Journal of the American Association for Laboratory Animal Science
Copyright 2024
by the American Association for Laboratory Animal Science

Vol 63, No 1
January 2024
Pages 20–33

Comprehensive Colony Health Management and Emerging Pathogens of the Annual Killifish Species *Nothobranchius furzeri*

Uta Naumann,[1] Jennifer L Brazzell,[2] Marcus J Crim,[2] and Beate Hoppe[1,*]

The Leibniz Institute on Aging has maintained killifish colonies for over 15 y. Our veterinarians, scientists, and animal technicians developed a fish health scoring system and routine colony health surveillance program for our colonies. Over a 4-y period, health data from the African turquoise killifish *Nothobranchius furzeri* colony were systematically collected and analyzed. The fish health assessment system facilitated categorization of clinical signs and differentiation of fish with mild clinical signs from fish that required euthanasia. This report provides new information on clinical signs and conditions that may occur in young and aged *N. furzeri*. To be comprehensive, a colony health surveillance program incorporates animal health at both the individual and the population levels. The quarterly routine health monitoring program identified *Mycobacterium* spp. as the most common agent in our facility and identified the killifish pathogen (*Loma acerinae*) for the first time. Taken together, these findings demonstrate the importance of a comprehensive colony health management system in a fish research facility. By improving the health and welfare of fish used for research, the scientific community will benefit from less variable and more reliably reproducible research results.

Abbreviations and acronyms: *A. hydrophila*, *Aeromonas hydrophila*; EU, epidemiologic unit; H&E, hematoxylin and eosin; *L. acerinae*, *Loma acerinae*; *M. abscessus*, *Mycobacterium abscessus*; *M. chelonae*, *Mycobacterium chelonae*; *M. fortuitum*, *Mycobacterium fortuitum*; *M. gordonae*, *Mycobacterium gordonae*; *N. furzeri*, *Nothobranchius furzeri*; wph, weeks posthatching

DOI: 10.30802/AALAS-JAALAS-23-000067

Introduction

The African turquoise killifish, *Nothobranchius furzeri*,[17,26] which originated from ephemeral ponds in Zimbabwe and Mozambique, has emerged as an important model organism for aging research in recent years.[24] *N. furzeri* has an unusually rapid life cycle that has been exploited for aging research, as it satisfies important criteria for animal model selection including the availability of molecular techniques and financial feasibility.[13] As this aging model, with a maximum lifespan of 6 to 12 mo, attracts the attention of a growing number of research groups, the number of institutional fish facilities that keep and breed *N. furzeri* is increasing. Few housing and breeding protocols have been published,[7,22,25] and the scientific community is far from standardizing housing and husbandry conditions of *N. furzeri*. The Leibniz Institute on Aging has maintained *N. furzeri* colonies for over 15 y. Our fish facility uses a continuous improvement process of husbandry practices to maintain a modern facility with a large healthy fish colony.

Recently, 2 publications from a FELASA/AALAS joint working group provided recommendations regarding biosecurity, prevention of zoonosis, and health monitoring for fish used in research, including killifish.[19,20] However, only a few pathogens have been previously reported in laboratory-maintained *N. furzeri*. These include the dinoflagellate *Piscinoodinium pillulare*,[25] the microsporidium *Glugea* spp.,[25] and *Mycobacterium* spp.[10,12] The knowledge base of disease-causing agents for killifish is still incomplete, especially as it pertains to the susceptibility of young and aged *N. furzeri* to well-known fish pathogens such as *Mycobacterium* spp. or microsporidia.

A significant challenge in keeping *N. furzeri* is the fact that healthy aged fish are needed for research. Aged animals are more susceptible to diseases[3] and can pose a colony health risk, since they are kept in large numbers and are present on all racks throughout the facility.[20] Because isolation of aged animals in separate areas is usually not feasible, appropriate tools are needed to monitor the health of individual aged fish. General fish health parameters are already available,[4,16] and attempts have been made to define specific humane endpoints for fish (piscine endpoints).[1] However, a killifish-specific health score sheet that is simultaneously applicable to young and aged fish does not yet exist. Score sheets, also known as welfare assessments, serve as an essential tool for assessing and documenting the health status of individual animals and define species-specific humane endpoints. In contrast to experiment-specific score sheets, health score sheets must encompass a wider range of clinical signs, including common conditions that can occur throughout an animal's lifetime. Furthermore, health score sheets should be conducive to daily use and recording, can be used to create a comprehensive picture of animal health status, and can provide a welfare diary.

The challenge of creating a killifish-specific scoring system is to distinguish signs that reflect natural aging traits, also referred to as hallmarks of aging,[14] from those that point to a disease.[9] Fish with acute clinical signs that indicate significant suffering should be euthanized immediately, regardless of the

Submitted: 11 Jul 2023. Revision requested: 17 Aug 2023. Accepted: 12 Nov 2023.
[1]Animal Facility Fish, Leibniz Institute on Aging – Fritz Lipmann Institute, Jena, Germany; and [2]IDEXX BioAnalytics, Columbia, Missouri
**Corresponding author. Email: beate.hoppe@leibniz-fli.de*

fish's age, whereas fish showing signs of aging are valuable for research. Our goal was to reduce the pain, suffering, distress, and harm of individuals by establishing clearly defined humane endpoints.[29] The consistent use of health score sheets results in an overall improvement in health and well-being and helps to protect against the transmission of diseases within the colony.[5] In terms of animal welfare, this approach serves as a part of the third R (Refinement) in the 3Rs Principle.[27]

The present report shows collected comprehensive data over a 4.5-y time period from daily health checks of all fish, revealing an age-stage-specific appearance of conditions and clinical signs. This resulted in the identification of appropriate humane endpoints for the different age groups. Data were complemented by quarterly colony health monitoring for a list of specific infectious agents. These results led to the identification of pathogens specific for *N. furzeri*. Combining daily individual health assessment using a fish health score sheet with routine colony health monitoring enabled rapid identification and containment of disease outbreaks. Consequently, daily health checks and quarterly colony health monitoring became an inherent part of standard routines in the fish facility.

Materials and Methods

Animals and husbandry. All fish were maintained in the animal facility fish of the Leibniz Institute on Aging – Fritz Lipmann Institute Jena consistent with the German Animal Welfare Law. All breeding and husbandry were covered by the license §11-003798, which was approved by the local authorities (Zweckverband Veterinär- und Lebensmittelüberwachungsamt Jena-Saale-Holzland).

The fish facility consisted of 26 fish racks of various sizes, each with its own water circulation, and 3 additional racks of static fish tanks (both Aqua Schwarz, Göttingen, Germany). For the purposes of this study, each rack was considered to be its own epidemiologic unit (EU). Given a total number of 2,677 tanks and a maximum capacity of 14,000 *N. furzeri*, the EUs were distributed across 5 rooms. Five wild-type lines (GRZ-D,[17] MZM-0410, MZCS-08/122,[8] MZM-0403, and MZM-0703) and several genetically modified lines were housed during the period of data collection and analysis. *N. furzeri* were housed either individually or in groups (maximum one fish per 1.7 L). Husbandry conditions included a water temperature of 26 °C ± 1 °C, conductivity of approximately 2.4 mS, and a 12:12-h light:dark cycle. Water quality parameters, including pH, temperature, and conductivity, were monitored and recorded continuously. Nitrate and nitrite were measured daily by using a quick test (Quantofix test stripes, Macherey-Nagel). Group-housed fish were supplied with certified contaminant-screened blue polycarbonate igloos, retreats, huts, and tents (Bio-Serv, Flemington) as enrichment. Breeding groups, which consisted of one male and 4 females, were provided with a sand bowl for oviposition. Embryos were collected on coconut fiber substrate (Dragon Coco-Ground, Zoo Zajac, Duisburg, Germany) and incubated at 23 °C. Freshly hatched larvae were fed twice daily with live first instar brine shrimp nauplii (*Artemia* sp.) and then weaned at the age of 4 to 6 wk onto live bloodworms (*Chironomidae*) once a day. Fish were tracked on an individual basis by using the commercially available database Tick@lab (a-tune, Darmstadt, Germany). Fish were visually inspected daily by animal technicians with reference to the killifish health score sheet. Health scores of fish exhibiting clinical signs were documented in Tick@lab.

Survival curve analysis. Survival curve analysis was performed using fish of the *N. furzeri* GRZ-D line that hatched between 2018 and 2021. In contrast to the use of preselected experimental fish that were individually housed, our study included predominantly group-housed fish that were designated for use as broodstock and for organ or tissue collection after they became too old for breeding. Fish that were euthanized due to reaching a predetermined humane endpoint or that died peracutely were included in the survival curve analysis. Aged *N. furzeri* that were euthanized as "too old for breeding" were included in the analysis as censored data. Kaplan-Meier plot statistics were performed to estimate the survival function GraphPad Prism software (Dotmatics, Boston, MA).

Development of a killifish health score sheet. During daily health inspection, the health status of *N. furzeri* fish was scored based on 3 primary categories: behavior, body condition, and appearance. To standardize and objectify the daily health check, veterinarians, scientists, and animal technicians developed a killifish-specific score sheet that summarized the different conditions in 2 or more subcategories per category, including a description of the most frequently observed health conditions (Figure 1). In the behavior category, the subcategories included swimming behavior, feeding behavior, and social behavior. In the body condition category, fish body shape was visually assessed. In the appearance category, eyes, jaw, operculum, skin, fins, and spine were visually inspected for any clinical signs. The conditions of circumferential increase and dropsy were recorded in separate subcategories. Rare clinical signs and unclear conditions were summarized in the category "vet."

Fish displaying clinical signs or abnormalities were categorized with the help of a "traffic light" system designed to indicate the animal's experience of pain, harm, or distress. The green state comprised physiologic conditions with no apparent signs of pain, harm, or distress. The yellow state initiated a closer observation of fish exhibiting clinical signs that indicated short-term mild pain, harm, or distress. These fish were evaluated in addition weekly by the veterinarian. The red state indicated conditions with acute clinical signs or severe conditions that were irreversible or progressive and were defined as humane endpoints requiring immediate euthanasia. The green/yellow/red states could be used for all subcategories, and simple abbreviations, such as "SW" for swimming behavior, were developed for easy documentation. The fish health score sheet was completed using numbers, where green was 0, yellow was either 1 or 2, and red was 3. In the category vet, only the veterinarian provided the score. *N. furzeri* showing yellow clinical signs in more than one category and reaching a summed score of 5 were euthanized immediately, because they were considered to be experiencing significant pain, harm, or distress.

