal response variable (score 0–4, numbered by one to avoid zeroes).
and sex (male/female) and health status (healthy/moribund) as parametric explanatory variables and age (continuous, k = 4) as a semi-parametric explanatory variable. Kidney tissue proliferation in the pooled sample of GRZ and MZM trials and age-dependent kidney tissue proliferation in the MZM trial were analysed by ordinal GAM with kidney tissue proliferation score as the response variable. Liver tissue proliferative changes were analysed by binomial generalized linear model (GLM) only for the GRZ trial, where a reasonable number of proliferative changes was detected. Proliferative change was the response variable (coded as absent/present; macrophage proliferation, mononuclear cell aggregates and necrosis were pooled together as “present”). The details of all full and most parsimonious models are provided in Table S1.

2.8 | Ethics

All methods and procedures were carried out in accordance with relevant guidelines and regulations of the Czech Republic. Experimental facility and handling protocols were approved according to national laws no. 246/1992 and no. 419/2012 and by Ministry of Agriculture (breeding facility no. CZ 62760203, permit approval document 62116/2017-MZE-17214 dated 20 October 2017). The study species are not endangered or legally protected in the wild. All examinations were completed on captive individuals.

3 | RESULTS

3.1 | Gross necropsy findings

Gross lesions observed in *Nothobranchius* individuals of MZM and GRZ strains included changes in the size, shape and colour of the liver, kidney, spleen and swim bladder. Hepatomegaly was regularly associated with a strikingly light/yellow colour of the parenchyma. Renomegaly was frequently found in old MZM adults from the age of 12 weeks, especially in moribund fish (Figure 1a, Table S2). The regular wheat-grain form of the normal kidney was frequently distorted into an asymmetric form with prominent light bulges. Splenomegaly and abnormalities of the swim bladder were found less frequently. Swim bladder abnormalities manifested as a thickening and whitening of the wall, a filling-up of the lumen with a semiliquid material and an enlargement of the gas gland. Detailed histopathological investigations on the swim bladder may be found in Dyková et al. (2020) and Dyková et al. (2021).

3.2 | Histopathological findings

The results of histopathological examinations are summarized for corresponding organs of all study sets despite slight differences in methodology, because the changes in corresponding organs coincided in type. In fact, the study of the MZM cohort based on light microscopy of semithin and electron microscopy of ultrathin sections, the results of which were obtained first, instigated questions about the aetiology of lesions and determined the choice of method for the GRZ study and re-examination of archived material.

3.2.1 | Kidney

Light microscopy of H&E and toluidine blue-stained sections (available from MZM cohort and archived material) revealed proliferation of haemopoietic tissue in the small cranial part of the kidney (corresponding to the head kidney of other bony fish) that was manifested as a monomorphic population of cells with markedly basophilic nuclei (Figures S1–S3). The same type of tissue proliferation was observed in the excretory region of the kidney, together with proliferation of less densely stained mononuclear cells strikingly reminiscent of macrophages (Figure S2a,b). The proliferating cell populations caused an atrophy of glomeruli, displacement of renal tubules and a substantial reduction of their number (Figure S1b–d, S2a). In the most advanced stages of this pathological process, the remnants of altered renal corporcles were surrounded by a narrow zone of more basophilic mononuclear cells and an abundant population of mononuclear cells resembling macrophages (Figure S2b). They predominated in the foci of bacterial infections seen clearly in the toluidine blue-stained sections. In total, bacterial infections were found in semithin sections of 26% of asymptomatic and 57% of moribund MZM fish, respectively. The intensity of interstitial kidney tissue proliferation reflected quite accurately the semi-quantitative scale of the grossly visible enlargement of this organ in a different manner for kidneys with bacterial presence (Ordinal GAM, edf = 2.871, $\chi^2 = 112.49$, p < .001, Figure 1b, Table S3 or without (edf = 2.321, $\chi^2 = 48.96$, p < .001). The extent of proliferative lesions was not a function of age in MZM individuals (ordinal GAM, edf = 1.00, $\chi^2 = 1.09$, p = .296, Table S4); however, the findings within a specific age group were not always uniform, suggesting variability in individual reactions.