Aged fish received age-related yellow health scores in certain categories like reduced feeding behavior, medium reduced social behavior, underconditioned body condition, moderate ocular abnormality, or an age-related mild spinal deformation. Young fish that displayed these signs received a higher score. The cases shown in the red state in Figure 2 had the most obvious severe clinical signs and were collected over several years. To facilitate fast scoring, fish tanks were labeled with the health score sheet abbreviations.

All animals were assessed regularly by the designated veterinarians. Veterinarians first trained animal technicians and scientists by using pictures and videos of sick fish. The next step was to train personnel in assessing the health status and scoring of living fish by using the health score sheet.

Analysis of the fish health score sheet. Fish health score sheet analysis was performed using a customized report function of the animal database Tick@lab. Health scores were assigned on the day of assessment and were saved in Tick@lab for all

Category		Severity	Clinical signs	Abbr. for documentation	Score
Behavior	Swimming	Physiological	Upright position, balanced, without tilting	SW0	0
		Altered	Sloping position, cannot hold stable	SW2	2
		Strongly altered	Negative or positive buoyancy, tilting	SW3	3
	Feeding	Physiological	Active feeding and hunting	FB0	0
		Reduced	Decreased response to food	young FB2; old FB1	2-(1)
		Stronly reduced	Starvation, completely unreactive towards food	FB3	3
	Social	Physiological	Responsive and purposed swimming	SB0	0
		Reduced	Very delayed reaction or very aggressive	young SB2; old SB1	2-(1)
		Strongly reduced	Apathy, no swimming movements	SB3	3
Body condition		Physiological	Body bigger than head, well-conditioned	BC0	0
		Underconditioned	Head, belly and base of anal fin in a line	young BC1; old BC0	1-(0)
		Severe emaciation	Body narrow than head, concave shape along the belly, juveniles: much smaller than mates	BC3	3
Appearance		Physiological	Clear eyes, scales attached to body, no lesions, intact fins, normal coloration, even skin	[Abbr.]0	0
	Eyes	Moderate damage	One protruding or deformed eye, loss of one eye, enophthalmos	young EY2; old EY1	2-(1)
		Severe damage	Bleeding, exophthalmus, both eyes missing	EY3	3
	Jaw	Moderate deformation	Slight changes, no influence on feeding behavior	JA2	2
		Severe deformationen	Deformed jaw, strongly influenced feeding behavior	JA3	3
	Operculum	Malformations	Missing or deformed gill cover	OP2	2
	Skin	Changed appearance	Small lesion, dark/white discolored foci, pale coloring	SK2	2
		Severe changes	Large discolored patch, hemorrhage, abscess wound with organ exposure, fungi infection	SK3	3
	Circumferential increase	Increased tissue growth	Possible tumor growth, possible spawning problem	CI3	3
	Dropsy	Severe changes in appearance	Protruding scales, massively enlarged abdomen and bulging eyes	DR3	3
	Fin	Mild damage	Slightly injured or partially missing fin	FI2	2
		Severe damage	Missing, severly injured or rotten fins	FI3	3
	Spine	Mild deformation	Moderate skeletal curvature or deformity of the spine	young SP1; old SP0	1-(0)
		Severe deformation	Strong deformity of spine	SP3	3
Other symptoms		Other reasons	Rarely occuring clinical signs, unclear conditions	VET	1-3

Figure 1. Killifish-specific health score sheet. The traffic light-based system with the categories behavior, body condition, and appearance determined the health status of the fish at the time of daily routine health inspection. Subcategories with descriptions of severity and clinical signs as well as shortcuts and numbers provided documentation of the condition and triggered follow-up of the health status of individual fish if warranted.

animals from hatching until death. Health scores were summed and graphed using Microsoft Excel (Version 365, Microsoft Corporation, Redmond, WA). Evaluation was performed with fish of the *N. furzeri* wild-type line GRZ-D that were born between 01/01/2018 and 24/06/2022 and that were produced either to serve as broodstock or to be used for organ harvesting and/or colony health monitoring. The age groups for GRZ were determined as follows: "Juvenile" = 0 to 5 wk posthatching (wph), "Adult" = 6 to 20 wph, and "Aged" > 20 wph.

Health monitoring program. Our routine quarterly killifish colony health surveillance program incorporates both molecular methods using whole fish (sentinels) and environmental samples (sump) for molecular analysis and whole fish for histopathology (sentinels). Adult postfiltration sentinel *N. furzeri* were placed in sentinel tanks in each EU for a minimum exposure period of 3 mo. Sentinels were euthanized by hypothermal shock (rapid chilling) or overdose with buffered tricaine methanesulphonate (MS-222, 1g/L) and submitted to IDEXX BioAnalytics (Columbia, MO) for individual evaluation by histopathology, pooled into groups of 5 for evaluation by real-time PCR, or analyzed individually in-house by conventional PCR for microsporidia. Each health monitoring round sampled 2.7% of the entire fish colony. In addition, biofilter samples were collected from the sump of each EU as environmental samples and were processed individually by real-time PCR. Data were collected and analyzed from all the above-mentioned *N. furzeri* lines between the third quarter of 2019 and the end of 2022.

Molecular analysis. Environmental samples and pooled samples of up to 5 whole frozen sentinel fish were submitted to IDEXX BioAnalytics to be tested for the following infectious agents by real-time PCR: *Aeromonas hydrophila*, *Edwardsiella ictaluri*, *Flavobacterium columnare*, *Ichthyophthirius multifiliis*, *Mycobacterium abscessus*, *Mycobacterium chelonae*, *Mycobacterium fortuitum*, *Mycobacterium haemophilum*, *Mycobacterium marinum*, *Mycobacterium peregrinum*, *Myxidium streisingeri*, *Piscinoodinium pillulare*, *Pseudocapillaria tomentosa*, *Pseudoloma neurophilia*,

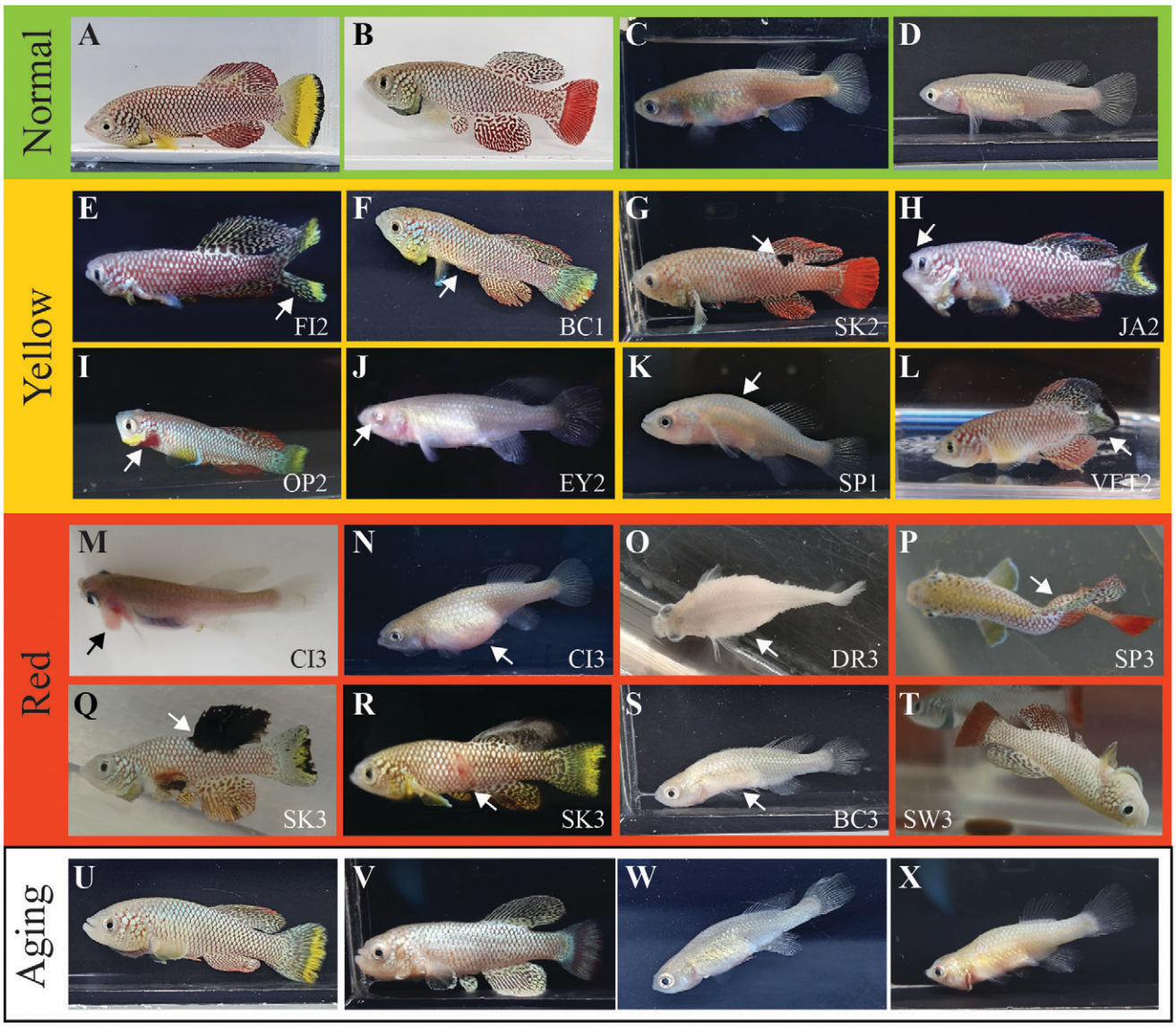

Figure 2. Examples of clinical signs recorded during daily health checks. Appearance of normal ("green") male (A, B) and female (C, D) adult *N. furzeri*. (E–L) *N. furzeri* with "yellow" clinical signs or conditions. (E) Appearance: split fin without bleeding; (F) body condition: underconditioned, head, belly, and base of anal fin in a line; (G) appearance: skin, dark discolored foci; (H) appearance: jaw, moderate deformation; (I) appearance: missing operculum; (J) appearance: eye, enophthalmos; (K) appearance: moderate spinal deformity; (L) appearance: altered body shape, short-tail. (M–T) *N. furzeri* with "red" clinical signs or conditions. (M) Appearance: circumferential increase, increased tissue growth near the gills; (N) appearance: circumferential increase, abdominal distension; (O) appearance: dropsy, protruding scales, massively enlarged abdomen and bulging eyes; (P) appearance: spine, strong deformity, kyphosis; (Q) appearance: skin, large dark discolored patch; (R) appearance: skin, fungi infection; (S) body condition: severe emaciation, body narrow than head, concave shape along the belly; (T) swimming: tilting. (U–X) Aged *N. furzeri* with typical aging traits. (U) Appearance: spine, mild spinal curvature; (V) appearance: pale coloring; (W) body condition: underconditioned; (X) appearance: spine, mild spinal curvature; body condition: underconditioned. Arrows pointed to sites of clinical signs.

Pseudomonas aeruginosa, and *Saprolegnia brachydanis*. Fish were homogenized and total nucleic acids were extracted using a commercially available platform (NucleoMag VET Kit, Macherey-Nagel, Düren, Germany). Fluorogenic real-time PCR assays were based on the IDEXX BioAnalytics proprietary service platform (IDEXX Laboratories, Westbrook, ME). Real-time PCR analysis was performed at IDEXX BioAnalytics using standard primer and probe concentrations (Applied BioSystems) and LightCycler 480 Probes Master (Roche Applied Science, Indianapolis, IN) in a commercially available instrument (LightCycler 480, Roche Applied Science). All IDEXX BioAnalytics real-time PCR assays have been validated to detect 10 template copies of target DNA per reaction. In addition to positive and negative controls for each real-time PCR assay, a multiplexed hydrolysis-probe-based real-time PCR assay targeting a eukaryotic gene (*18S rRNA*) and bacterial gene (*16S rRNA*) was used to ensure nucleic acid recovery and the absence of PCR inhibitors in the extracted nucleic acids.

Conventional PCR was performed in-house to test for microsporidia (*Glugea* spp., *Loma* spp.). PCR was performed as follows: DNA was extracted from killfish gut and swim bladder by using a commercially available kit (QIAamp DNA Mini Kit, Qiagen, Hilden, Germany). Standard PCR (Phusion High-Fidelity PCR Master Mix with HF Buffer, NEB, Frankfurt am Main, Germany) was performed using primers that targeted the small subunit ribosomal RNA gene of microsporidia species (forward primer: 5′-TAT TAA GCG ACG AGG GGT GAA-3′ and reverse primer: 5′-GAG GGC ATC ACT GAC CTG TTC-3′).

The resulting amplicons were sequenced using Sanger sequencing on a commercially available service platform (Eurofins Genomics, Ebersberg, Germany) and compared with GenBank sequences using BLAST software (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/).

Histopathology. Whole formalin-fixed sentinels were submitted to IDEXX BioAnalytics for preparation, reading, and interpretation of histologic slides. Fixed fish were sectioned parasagittally, placed in tissue cassettes (Tissue-Tek Uni-Cassette, Sakura Finetek, Nagano, Japan), and decalcified for 4 to 12 h in a solution of 25% formic acid and 10% sodium citrate, embedded in paraffin using routine methods, sectioned at 5 µm, and stained using hematoxylin and eosin (H&E) and Ziehl-Neelsen acid-fast stains (StatLab Medical Products, McKinney, TX) before evaluation using light microscopy. The tissues evaluated were brain, skin, gills, heart, intestines, kidneys, liver, pancreas, reproductive organs (ovary or testis), skeletal muscle, spinal cord, spleen, swim bladder, and vertebral column. A light microscopic screen of the Ziehl-Neelsen-stained slides was evaluated for acid-fast mycobacterial organisms in all fish.

Results

Daily health check and application of the killifish health score sheet. Healthy adult *N. furzeri* normally displayed behaviors, such as responsive and purposeful swimming, maintaining an upright swimming position without tilting, robust appetite and eager food consumption during feeding times, and regular and rhythmic respiratory patterns. The typical appearance included bright and vibrant coloration of the males (Figure 2A, B) and inconspicuous silver coloration of the body with transparent fins of the females (Figure 2C, D). Healthy fish had intact fins and scales, a well-rounded body shape without any signs of bloating or deformities, and even, regular skin without lesions.

Mild clinical signs could indicate potential health issues. Examples of fish with different conditions in the yellow state are summarized in Figure 2E to L. Fish showing a torn or split fin without bleeding were categorized as mild fin damage, FI2 (Figure 2E). Fish showing the head, belly, and base of the anal fin in a straight line (Figure 2F) were scored as underconditioned in the category body condition, using the abbreviation BC1. Skin abnormalities, such as foci of hyperpigmentation, were categorized as changed appearance of the skin, SK2 (Figure 2G). Moderate deformities of the jaw that did not impact feeding behavior, JA2 (Figure 2H), or opercular malformations, OP2 (Figure 2I), were scored as soon as fish were large enough for identification of these conditions. These signs were only observed in young fish. Mild clinical signs, such as enophthalmos or loss of one eye, EY2 (Figure 2J), did not impact overall health and occurred more often in aged fish. Assessment of severity of moderate spinal alterations/deformities, SP1 (Figure 2K), was performed with regard to the age of the individual fish. Rare conditions like altered body shapes (Figure 2L) were classified in the category VET. Veterinarians performed severity assessments for these cases.

In daily routine health assessments, the red state identified conditions that were considered to indicate a humane endpoint and required immediate euthanasia. Fish that displayed lesions of increased tissue growth in any part of their body, including those close to the gills (Figure 2M), were scored for circumferential increase, CI3, and euthanized. This category also included fish with abdominal distensions, such as females with egg retention, CI3 (Figure 2N), and abdominal distensions of unknown origin. Dropsy, DR3, with the signs of protruding scales, massively enlarged abdomen, and bulging eyes (Figure 2O) was scored in a separate category for tracking purposes. Cases of severe skeletal curvature, SP3, such as the scoliosis shown in Figure 2P, were not seen as an aging trait. In contrast, cases of kyphosis (Figure 2U) were clustered in aged fish. The red category for skin appearance was defined by severe changes or lesions of the skin, including large, discolored patches of the skin, such as hyperpigmented areas (Figure 2Q). Other observed conditions were fungal infections (Figure 2R) and wounds with organ exposure and hemorrhage. Fish showing a concave shape along the belly (Figure 2S) were classified as severely emaciated and euthanized with the score BC3. Loss of body condition developed gradually and was not always associated with reduced feeding behavior. Abnormal swimming behaviors, such as tilting or displaying negative or positive buoyancy with unknown etiologies, were grouped under the score SW3 (Figure 2T).

Typical age-related traits seen in aged fish were mild spinal curvature (e.g., mild kyphosis; Figure 2U, X), pale coloration (Figure 2V), and mild underconditioning (Figure 2W, X). Other age-related signs were ocular abnormalities and a reduction in feeding or social behavior. If a young fish exhibited any of these signs, like a mild spinal curvature (Figure 2K), it was considered to reflect an infectious or noninfectious disease.

Analysis of the fish health score sheet in short-lived GRZ-D. *N. furzeri* wild-type lines with different life spans were housed in the fish facility. Mortality was recorded from hatching onward to gain an insight into age-related mortality. The health score sheet was maintained for all fish from hatching onward; data are shown for the short-lived GRZ-D line (Figure 3B), which has a median survival of 49 d (Figure 3A) and a fertility period of 6 to 20 wk after hatching. A comparatively high mortality rate was observed in the juvenile stage, whereas the survival rate was higher in the adult and aged fish (Figure 3A).

Fish displaying clinical signs during the daily health checks received a yellow or red score. For GRZ-D, 50% of fish developed clinical signs or conditions that were scored and observed further or required euthanasia (Figure 3C). Analysis of the individual score categories revealed that the score OP2, opercular malformations, was one of the most frequently assigned health scores of the yellow category, occurring in 26% of GRZ-D fish (Figure 3C). Opercular malformations were first identified in adolescent fish around 3 wph, as soon as the opercula were large enough to be seen without using a microscope. This condition did not seem to develop later in life. Abnormal growth of the gill cover with varying levels of severity was a common characteristic of GRZ-D fish. Mild to moderate opercular malformations ranged from minor shortening of the gill cover to complete exposure of the gills due to the lack of the operculum, but this did not appear to affect overall health. These fish were excluded from breeding but could be used for scientific purposes. In 24% of the cases, the fish were assigned either other yellow or red scores (Figure 3C).

Analysis of mortalities of all fish showed that 49% died peracutely, whereas 27% of the fish received a red score and were euthanized based on reaching a humane endpoint (Figure 3D). The remaining 24% of fish were euthanized for nonhealth-related reasons.