The parallel H&E and ZN stainings of semiserial sections confirmed the association of proliferative lesions with mycobacterial infections in GRZ (ordinal GAM, estimate [SE] = 1.17 [0.43], z = 2.70, p = .007, Table S5; Figure 2a,b), and females were more affected by kidney proliferation (1.00 [0.33], z = 3.05, p = .002). The intensity of mycobacterial infections (visually estimated density of bacteria in histological sections) varied from quantities in the field of view only just countable to innumerable mycobacteria forming densely packed masses (Figure S3a–c). Maximum densities of mycobacteria were observed in severely proliferated interstitial kidney tissue with predominating macrophages (Figure 2a,b, Figure S3). The latter represented an intense host defence reaction and simultaneously caused the displacement and effacement of renal tubules. The areas of interstitial tissue proliferation were not sharply delimited; they consisted of mononuclear cells with densely stained nuclei (supposed precursors of macrophages) closely surrounding renal tubules, mononuclear
macrophages with mycobacteria in the cytoplasm and cell debris intermixed with extracellularly localized mycobacteria. In the course of infection, a misbalance between intensely replicated bacteria and their phagocytic killing was observed. At moderate-to-advanced stages of infection, the host reaction dominated by macrophages was supplemented with atypical granulomatous inflammatory foci. They were characterized by a pronounced alterative/necrotic core and a poorly organized periphery with a minimum walling-off and absence of epithelioid cells. Severe lesions were also observed in the nephrons. Mycobacteria were found to accumulate in dilated Bowman’s spaces of the renal corpuscles (Figure S3d,e) and also formed dense clusters/casts in the renal tubules (Figure S3f). Similar findings in the collecting ducts implied an important means of spreading infection.

3.2.2 | Spleen

Spontaneous lesions grossly manifested as splenomegaly and colour changes were found only in adult fish, after the age of 9 weeks (Figures S4,S7). Compared with age-matched apparently healthy fish of three Notobranchius spp. (Figure S4a), the inner architecture of the spleen in infected MZM individuals was significantly changed in that the red pulp was substantially reduced while the white pulp proliferated diffusely (Figure S4b). The distinction between the subcapsular layer of the red versus the white pulp was not clear due to the replacement of erythrocytes in sinusoids by a relatively uniform population of mononuclear cells (Figure S4c). Advanced necrotic changes, together with numerous macrophages containing remnants of senescent cells removed from circulation or engulfed bacteria, were frequently observed in central areas of the organ (Figure S4d). As in the kidney, histopathological lesions in the spleen indicated a severe impairment of its haemopoietic function. The mycobacterial aetiology of the lesions inferred from electron microscopical examination of MZM (Figure S7d) and several extra GRZ samples was confirmed by positive ZN staining in all paraffin sections of the GRZ set. Mycobacteria were responsible for destruction of splenic architecture manifested by an almost complete disappearance of the red pulp, loss of vascular integrity, alteration of ellipsoids that trapped bacteria and domination of large atypical granulomatous lesions characterized by a conspicuous necrotic core with a minimum host tissue reaction rim on their periphery.

3.2.3 | Liver

The examination of the liver parenchyma revealed a series of non-proliferative and proliferative changes, both types usually including disappearance of the polygonal configuration of hepatocytes and loss of their cord arrangement (Figures S5–S8). The most commonly observed (85% of GRZ and MZM trials pooled) non-proliferative lesions were micro- and macro-vesicular vacuolations of hepatocytes consistent with rounded, sharply outlined lipid vacuoles, a glycogen-type vacuolation characterized by large, irregular vacuoles and hydropic degeneration (see figure 3 in Žák et al., 2020). Non-uniform staining of the hepatocyte cytoplasm was rarely seen.

Contrary to expectations, nodular proliferative hepatocellular lesions were found in only 2 of the 411 fish examined (Figure S5a). Light microscopy allowed for identification of these lesions as hepatocellular
adenomas or focal nodular hyperplasia (cf., Goodman, 2007). There was a simultaneous mononuclear/monocytic cell proliferation in and among organs of the body cavity in both these cases (Figure S5a,b).