Fish health scores were analyzed based on the age of the fish and are reported separately for yellow (Figure 3E) and red scores (Figure 3F). Hatchlings of GRZ-D with clinical signs received either altered (46%, yellow score) or strongly altered swimming behavior (larval "belly sliders," 51%, red score) due to insufficient inflation of the swim bladder, typically between

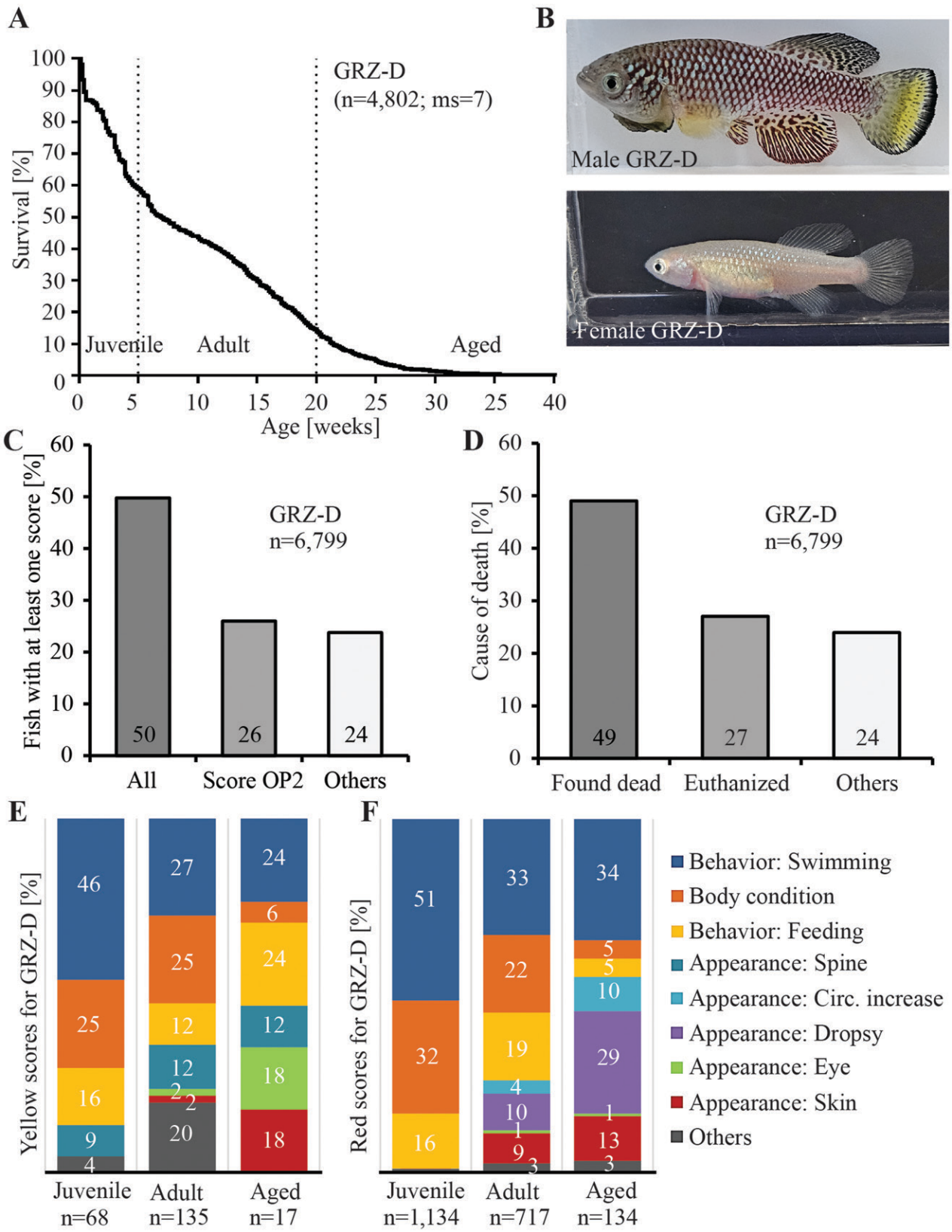

Figure 3. Analysis of health score sheet of *N. furzeri* GRZ-D wild-type line. (A) Survival curve of short-lived GRZ-D; ms, median survival; *n* = animal number. (B) Normal color patterns of male GRZ-D with yellow caudal fin and black terminal line and female GRZ-D with inconspicuous silver coloration. (C–F) Distribution of scores and conditions in the analysis period between January 2018 and June 2022. (C) Number of fish with at least one score: all score categories, opercular malformations score (OP2), and scores other than OP2. (D) Number of fish categorized for causes of death. Found dead: fish died peracutely with no preceding clinical signs; euthanized: fish reached a humane endpoint; others: fish were euthanized for other, nonhealth related reasons; *n* = number of all GRZ-D that were present during the analysis period. (E, F) Age-specific trends of scores in the (E) yellow and (F) red categories for the short-lived GRZ-D line. Age categories were defined based on fertility; *n* = number of fish with the given health scores. The OP2 score (opercular deformation, yellow score) was not included in the calculations.

1 and 5 d after hatching. Other frequent abnormalities in the first weeks after hatching were an underconditioned appearance (25%, yellow score), severely reduced growth as compared with other fish of the same age (32%, red score), and either reduced (16%, yellow score) or substantially reduced feeding behavior (16%, red score). In general, hatchlings that exhibited clinical signs were more likely to be assigned to a red score, as evidenced by the number of health scores in the yellow state ($n = 68$) as compared with the red state ($n = 1,134$). A few juvenile fish showed mild spinal deformities (9%, yellow score). In adult GRZ-D fish, the majority of yellow and red scores were assigned in the categories swimming (27% yellow score, 33% red score), body condition (25% yellow score, 22% red score), and feeding (12% yellow score, 19% red score). Some clinical signs that were not found in the juveniles were seen in adults. For example, 2% of adult fish receiving a yellow score had mild clinical signs affecting the eyes (e.g., unilateral enophthalmos) or the skin (e.g., discolored foci). The red score of increased body volume was found in 4% of adult fish showing clinical signs that included distention resulting from neoplasia, localized circumferential increase of unknown etiology, and reproductive distention in female fish due to spawning problems. Dropsy was identified in 10% of adult fish that showed clinical signs. Hemorrhage and severe skin changes were associated with 9% of cases leading to euthanasia. Aged fish with clinical signs showed either an unstable swimming position (24%, yellow score) or a positive buoyancy or tilting (34%, red score). Negative buoyancy (belly slider) was observed only rarely in aged fish. Mild ocular and skin lesions were age related in GRZ-D and were each identified in 18% of aged fish receiving a yellow score. Dropsy was the second most common clinical sign in aged fish and resulted in 29% of red cases that required euthanasia. Other red cases included severe changes in the skin (13%) and circumferential increases (10%).

Quarterly health monitoring program. In the current study, 17 fish EUs were analyzed over a 3.5-y period. Samples collected from each unit were analyzed for infectious agents, including bacterial pathogens and opportunists, endo- and ectoparasites, and fungi (Figure 4A). The results were summarized in a health monitoring report. Table 1 shows an excerpt of a health monitoring report with positive test results, including the diagnostic laboratory and detection method. The most recent test results and positive results from the prior 2 y are displayed, distinguishing between pooled fish samples and environmental samples. Results are expressed as the number of positive samples/total number of samples submitted. *Mycobacterium chelonae* (115/140), *M. fortuitum* (109/140), and *M. gordonae* (34/140) were commonly identified in environmental samples. *Mycobacterium abscessus* (3/140) and the opportunists *Pseudomonas aeruginosa* (6/140) and *Aeromonas hydrophila* (1/140) were sporadically detected in environmental samples. *M. chelonae* (6/51) and *A. hydrophila* (1/51) were also occasionally detected in pooled sentinel fish samples. *M. abscessus*, *M. gordonae*, and *M. fortuitum* were identified in environmental samples but were never identified in pooled fish samples. Over a course of 2 y, 5 of 189 sentinels tested positive for microsporidia, detected in-house by conventional PCR. Environmental samples were not tested for microsporidia. The following infectious agents have never been detected in our killifish facility in routine testing: *Edwardsiella ictaluri*, *Flavobacterium columnare*, *Mycobacterium haemophilum*, *Mycobacterium marinum*, *Mycobacterium peregrinum*, *Mycobacterium saopaulense*, *Ichthyophthirius multifiliis*, *Myxidium streisingeri*, *Piscinoodinium pillulare*, *Pseudocapillaria tomentosa*, *Pseudoloma neurophilia*, and *Saprolegnia* spp.

Although the health monitoring report includes results from the last 2 y, a more detailed report on *Mycobacterium* spp. is presented in Figure 4B to E. *M. abscessus* was detected only rarely in environmental samples in quarters 1/2021 and 4/2022. In the other quarters, this agent was not detected. *M. gordonae* was identified immediately after starting the EUs in environmental samples and was detected regularly with a frequency of up to 60%. *M. fortuitum* and *M. chelonae* were identified regularly in environmental samples with a detection frequency of up to 100%. Only *M. chelonae* was identified regularly in sentinels, albeit at a low prevalence. Positive findings in sentinel fish or EUs did not appear to be associated with increased mortality, suggesting that most *M. chelonae* infections were subclinical. In contrast to *M. chelonae*, *M. fortuitum* was identified in environmental samples but was never identified in pooled fish samples.

Complementing real-time PCR analysis, histopathology was an integral part of the quarterly colony health monitoring program that helped to identify emerging pathogens, characterize noninfectious lesions, and associate clinical signs with various etiologies.

A total of 127 fish were submitted for histopathologic analysis. No histopathologic changes were identified in the brain, the spinal cord, or vertebral column. A list of all recorded findings is presented in Table 2. Findings were categorized by organ/tissue and type of lesion and separated by age. The most frequent findings were nephrocalcinosis and dilated, cystic tubules, fibrosis, and interstitial nephritis in the kidney of aged *N. furzeri*.

Overall, aged *N. furzeri* fish had a higher prevalence of lesions than younger fish. The difference included more histiocytic infiltrates and granulomas in various organs, including the heart, kidney, liver, ovary, and testis, in aged fish. One adult fish exhibited these lesions in histopathologic analyses, but they were seen in 14 aged fish. Other age-related lesions were nephropathies (8 adult fish, 52 aged fish), degenerated ova with egg-associated inflammation with fibrosis (5 adult fish, 8 aged fish), and proteinaceous content or epithelial tumor in the swim bladder (0 adult fish, 4 aged fish). Lesions seen in aged fish include lipidosis in the liver (2 adult fish, 4 aged fish), testicular atrophy (0 adult fish, 3 aged fish), and atrophy of the exocrine pancreas (0 adult fish, 2 aged fish). In addition, 9 aged *N. furzeri* fish showed histopathologic evidence of mycobacterial infections, and 3 had microsporidian infections. These conditions were not seen in adult fish.

Histopathologic findings were grouped into infectious, inflammatory noninfectious, proliferative nonneoplastic, neoplastic, degenerative, and other categories and were separated by sex and age (Table 3). In general, aged fish had more lesions of all categories compared with adult fish. The frequency of lesions was slightly higher in males than in females, except for infectious lesions. The observed lesions were primarily in inflammatory noninfectious, infectious, and degenerative categories. Lesions classified as neoplastic or proliferative nonneoplastic were found less frequently, particularly among female fish.

Inflammatory noninfectious pathologies were the most observed lesions in the analyzed *N. furzeri* lines. In both sexes, 6% to 23% of adult and 48% to 66% of aged fish had abnormalities. These included intestinal mucosal histiocytic infiltrate, nephropathies, liver hyperplasia, and degenerate ova with egg-associated inflammation. Granulomas and histiocytic infiltrates were also seen in various organs. Degenerative lesions were seen in 5% of adult and 13% of aged females. Among males, 22% of adult fish and 44% of aged fish had degenerative lesions, predominantly nephrocalcinosis, as well as liver lipidosis and testicular atrophy. Histopathologically identified

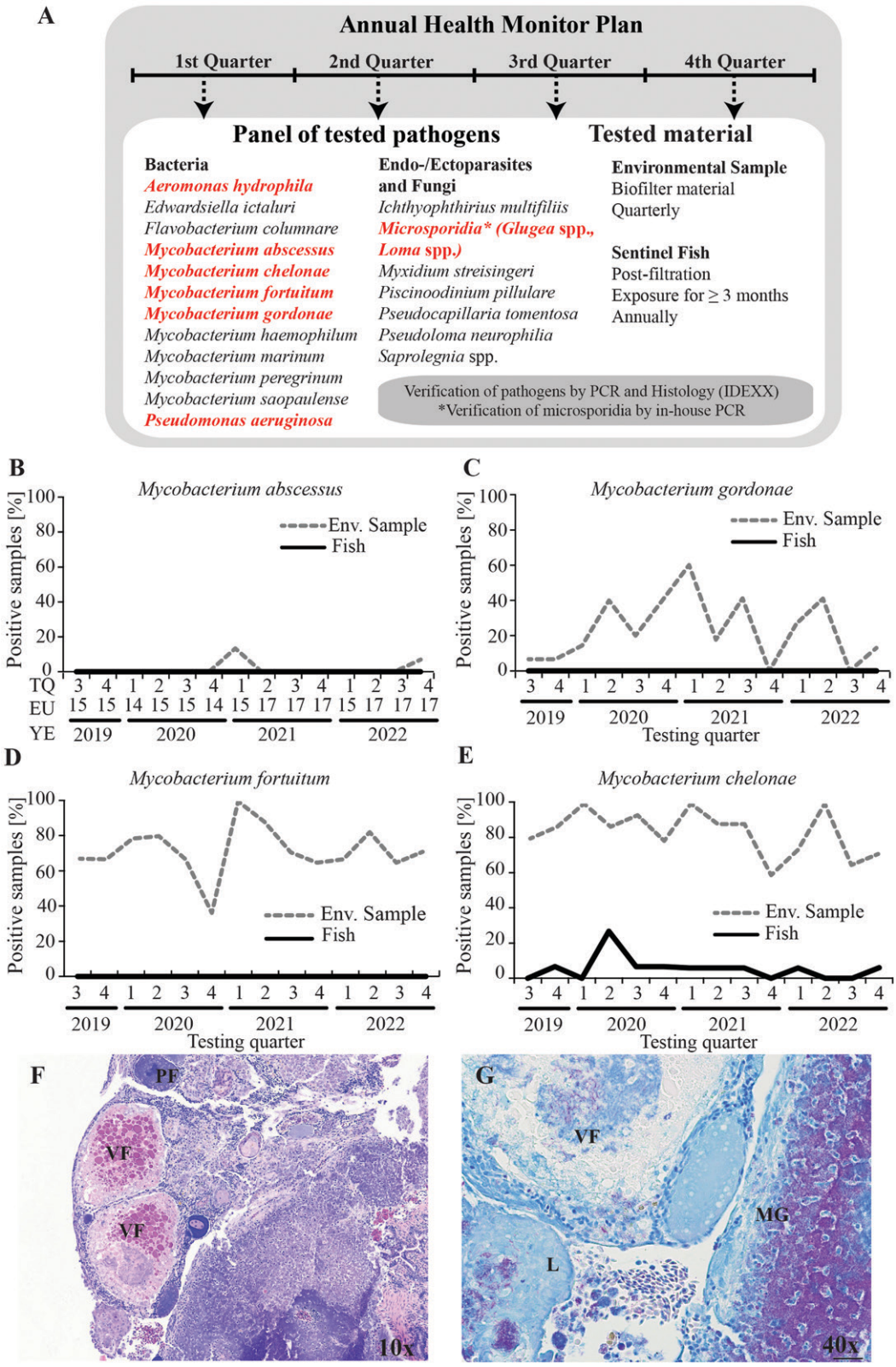

Figure 4. Quarterly health monitoring and mycobacteriosis. (A) Overview of the annual health monitoring schedule and panel of tested pathogens. (B–E) PCR results for *Mycobacteria* spp. assessment of environmental samples and sentinel fish for the quarterly testing intervals between 2019 and 2022. EU, number of EUs tested; TQ, testing quarter; YE, test year. Number of tested EUs shown for (B) are the same for (B–E). (F–G) Histologic analysis indicated oophoritis caused by mycobacteria. (F) H&E stain of ovary shows obliteration of ovarian tissue by coelomic granulomatous inflammation. PF, previtellogenic follicles; VF, vitellogenic follicles. (G) Ziehl-Neelsen stained ovary tissue shows numerous acid-fast positive (magenta) bacilli, suggestive of mycobacteria species. L, in the center of granulomas and within; MG, bacilli in macrophages in the coelomic granulomatous inflammation.

Table 1. Extract from the health certificate for export of fish

Infectious Agents	Testing Lab*	Historic Results (01/2021–11/2022)		Most Recent Test Results (test date: 23/11/2022)	
		Fish	Environmental Sample	Fish	Environmental Sample
Bacteria					
Aeromonas hydrophila	IDEXX	1 of 51 (2%)	1 of 140 (1%)	1 of 6 (16%)	0 of 17 (0%)
Mycobacterium abscessus	IDEXX	0 of 51 (0%)	3 of 140 (2%)	0 of 6 (0%)	1 of 17 (6%)
Mycobacterium chelonae	IDEXX	6 of 51 (12%)	115 of 140 (82%)	1 of 6 (16%)	12 of 17 (71%)
Mycobacterium fortuitum	IDEXX	0 of 51 (0%)	109 of 140 (78%)	0 of 6 (0%)	12 of 17 (71%)
Mycobacterium gordonae	IDEXX	0 of 51 (0%)	34 of 140 (24%)	0 of 6 (0%)	2 of 17 (12%)
Pseudomonas aeruginosa	IDEXX	0 of 51 (0%)	6 of 140 (4%)	0 of 6 (0%)	2 of 17 (12%)
Parasites and fungi					
Microsporidia (Glugea spp., *Loma acerinae)*	In-house	5 of 189 (3%)	Not tested	0 of 6 (0%)	Not tested

The table shows the frequency of positive hits over a 2-y period. Results are expressed as the number of positive samples and total number of samples submitted.
*Test frequency: quarterly; test method: PCR.

infectious lesions included mycobacterial infections of the ovary that caused degeneration or necrosis. One female breeder fish had severe mycobacterial oophoritis with destruction of normal histologic structures, granulomatous inflammatory infiltrates, and numerous acid-fast bacilli in macrophages (Figure 4F, G). Microsporidial infections involved changes in the swim bladder (Figure 5F, G). Fungal bacterial aerocystitis was also seen (Figure 5H, I).

Infectious swim bladder diseases. In the beginning of 2020, young adult GRZ-D fish suddenly began to present a negative buoyancy disorder in 2 EUs. *N. furzeri* displaying negative buoyancy disorders are sometimes referred to as belly sliders.[12] Other EUs in the fish facility were not affected. Clinical signs appeared acutely and were categorized as strongly altered swimming behavior (SW3, red score). Affected EUs were inspected several times a day, and fish displaying clinical signs were euthanized immediately. The respective room was isolated from the rest of the facility and quarantined. At the peak of the epizootic, approximately 13% of GRZ-D fish that had hatched in Q2-20 were affected (Figure 5A). Swim bladders of normal healthy *N. furzeri* were inflated and transparent at necropsy (Figure 5B). In contrast, affected animals displayed obvious gross swim bladder lesions at necropsy, described as a milky flat layer overlaying the swim bladder (Figure 5C, arrow) or a swim bladder lumen filled with a white mass (Figure 5D, arrow).

In line with the quarterly health monitoring, whole fish, coelomic swabs, and swabs collected from the white swim bladder lumen masses were submitted for molecular analysis and whole fish for histopathology. Molecular diagnostic results were negative for mycobacteriosis. Histopathology revealed a thickened swim bladder wall due to epithelial hyperplasia and a swim bladder lumen filled with histiocytes (Figure 5E) containing intracellular ovoid to pyriform organisms with posterior vacuoles, consistent with microsporidia (Figure 5F, G). In some cases, both fungal and bacterial organisms accompanied by inflammatory infiltrates and cell debris were observed in the lumen of the posterior chamber of the swim bladder, although no acid-fast bacilli were observed in the affected swim bladders (Figure 5H, I).

Samples of affected fish were also included in the routine quarterly molecular biologic evaluation for microsporidia by in-house conventional PCR targeting the SSU rRNA gene. Subsequent sequence analysis of the amplicon matched *Loma acerinae* with 100% identity over 428 base pairs of sequence (Figure 5J). Immediate removal of all fish with clinical signs resolved the *L. acerinae* epizootic within a few months, without contamination of any other EUs. During the course of the outbreak, microsporidia PCR was performed monthly. After the frequency of swim bladder disease returned to preoutbreak levels, PCR was again performed on a quarterly basis.

L. acerinae infections were detected in an unusually high number of positive test results in 2020 (6 of 103) and in 2021 (5 of 138 tested fish). Before the epizootic, *L. acerinae* had not been identified in the fish facility. Sequence analysis of the PCR products from microsporidium PCR closely matched *Glugea* spp. in 2019 in one of 132 tested fish (Figure 5K).

Most frequent cases of noninfectious diseases. Different age groups of *N. furzeri* showed a number of typical, noninfectious conditions. Aged male sentinels that were submitted for histopathologic analysis as part of our routine health monitoring showed high frequencies of degenerative and inflammatory noninfectious nephropathologies in all lines (Figure 6A). Based on the fish health score sheets, these fish did not show clinical signs of disease, even though they did have marked degenerative nephrocalcinosis. Off-white mineral casts filling tubular structures were found in whole renal tissue, leading to swelling of the kidney (Figure 6B). The findings were confirmed in histologic sections. Dilated tubules with calcified material in the lumens were seen next to healthy structures (Figure 6C).