The most frequent (34% from GRZ and MZM together) and typical finding of proliferative changes in the liver parenchyma was a proliferation of cellular components other than hepatocytes (Figure S6). In the liver of young individuals, always in close association with blood vessels, solitary structures composed of cells of uniform size were observed, with a delicate capsule and minimum pigment content. These were mononuclear cell aggregates (MAs) morphologically closely related to macrophage centres (MMC sensu Roberts, 1978) alternatively known as pigmented macrophage aggregates (PMA sensu Wolf et al., 2015) or mononuclear phagocyte centres (MPC sensu Wittamer et al., 2011) belonging to the teleost system of innate/nonspecific immunity (Figure S6c). A noticeably increased (albeit not statistically analysed) number of enlarged MAs were observed in older individuals (less frequently in the MZM fish cohort than in NF 222, NF 121 and NK 91). The shape of these MAs was either indistinctly defined or had distinct contours due to the surrounding oedematous changes. A lymphoid cell rim on the MA periphery was found in only one case. In large MAs, there were mild-to-severe oedematous changes leading in advanced stages to the development of multilocular or large unilocular pseudocysts in the liver parenchyma. In some MAs, there were phagocytosed bacteria even if a focus of bacterial infection was not found in the liver (Figure S7a,b). Bacterial infections were confirmed in 26% asymptomatic and 57% moribund MZM fish, respectively. Some cell aggregates containing phagocytosed bacteria were necrotic; however, typical granulomas (a common fish reaction to antigens concentrated by macrophages) were observed only once.

As in the kidney, mycobacterial infection diagnosed in the GRZ cohort was also found to be responsible for proliferative histopathological lesions in the liver (Binomial GLM, estimate [SE] = 2.59 [0.41], z = 6.38, p < .001, Table S6). Ziehl–Neelsen staining visualized a transformation of MAs into typical macrophage centres (MC/MPC) in which a high proportion of macrophages characterized by indistinct cytoplasmic borders and abundant cytoplasm with phagocytosed mycobacteria prevailed (Figure 3a–d). Within

FIGURE 2  Semiserial sections through kidney parenchyma with two renal corpuscles (RC), proliferated interstitial tissue, several convoluted tubules and blood vessel (BV). (a) Section is stained with H&E; parallel section (b) stained with ZN shows dense concentration of mycobacteria in proliferated interstitial tissue rich in macrophages.
these structures, densities of mycobacteria ranged from minimal to moderate, but in general, densities were lower than those in corresponding kidney interstitial tissues (compare Figure 3 and Figure S3). The activation of the mononuclear phagocyte system was manifested in the size of MC and, even more, in their increased abundance. Low numbers of MC characteristic of the initial stages of infection explain lower prevalence of proliferative lesions in the liver (53%; 90 of 169 in GRZ) compared with the kidney (70%). Despite the fact that the changes in the liver and kidney developed in parallel and were dominated by macrophages in both organs, histopathological features differed in the initial stages of infection. They were of diffuse nature in the kidney, whereas in the liver, the less intensive changes were of focal nature, being restricted to MC. In advanced stages dominated by necrotic changes of formerly proliferating cells, lesions in both organs were similar. They represented atypical granulomatous lesions known in infections caused by non-tuberculous mycobacteria.

3.2.4 | Other body cavity organs

Mononuclear cells were also frequently found to proliferate in the body cavity, with devastating impact on the gonads and swim bladder (Figure S9a–c, S10). As in the kidney and liver, the proliferation of monocytes/macrophages was associated with mycobacterial infection. In swim bladders filled almost completely with macrophages containing mycobacteria, proliferation of gas gland epithelium was also observed. In exceptional cases, this proliferation was of neoplastic nature (Figure S11). The connective tissue surrounding an altered swim bladder usually contained a granuloma bearing a striking resemblance to cholesterol granulomas described, for example, in the rat liver (cf. Thoolen et al., 2010, Figure S10a). Among the details which support the systemic character of mycobacterial infections, an incidental finding of severe alteration of the cardiac muscle points to the need for systematic study of the heart (Figure S10b).