Juvenile GRZ-D fish frequently developed a combination of different clinical signs. These were most apparent at the age of 6 wph (Figure 6D, E) and were equally distributed among male and female fish. According to the fish health score sheet, affected juvenile fish showed opercular malformations (OP2), spinal deformity (SP1), and severe growth retardation (BC3). Due to the presence of several clinical signs, these fish were viewed as significantly impaired and they were euthanized. This phenotype was seen in 0.2% of all juveniles from the more outbred MZCS-08/122 line, as compared with 5.4% of juveniles in the inbreed GRZ-D line (Figure 6F).

Proliferative masses on the lower lip were commonly observed and scored as SK2 (changed appearance of the skin) on the health score sheet (Figure 6G). Evaluation of H&E-stained tissue sections (Figure 6H, I) revealed expansion of the dermis with fibrous connective tissue, mucinous matrix, and neovascularization. Histologic analysis of several fish indicated that this phenotype was associated with variably minimal to mild inflammatory infiltrates. The surface epithelium was variably

Table 2. Summary of histopathology findings for wild-type *N. furzeri* lines

Age Histopathological Findings		Adult	Aged
Total number of fish submitted for histologic analysis		54	73
Mycobacterium screen		0	9
Epidermis	Chromatophoroma	1	0
	Mixed tumor of subcutis of head with histiocytic infiltrate	0	1
Gills	Curled operculum	0	1
	Proliferative branchitis	2	3
Heart	Granuloma	0	2
Intestine	Intestine mucosal histiocytic infiltrate	0	1
	Amyloid	0	1
	Crypt dilation/goblet cell hyperplasia/mucous	0	1
Kidney	Nephrocalcinosis	4	20
	Scattered dilated tubules	2	3
	Scattered to many dilated, cystic tubules, fibrosis, interstitial nephritis	2	29
	Histiocytic infiltrates/granuloma	0	5
	Hematopoiesis	0	1
	Renal tubular neoplasia	0	1
Liver	Adenomatous change	0	1
	Biliary hyperplasia with fibrosis, mononuclear infiltrates, pigment laden macrophages	0	1
	Lipidosis	2	4
	Basophilic focus	0	1
	Histiocytic infiltrate/granuloma	1	3
	Chromatophoroma	1	0
Ovary	Degenerate ova with egg-associated inflammation with fibrosis	5	8
	Degeneration/necrosis mycobacteria	0	1
	Granuloma	0	3
Skeletal muscle	Mononuclear infiltrate	0	1
	Lipidosis	0	1
Spleen	Increased stromal compartment with pigment-laden cells	0	1
	Decreased hematopoiesis	0	1
	Heterotopic thyroid	0	1
	Chromatophoroma	1	0
Swim bladder	Microsporidia	0	3
	Fungal/bacterial aerocystitis	1	1
	Proteinaceous content	0	2
	Epithelial tumor	0	2
Testes	Testicular atrophy	0	3
	Granuloma	0	1
Pancreas*	Exocrine pancreatic atrophy	0	2
	Hyperplasia of pancreatic islets	Many	Many

Results are reported as the number of occurrences by age.
*Many fish also had large pancreatic islets consistent with Brockmann bodies; this was considered within normal limits.

absent with minimal associated inflammation. Histopathology did not identify a cause of the lesion, suggesting an extrinsic etiology. The incidence of this lesion increased with age in both males and females and was observed more frequently in group-housed fish as compared with singly housed fish. No aggressive behavior was observed among the fish. Fish with small proliferative masses were closely monitored by animal technicians and veterinarians. Fish with expanding lesions that interfered with normal feeding behavior or with ulcerated lesions were euthanized due to reaching the humane endpoint.

Discussion

This study summarizes the results of a continuous 4.5-y screening program that monitored the health parameters of *N. furzeri* in a fish research facility. The continuous recording of clinical signs provided valuable new information about the health conditions of *N. furzeri*, leading to the development of an image catalog that highlights clinical signs and effectively differentiates them from age-related traits. The observed clinical signs were classified based on their severity and were evaluated and analyzed across different life stages. The present study examined the prevalence of various pathogens at our institution and identified the novel pathogen *L. acerinae* through observation of clinical signs and the examination of affected tissue. We also identified a number of species-specific histopathologic noninfectious lesions that we categorized based on the age and sex of affected fish. Daily visual inspection of all fish in the facility was crucial to ensure the well-being of the individual animals and the health of the colony. Existing welfare assessment methods

Table 3. Frequency of histopathologic findings for wild-type *N. furzeri* lines

	Female (% affected)*		Males (% affected)*	
	n = 45 fish		*n* = 82 fish	
	Adult	Aged	Adult	Aged
All lesions	27	61	34	82
Infectious	5	26	0	10
Inflammatory noninfectious	23	48	6	66
Proliferative nonneoplastic	0	9	6	8
Neoplastic	0	0	3	8
Degenerative	5	13	22	44
Other†	0	9	0	12

Results are categorized by disease category, sex, and age.
*Percentages may not add up to >100%; some animals with lesions had more than one type of lesion.
†Other refers to hyper- or hypopigmentation, opercular changes, intestinal amyloid, renal hematopoiesis, decreased splenic hematopoiesis, heterotopic thyroid tissue in spleen, hepatic basophilic focus, and proteinaceous content in swim bladder.

were not adequately specific and did not address the unique requirements of *N. furzeri*. To overcome these limitations, we developed a comprehensive and practical fish health score sheet that incorporates species-specific parameters and conditions.

We now present the first systematic evaluation of clinical signs and survival data that appear in these fish across their lifespan. This analysis was the basis for our development of a health profile that included descriptions of abnormalities and euthanasia criteria for different age stages of this killifish species. The use of this system in concert with systematic evaluation of the collected data has provided a better understanding of the differences between the lesions associated with the natural aging process and clinical signs indicating compromised well-being of individual fish due to nonage-related conditions. Information about early life-stage morbidity and mortality in the first weeks after hatching had been sparse for *N. furzeri*. Juvenile mortality was observed in the first 4 wk after hatching and could be due to a few main factors that include altered swimming behavior due to a noninflated swim bladder,[32] severe growth retardation, and strongly reduced feeding behavior due to developmental defects. In general, a continuous recording of age-specific mortality is crucial for obtaining precise projections of lifespan.[23]

Growth retardation and opercular defects were specific to the GRZ-D line. The reason for this could be either extrinsic or intrinsic. One potential extrinsic factor is a nutritional imbalance, such as vitamin C deficiency.[28,30] Intrinsic factors like accumulation of deleterious genetic traits and decreased genetic diversity in a population can lead to inbreeding depression.[3,21] Due to the lack of options for outcrossing this fish line, directed mating strategies with selection of healthy breeder fish is a possible strategy for reduction of these problems. Moreover, formulation of a species-specific diet[33,34] for these fish could help to prevent a nutritional imbalance that leads to malformations.

Healthy fish can carry subclinical infections caused by obligate, opportunistic, or facultative pathogens. Routine monitoring for a predetermined list of infectious agents is an important strategy to detect breaks in biosecurity and minimize the impact of infectious agents on research. Because subclinical infections are relatively common, the prevalence of infectious agents must be determined based on routine health monitoring of apparently healthy animals, rather than focusing on animals with morbidity. Consistent with reports from zebrafish colonies,[2,6,18] we found that environmental samples collected from the sump/biofilter were more sensitive than samples from sentinel fish for detection of facultative pathogens such as *Mycobacterium* spp.

The identification of fish showing clinical signs in daily visual inspection and parallel molecular and histopathologic diagnostics led to the identification of a new pathogen of *N. furzeri*. For the first time, we provide data showing that the microsporidian parasite *L. acerinae* infects the swim bladder, which presents clinically as an irreversible negative buoyancy disorder. Rapid identification, containment, and eradication of this pathogen was critically important to protect other EUs in the fish facility. *L. acerinae* has a broad host range among fish species and a widespread distribution,[31] which made an identification of the source difficult. The identified *Loma* outbreak proved that the combination of daily visual inspection of all animals and use of the health score sheet, together with the quarterly health monitoring program, allowed rapid detection. Health measures were taken quickly, and containment of the epizootic was managed without massive culling of the colony.

Although the normal histology of *N. furzeri* histology has been described,[11] the current report describes histopathologic lesions that appear to develop spontaneously in *N. furzeri* of different age groups. An aging phenotype was established based on the type of lesions in aged *N. furzeri*. We did not find an accumulation of proliferative nonneoplastic or neoplastic lesions in aged fish. Instead, the lesions were inflammatory noninfectious, infectious, or degenerative. The most prevalent lesion of aged fish was subclinical nephrocalcinosis. Severe nephrocalcinosis is known to be a cause of dropsy,[15] a condition that was prevalent in aged fish in our colony.

A further age-related macroscopic lesion was the proliferative mass at the lower lip. Histopathology indicated that the lesion appeared due to a noninfectious process of an unknown etiology. Possible etiologies include mechanical trauma due to feeding behavior, environmental factors, or genetics. Small lesions did not interfere with feeding or well-being of the fish, whereas ulcerated lesions might provide an entry portal for pathogenic agents and affect the overall health of the fish.

The etiology of clinical signs was not determined for each individual fish. Consequently, the understanding of underlying factors contributing to abnormalities and clinical signs remains incomplete. The present study used an accumulation of conditions to make conclusions about the colony health status, with potentially delayed identification of subclinical infections. For this reason, a complementary screening method was used. However, the scope of monitored infectious agents is determined by very limited information on pathogens of *N. furzeri*. A more comprehensive evaluation of potential pathogens and risk factors is needed, particularly with regard to the infectivity and pathogenicity of infectious agents in this killifish species. Targeted studies are required to extend our understanding of pathogenesis, infection dynamics, and pathogen transmission for both known and unidentified infectious agents of killifish.