**FIGURE 3** Liver parenchyma in GRZ strain of *Nothobranchius furzeri* infected with mycobacteria (a–d), invisible in H&E-stained monocyte/macrophage centres/cell aggregates (a,b), suspected in semithin section stained with toluidine blue (c), and well demonstrated in (d) section parallel to (b) that was stained with ZN.
3.3 | Agents of mycobacterial infections

In kidney tissue samples of the 11N. furzeri (4 GRZ and 7 MZM), three species were determined. *Mycobacterium marinum* was determined in all 7 MZM and one (25%) GRZ samples based on identity of full-length 16S sequences with *M. marinum* CCUG 20998 and DSM 44344. Determination in the GRZ individual was confirmed by analysis of variable V3/V4 regions. In 3 (75%) GRZ individuals, more than 99.9% of 16S reads completely mapped to 16S rRNA of *M. chelonae* subsp. *gwanakae* (MOT36W, the type strain) and of *M. saopaulense* (ID EPM 10906 and CCUG 66554).

4 | DISCUSSION

Histopathological lesions observed in our assemblage of *Nothobranchius* fishes were relatively easy to classify as either non-proliferative or proliferative. The interpretation of lesions within each of these categories was more difficult and required a careful evaluation of findings, comparison with lesions described in previous papers on histopathology of *Nothobranchius* and other fish species, and also selection of appropriate diagnostic terminology consistent with internationally harmonized nomenclature and diagnostic criteria for rat and mouse (Keenan et al., 2015; Thoolen et al., 2010) and fish (Boorman et al., 1997; Wolf et al., 2015; Wolf & Wolfe, 2005).

The interpretation of the predominating non-proliferative lesion, that is the accumulation of lipids and glycogen in hepatocytes, remained open for several reasons. In general, fish accumulate considerably more lipids/glycogen than mammals (Wolf & Wolfe, 2005) and the energy demands of annual killifish have not been specified for different periods of their short life, although sex-related differences were documented (Di Cicco et al., 2011; Vrtílek et al., 2018). The usual level of lipid accumulation observed in hepatocytes of *Nothobranchius* fishes would be classified into other fish species as dystrophy/degenerative pathological process. Considering that a high level of lipid storage was also described in individuals from wild populations (Vrtílek et al., 2018), the border between the natural/physiological condition and a pathological process is difficult to determine. Recently, the hepatocellular accumulation of foreign materials has received attention in connection with attempts to standardize the *Nothobranchius* fish diet (Zák et al., 2020).

Light microscopically discernible lesions in the kidney, spleen and liver of *Nothobranchius* fishes had in common the proliferation of mononuclear cells which either formed aggregates strikingly reminiscent of macrophage centres (MC) (predominantly in the liver) or proliferated diffusely (mostly in the kidney interstitium). Similar changes were described in earlier studies (Cooper et al., 1983; Markofsky, 1979; Markofsky & Milstoc, 1979) as "nodular degeneration progressing to cyst-like formations" (in the liver) and "nodular type of histiocytic lymphoma" (in the kidney). Aggregates of mononuclear macrophages (MAs), light microscopically identical with those documented by authors in the last decade (Blážek et al., 2017; Cellerino et al., 2016; Di Cicco et al., 2011), were presented earlier in an overview on medaka lesions induced in a carcinogenetic study (Boorman et al., 1997). In the latter study, the presence of MAs in the liver was termed “focal histiocytosis” – however, a clear distinction between the MAs and the hepatic granulomas was not presented (see figure 7 in Boorman et al., 1997, where an aggregate of macrophages is called a hepatic granuloma). A convincing example that the MAs can be distinguished from the granulomas in the fish liver was given by Wolf and Wolf (2005).