A steadily increasing number of investigators use *N. furzeri* as a model organism. The maintenance of this species for scientific purposes is not trivial, because species-specific biology, pathogens, and the maintenance of very old fish pose special challenges for colony health management. The ability to assess the health and well-being of the fish is also beneficial from a scientific perspective. Our data and our approach provide the opportunity to more easily evaluate both the health status of killifish and the influence of the health status on the research

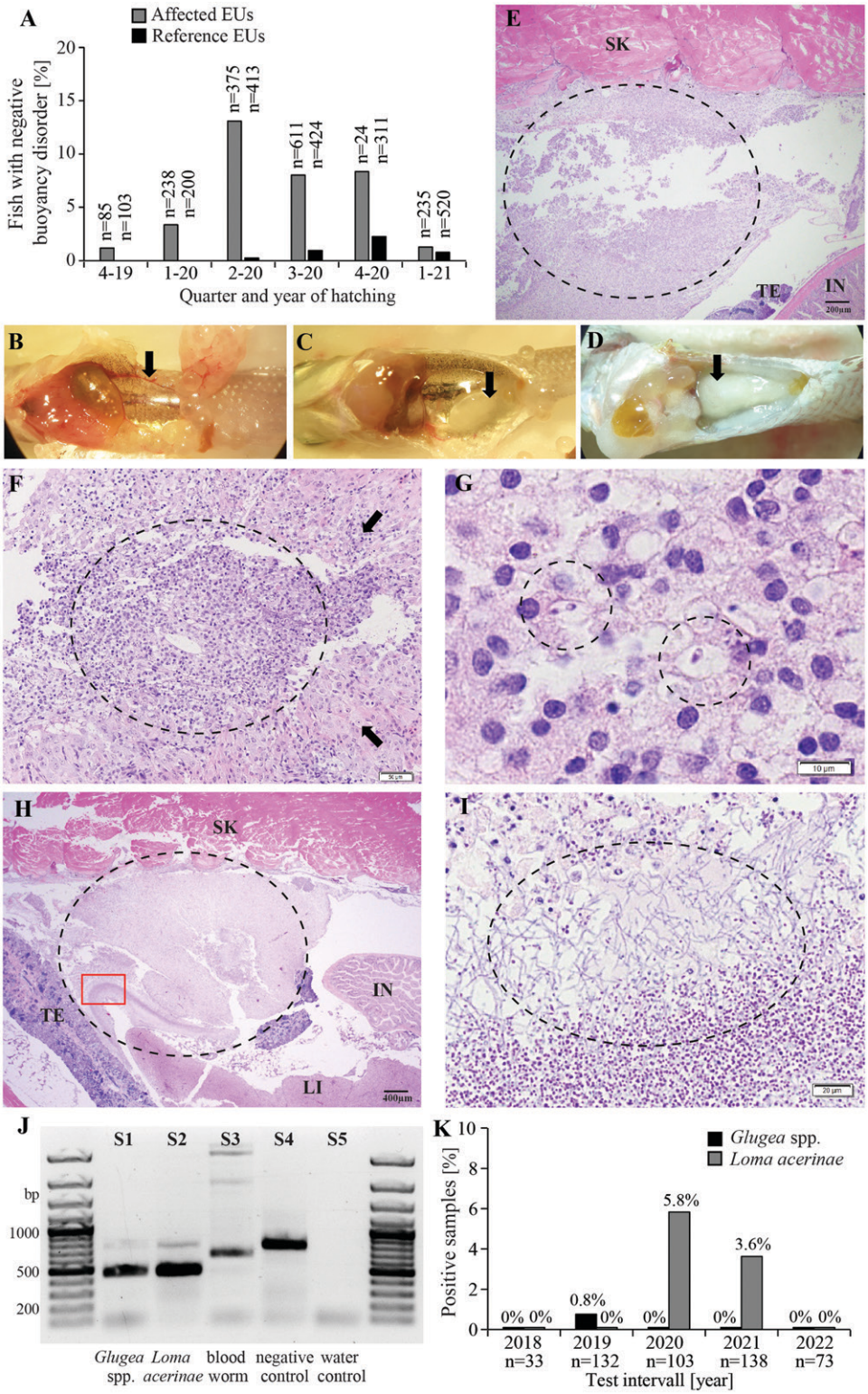

Figure 5. Swim bladder diseases in *N. furzeri*, case study microsporidiosis. (A) Number of adult fish with negative buoyancy disorder (adult belly slider); *n* = total number of hatchlings in the respective quarters and EUs. (B) Typical morphology of swim bladder. (C, D) Dissection of fish with changes of swim bladder integrity, (C, arrow) milky flat overlay on the swim bladder or (D, arrow) swim bladder lumen filled with white mass. (E) H&E-stained section of the coelomic cavity. Dotted circle surrounding a large accumulation of histiocytes filling the area of the swim bladder lumen. IN, intestine; SK, dorsal body wall (skeletal muscle); TE, testis. (F, G) H&E staining indicating microsporidian infiltrates. (F) Section of the coelomic cavity. Encircled region shows the swim bladder lumen filled with histiocytes and intracellular microsporidian organisms. Arrows point to markedly thickened swim bladder wall due to epithelial hyperplasia. (G) Section of the swim bladder lumen. Dotted circles: histiocytes infected with unicellular ovoid to pyriform organisms (spores) that have a posterior vacuole. (H, I) H&E staining indicating fungal/bacterial infiltrates. (H) Section of the coelomic cavity. Dotted circle surrounds filling of swim bladder lumen with cell debris and fungal/bacterial organisms. Red box indicates location of magnification image. (I) Magnification of (H). Section of the swim bladder lumen shows fungal hyphae and bacterial infection (dotted circle). (J) PCR for microsporidia. S1, S2, tissue lysates from fish infected with *Glugea* spp. or *Loma acerinae* showed specific PCR product; S3, lysate of red bloodworms; S4, fish tissue lysate showed unspecific PCR product. (K) Overview of PCR results for *Glugea* spp. or *L. acerinae*; *n* = number of tested fish samples between 2018 and 2022.

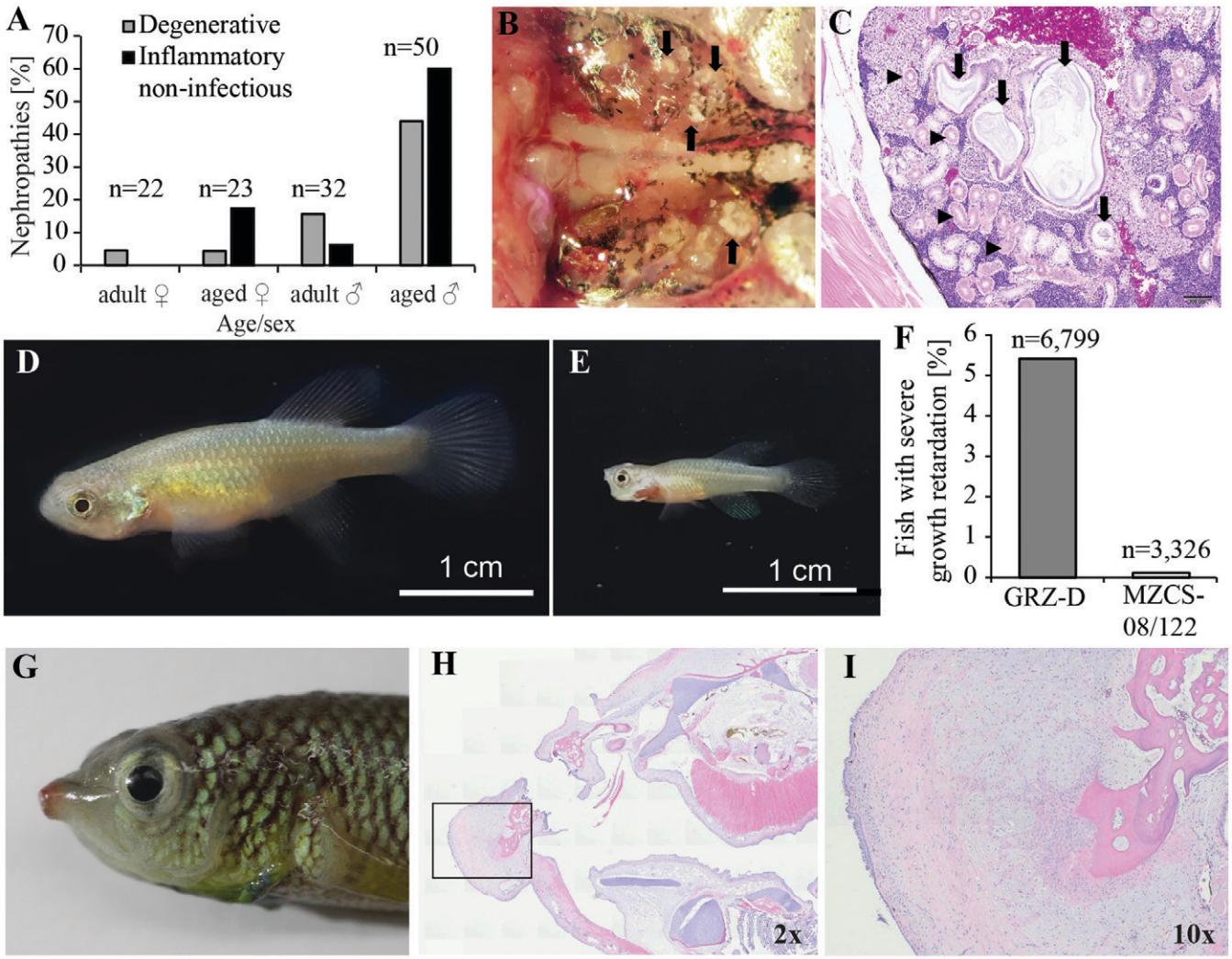

Figure 6. Noninfectious diseases of *N. furzeri.* (A) Sex- and age-specific predisposition to nephropathies. Graph summarizes findings from histologic analysis between 2021 and 2023; *n* = number of analyzed fish. (B) Dissection of an aged male fish shows indurated mass filling tubular structures (arrows) in the kidney. (C) H&E stain of kidney sections shows dilated tubules with calcified structures in lumen (arrows), next to histologically normal tubules (arrowheads). (D, E) Female GRZ-D at 6 wph. Female in (D) displays normal developed phenotype, while female in (C) shows spinal deformity and severe growth retardation. (F) Number of GRZ-D and MZCS-08/122 fish with severe growth retardation. (G) Male *N. furzeri* with proliferative mass on the lower lip. (H, I) H&E stain of head sections shows expanding of the dermis with fibrous connective tissue, mucinous matrix, and neovascularization. The surface epithelium was variably absent with minimal associated inflammatory cells. Black box indicates locations of magnification image (I).

results. With regard to concerns about the reproducibility of research results, the well-being of the model organisms must be carefully considered. The importance of meaningful health monitoring on a daily basis with a fish health score sheet, complemented with routine screening for pathogenic agents, should not be underestimated as an aid to successful colony maintenance. Here we provide a comprehensive colony health management protocol that we have established and used successfully in our facility over several years. These data increase the available knowledge about pathogens in *N. furzeri* and provide a valuable resource for successful maintenance of this fish species for the rapidly growing killifish research community.