A comparison of the data obtained from the material newly collected by ourselves, those obtained by our re-examination of the material archived from the study by Blážek et al. (2017) and those published by Cooper et al. (1983), Markofsky (1979) and Markofsky and Milstoc (1979), showed that all these studies dealt with an identical type of cell aggregates in the liver, which were classified as hepatomas and hepatocarcinomas by Di Cicco et al. (2011) and later adopted in other studies (Blážek et al., 2017; Cellerino et al., 2016; Godoy et al., 2019; Součková et al., 2018). We suggest designating the cell aggregates of this type mononuclear cell aggregates (MAs) because there is no evidence of their hepatocellular origin (cf., Goodman, 2007; Hruban, 1979; Quaglia, 2018). In histopathological evaluation of proliferating cells, we decided to keep to the Wittamer et al. (2011) terminology, leaving a more detailed determination of monomorphic mononuclear cells of low-level differentiation to immunogenetic markers of a further study. We believe that this approach better corresponds to the results that could potentially be obtained on the cell types of *Nothobranchius* fishes using the light microscope.

Although the results of our study and comparison with published data prompt us to present a different opinion on the nature of the lesions, all the above-mentioned studies strongly influenced our lesion assessment approach, especially in the initial stage of research. We took into consideration non-neoplastic processes related to hematologic and immune competency of ageing fish and neoplastic processes triggered by an antigenic impulse, specifically case studies on the aetiology of plasmacytoid leukaemia in netpen-reared and wild-caught Chinook salmon (*Oncorhynchus tshawytscha*). These studies demonstrated the complexity of diagnostics and limits of histology in diagnosis of this leukaemia (Eaton et al., 1994; Eaton & Kent, 1992; Kent & Dawe, 1993; Stephen et al., 1995). The safe determination of virus aetiology of this disease required the exclusion of other suspected agents that were associated with histopathological lesions and, finally, the experimental verification of viral aetiology. Despite the dissimilarity of the fish species studied, the disease conditions histologically proven in Chinook salmon do not appear to be different from our *Nothobranchius* study. In both taxa, Chinook salmon and *Nothobranchius* fishes, the proliferation of mononuclear blasts occurred in both the haemopoietic organs and a non-haemopoietic one, suggesting leukaemia type of proliferation. The suspected neoplastic process observed in *Nothobranchius* H&E-stained sections resembled mononuclear cell leukaemia (MNCL) described in aged rats of the inbred F-344 strain by Caldwell (1999) and also mentioned in the review of splenic histopathology by Suttie (2006).
Histological examination plays a pivotal role in the accurate diagnosis of many disease processes including some tumours. In the diagnosis of others, for example, haematopoietic neoplastic diseases, histology is seriously limited. Its informative value has improved but not changed substantially since Harshbarger (1984) analysed similarities between certain non-neoplastic lesions/pseudoneoplasias and neoplasms in fish, amphibians and reptiles at The Symposium on the Use of Small Fish Species in Carcinogenicity Testing (Bethesda, MD, December 8–10, 1981).

Immunohistochemistry can support histological diagnoses and is widely used in traditional animal models and human oncology but faces difficulties in fish due to the lack of appropriate markers, antibodies and reference sections.

The combined presence of the neoplasms, bacteria and viruses was widely discussed in a comprehensive review on neoplasms of the urinary tract in fish by Lombardini et al. (2014) and a study by Burns et al. (2018). These authors drew attention to infectious aetiologies of neoplastic proliferations and agents suspected of inducing tumours in fish. Association of chronic fish mycobacterial infections with cancer was tested by Broussard et al. (2009) in a series of experimental M. marinum infections in Japanese medaka. M. marinum infections only acted as promoters potentiating hepato-cellular proliferative lesions when supported with low doses of the mutagen benzo[a]pyrene. To date, neither mycobacterial infections of Nothobranchius fishes nor their association with neoplasias (as described previously) have been studied systematically.