Acknowledgments

We thank the animal technicians of Animal Facility Fish at Leibniz Institute on Aging – Fritz Lipmann Institute Jena for their passionate work in fish caring, Viviane Ständert and Patricia Schmidt for meaningful discussions and proofreading, and our former veterinarian Alessia Montesano and current veterinarian Anne Süßenguth for their veterinary care for our fish. We thank Shari Hamilton for the analysis of the histopathologic results.

References

1. **Andersen L, Rønneseth A, Powell MD, Brønstad A.** 2023. Defining piscine endpoints: towards score sheets for assessment of clinical signs in fish research. Lab Anim **57**:455–467. https://doi.org/10.1177/00236772231156031.
2. **Borges AC, Pereira N, Franco M, Vale L, Pereira M, Cunha MV, Amaro A, Albuquerque T, Rebelo M.** 2016. Implementation of a zebrafish health program in a research facility: a 4-year retrospective study. Zebrafish **13**:S115–S125. https://doi.org/10.1089/zeb.2015.1230.
3. **Charlesworth D, Willis JH.** 2009. The genetics of inbreeding depression. Nat Rev Genet **10**:783–796.
4. **Clark TS, Pandolfo LM, Marshall CM, Mitra AK, Schech JM.** 2018. Body condition scoring for adult zebrafish (*Danio rerio*). J Am Assoc Lab Anim Sci **57**:698–702. https://doi.org/10.30802/AALAS-JAALAS-18-000045.

5. **Collymore C, Crim MJ, Lieggi C.** 2016. Recommendations for health monitoring and reporting for zebrafish research facilities. Zebrafish **13**:S138–S148. https://doi.org/10.1089/zeb.2015.1210.
6. **Crim MJ, Lawrence C, Livingston RS, Rakitin A, Hurley SJ, Riley LK.** 2017. Comparison of antemortem and environmental samples for zebrafish health monitoring and quarantine. J Am Assoc Lab Anim Sci **56**:412–424.
7. **Dodzian J, Kean S, Seidel J, Valenzano DR.** 2018. A protocol for laboratory housing of turquoise killifish (*Nothobranchius furzeri*). J Vis Exp **134**:57073. https://doi.org/10.3791/57073.
8. **Dorn A, Ng'oma E, Janko K, Reichwald K, Polačik M, Platzer M, Cellerino A, Reichard M.** 2011. Phylogeny, genetic variability and colour polymorphism of an emerging animal model: the short-lived annual Nothobranchius fishes from southern Mozambique. Mol Phylogenet Evol **61**:739–749. https://doi.org/10.1016/j.ympev.2011.06.010.
9. **Dudis RS, Wong TY, Escatte MG, Alamneh YA, Abu-Taleb R, Su W, Czintos C, Fitzgerald TA, Le Breton Y, Zurawski DV.** 2023. Longitudinal temperature measurement can determine humane endpoints in BALB/c mouse models of ESKAPEE infection. Virulence **14**:2186331. https://doi.org/10.1080/21505594.2023.2186331.
10. **Dykova I, Zak J, Reichard M, Souckova K, Slaby O, Blazek R.** 2021. Swim bladder as a primary site of mycobacterial infection in Nothobranchius "belly sliders". Dis Aquat Organ **145**:111–117. https://doi.org/10.3354/dao03601.
11. **Dyková I, Žák J, Blažek R, Reichard M, Součková K, Slabý O.** 2022. Histology of major organ systems of Nothobranchius fishes: short-lived model species. J Vertebr Biol **71**:21074.1-50. https://doi.org/10.25225/jvb.21074.
12. **Dyková I, Žák J, Reichard M, Součková K, Slabý O, Bystrý V, Blažek R.** 2021. Histopathology of laboratory-reared Nothobranchius fishes: mycobacterial infections versus neoplastic lesions. J Fish Dis **44**:1179–1190. https://doi.org/10.1111/jfd.13378.
13. **Ericsson AC, Crim MJ, Franklin CL.** 2013. A brief history of animal modeling. Mo Med **110**:201–205.
14. **Folgueras AR, Freitas-Rodríguez S, Velasco G, López-Otín C.** 2018. Mouse models to disentangle the hallmarks of human aging. Circ Res **123**:905–924. https://doi.org/10.1161/CIRCRESAHA.118.312204.
15. **Harrison JG, Richards RH.** 1979. The pathology and histopathology of nephrocalcinosis in rainbow trout Salmo gairdneri Richardson in fresh water. J Fish Dis **2**:1–12. https://doi.org/10.1111/j.1365-2761.1979.tb00134.x.
16. **Hawkins P, Dennison N, Goodman G, Hetherington S, Llywelyn-Jones S, Ryder K, Smith AJ.** 2011. Guidance on the severity classification of scientific procedures involving fish: report of a Working Group appointed by the Norwegian Consensus-Platform for the Replacement, Reduction and Refinement of animal experiments (Norecopa). Lab Anim **45**:219–224. https://doi.org/10.1258/la.2011.010181.
17. **Jubb R.** 1971. A new Nothobranchius (Pisces, Cyprinodontidae) from Southeastern Rhodesia. J Am Killifish Assoc **8**:12–19.
18. **Miller M, Sabrautzki S, Beyerlein A, Brielmeier M.** 2019. Combining fish and environmental PCR for diagnostics of diseased laboratory zebrafish in recirculating systems. PLoS One **14**:e0222360. https://doi.org/10.1371/journal.pone.0222360.
19. **Mocho JP, Collymore C, Farmer SC, Leguay E, Murray KN, Pereira N.** 2022. FELASA-AALAS recommendations for biosecurity in an aquatic facility, including prevention of zoonosis, introduction of new fish colonies, and quarantine. Comp Med **72**:149–168. https://doi.org/10.30802/AALAS-CM-22-000042.
20. **Mocho JP, Collymore C, Farmer SC, Leguay E, Murray KN, Pereira N.** 2022. FELASA-AALAS recommendations for monitoring and reporting of laboratory fish diseases and health status, with an emphasis on zebrafish (*Danio rerio*). Comp Med **72**:127–148. https://doi.org/10.30802/AALAS-CM-22-000034.
21. **Monson CA, Sadler KC.** 2010. Inbreeding depression and outbreeding depression are evident in wild-type zebrafish lines. Zebrafish **7**:189–197. https://doi.org/10.1089/zeb.2009.0648.
22. **Nath RD, Bedbrook CN, Nagvekar R, Brunet A.** 2023. Husbandry of the African turquoise killifish *Nothobranchius furzeri*. Cold Spring Harb Protoc **2023**:pdb.prot107738. https://doi.org/10.1101/pdb.prot107738.
23. **North EW, Gallego A, Petitgas P.** 2009. Manual of recommended practices for modelling physical–biological interactions during fish early life. ICES cooperative research report 295. http://dx.doi.org/10.25607/OBP-183
24. **Platzer M, Englert C.** 2016. *Nothobranchius furzeri*: a model for aging research and more. Trends Genet **32**:543–552. https://doi.org/10.1016/j.tig.2016.06.006.
25. **Polačik M, Blažek R, Reichard M.** 2016. Laboratory breeding of the short-lived annual killifish *Nothobranchius furzeri*. Nat Protoc **11**:1396–1413. https://doi.org/10.1038/nprot.2016.080.
26. **Reichard M, Polačik M.** 2019. *Nothobranchius furzeri*, an "instant" fish from an ephemeral habitat. eLife **8**:e41548. https://doi.org/10.7554/eLife.41548.
27. **Russel W, Burch R.** 1959. The principles of humane experimental technique. Med J Aust **1**:500.
28. **Sandnes K.** 1991. Vitamin C in fish nutrition–a review. Fiskeridir Skrift Ser Ernær **IV**:3-32.
29. **Toth LA.** 2018. Identifying and implementing endpoints for geriatric mice. Comp Med **68**:439–451. https://doi.org/10.30802/AALAS-CM-18-000022.
30. **Xu HJ, Jiang WD, Feng L, Liu Y, Wu P, Jiang J, Kuang SY, et al.** 2016. Dietary vitamin C deficiency depressed the gill physical barriers and immune barriers referring to Nrf2, apoptosis, MLCK, NF-κB and TOR signaling in grass carp (*Ctenopharyngodon idella*) under infection of *Flavobacterium columnare*. Fish Shellfish Immunol **58**:177–192. https://doi.org/10.1016/j.fsi.2016.09.029.
31. **Yurakhno VM, Voronin VN, Sokolov SG, Malysh JM, Kalmykov AP, Tokarev YS.** 2021. Genetic diversity of Loma acerinae (Microsporidia: Glugeida) from different fish hosts and localities–short communication. Acta Vet Hung **69**:38–42. https://doi.org/10.1556/004.2021.00012.
32. **Žák J, Anil AN, Dyková I.** 2023. Dissolved oxygen saturation is crucial for gas bladder inflation in turquoise killifish (*Nothobranchius furzeri*). Environ Biol Fishes **106**:673–683. https://doi.org/10.1007/s10641-023-01405-1.
33. **Žák J, Dyková I, Reichard M.** Good performance of turquoise killifish (Nothobranchius furzeri) on pelleted diet as a step towards husbandry standardization. Sci Rep. 2020 Jun 2;10(1):8986**.** doi: 10.1038/s41598-020-65930-0.
34. **Žák J, Roy K, Dyková I, Mráz J, Reichard M.** 2022. Starter feed for carnivorous species as a practical replacement of bloodworms for a vertebrate model organism in ageing, the turquoise killifish *Nothobranchius furzeri*. J Fish Biol **100**:894–908. https://doi.org/10.1111/jfb.15021.