Our detection of rod-shaped bacteria in ultrathin sections of MZM individuals led us to recognize the limits of H&E staining in detection of suspected agents of lesions and focus on the ZN staining method that facilitated recognition of mycobacteria as agents of lesions. This staining dispelled our remaining uncertainties about the nature of mononuclear cells in the liver aggregates, allowed us to classify them as phagocytic macrophages and, despite the low level of pigmentation, assign aggregates of these cells to the category of melano-macrophage centres studied in detail in other bony fishes for almost 50 years (Agius, 1985; Roberts, 1978; Steinel & Bolnick, 2017; Wolke, 1992).

Great interest in fish mycobacterioses evidenced by the large number of original papers and specifically focused reviews permits the analysis of our results from several perspectives. Of these, the type of host response is the most important. The reaction of interstitial kidney tissue associated with mycobacterial infections in the GRZ assemblage does not differ from bacterial kidney disease (BKD) in Atlantic salmon (Bruno et al., 2013; Noga, 2006). As in BKD caused by the diplobacillus Renibacterium salmoninarum, the proliferation of haematopoietic kidney tissue caused by mycobacteria is considerable and has a substantial indirect effect on the excretory structures of this organ. Injury caused by the host’s reaction, mentioned previously by Thompson (1984), has also been observed in an advanced stage of mixed Glugea-Mycobacterium infections of Nothobranchius fishes obtained from hobby fish breeder (unpublished).

The comparison of GRZ infections with data accumulated on spontaneous mycobacterial infections in other bony fishes shows considerable similarity with those of captive syngnathids (Fogelson et al., 2017). Host reactions in syngnathid fishes were dominated by macrophages and “atypical” inflammatory reactions sensu Gauthier et al. (2003) or “defective” inflammatory reactions sensu Fogelson et al. (2017). Special terms were used to emphasize that inflammatory reactions differ from typical chronic granulomas described in other teleosts including zebrafish (Kent et al., 2004) in the absence of concentrically arranged connective tissue/spindle-cell capsule demarcating the necrotic core of the lesion from surrounding tissue. By understanding histopathological changes as indicators of immune functions in vertebrates (including fish), we can assume that manifestations of innate immunity dominated in short-lived Nothobranchius fishes, whereas adaptive immune cells probably did not receive strong enough signals to induce a typical granulomatous inflammatory reaction.

Spontaneous and experimental mycobacterial infections in fish are not easy to compare, because the entry routes of infection are different (spontaneous/natural vs. intramuscular or intraperitoneal) and also infectious doses differ (unknown vs. precisely calculated doses of agents administered in experiments). Comparison of our findings with published studies demonstrates an exceptionally high level of mycobacterial infectivity, that is transmissibility and the ability of mycobacteria to reproduce in the organs of Nothobranchius fishes. Whether or not these characteristics are specific to Mycobacterium species that contaminate the Nothobranchius breeding system remains to be determined. Compared with the ample data published on mycobacteria in zebrafish colonies (Kent et al., 2004; Watral & Kent, 2007; Whippy et al., 2012), our Mycobacterium species determination in 11 fish provides only a preliminary indication of possible mycobacterial agents. We intend to make an extensive determination of Mycobacterium species in our large set of cryopreserved tissue samples of both Nothobranchius strains (MZM and GRZ) of this study, expected to bring representative data for comparison with the above-mentioned comprehensive studies in zebrafish colonies.

The potential role of mycobacteria as promoters of neoplastic proliferation, as well as the possibly enhanced susceptibility to infection of inbred fish lines (such as GRZ) requires further investigation.

Considering the ubiquity of non-tuberculous mycobacteria, our results demonstrate the importance of preventing their introduction into Nothobranchius breeding facilities by any means. This includes preventing infection from biofilms on aquarium sides by careful disinfection of eggs prior to hatching (Chang et al., 2015), elimination of alimentary contamination by live or defrosted food (Chang et al., 2019; Nenoff & Uhlemann, 2006) and the development and use of practical dry food for Nothobranchius fish (Zák et al., 2020). Examples of numerous additional measures are detailed in Mason et al. (2016).

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CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT
The datasets used for the current study are available from Iva Dyková (histopathology), Jakub Žák (statistical analysis) and Kamila Součková (molecular analysis) upon request.

ORCID
Iva Dyková https://orcid.org/0000-0001-9254-5582

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