**Welfare assessment of adult laboratory zebrafish: a practical guide.**

Sibylle Sabrautzki1\*,Manuel Miller1\*, Erika Kague2, Markus Brielmeier1

1Research Unit Comparative Medicine, Helmholtz Zentrum Muenchen - German Research Center for Environmental Health GmbH, Neuherberg, Germany

2 School of Physiology, Pharmacology and Neuroscience, Biomedical Sciences, University of Bristol, BS8 1TD, UK.

\*Equal contribution of authors; corresponding authors

**Abstract**

Teleost fish as *Danio rerio* (zebrafish) are successfully used in biomedical research since decades. Genetically altered fish lines obtained by state-of-the-art genetic technologies are serving as well-known model organisms. In Europe, following Directive 2010/63/EU, the generation, breeding and husbandry of new genetically altered lines of laboratory animals require governmental state approval in case that if pain, suffering, distress or long-lasting harm of to the offspring derived by breeding of these lines cannot be excluded. The identification and assessment of pain, distress or harm according to a severity classification as “mild”, “moderate“, “severe“ or “break-up” became a new challenging task for all scientists, animal technicians and veterinarians for daily work with laboratory zebrafish. Here, we describe the performance of the assessment of welfare parameters of selected pathologic phenotypes and abnormalities frequently found in laboratory fish facilities based on veterinary, biological and physiological aspects by using a dedicated score sheet. In a colony of zebrafish we evaluated the frequency of genotype-independent abnormalities observed within three years. We give examples for severity classification and measures once an abnormality has been identified according to the 3Rs.

**Introduction**

Since decades, teleost fish as *Danio rerio* (zebrafish) are serving as well-known laboratory model organisms for experimental research [1,2]. In Europe, Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes serves as a frame regulation for welfare of all vertebrates and cephalopods used as laboratory animals in research [3]. Annex VIII of the directive defines severity classification of procedures on laboratory animals but not for deleterious phenotypes.

At national level, in Germany the requirements of the Directive 2010/63/EU were transposed into national law such as the German Animal Protection Act (*Tierschutzgesetz, TierSchG*) and the German regulations on the welfare of animals used for laboratory animal experiments (*Tierschutz-Versuchstierverordnung, TierSchVersV*). The latter is defining the requirements for husbandry, euthanasia, knowledge and skills or organizational duties when working with laboratory animals. TheGerman National Committee for the Protection of Animals used for Scientific Purposes settled at the German Federal Institute for Risk assessment (*Bundesinstitut für Risikobewertung*, *BfR*) organized a workshop on criteria for assessment of adverse phenotypes in teleost fish [4]. The resulting document serves as a practical guide for assessment and documentation of adverse phenotypes and abnormalities in genetically altered larval and adult teleost fish. For approval of laboratory animal experimental protocols in Germany, measures and humane endpoints must meet legal requirements in case of abnormalities. Severity classification as “mild”, “moderate”, “severe” or “break-up” (humane endpoint) impairment of welfare is mandatory for experimental laboratory animal protocols.

Johansen et al. already stated - by reviewing the available literature - that guidelines for assessment of welfare in laboratory fish are only rare [5]. Severity assessment is based on the individual knowledge and experience of the scientists, animal keepers and veterinarians and is a challenge for all staff members working with laboratory fish. As with all species used as experimental laboratory animals, phenotypes of mutant fish lines that are due to the genetic alteration must be distinguished from wild-type fish showing any clinical signs of disease. For example, genetically altered fish may show external morphologic or pigmentation abnormalities due to genetic alterations [6] that phenotypically may also derive from infection or illness.

While for genetically altered rodents some suggestions for welfare control and severity classification of clinical signs of pain, suffering, distress and lasting harm are available [7-9], similar information for fish is still rare. However, some suggestions for standardization of welfare terms for zebrafish including severity classification for selected parameters are provided by the Zebrafish Information Network (ZFIN) health and welfare glossary [10-11]. Further, precise descriptions for distinguishing normal from pathologic behavior in zebrafish are available. They are also suitable to reveal abnormal behavior due to pain or distress [12-13].

Here, we present examples for severity classification of abnormalities routinely found in adult zebrafish by using a dedicated score sheet as a practical guide for regular welfare investigation of laboratory fish (Table 1). These examples may also serve to classify phenotypes in the frame of severity assessment of a new generated genetically altered zebrafish line. We further evaluated the frequency of abnormalities found in our fish facilities within 3 years to show the rate of abnormal phenotypes to be expected by welfare inspections in laboratory zebrafish husbandries (Fig. 1).

**Material and Methods**

*Housing and husbandry*

Housing conditions and hygienic monitoring in our fish facilities are as published recently [14]. Briefly, zebrafish are kept under specified pathogen-free (SPF) conditions for a 14/10 hour light cycle in racks containing glass tanks (10 L) derived by Aqua-Schwarz GmbH, (Göttingen, Germany) or plastic mouse Type I cages converted into fish tanks (2.1 L) within a recirculating water cycle. Housing density is 5 fish/L. The holding tanks regularly do not have any enrichment. However, this is added when aggression is observed and is also provided in mating tanks. The nominal values of the water temperature are set to 26.5 - 27°C. An automatic water change takes place several times a day in order to renew at least 5% but not more than 20% of the total water volume per day. Depending on the stage of development, the fish are fed with rearing food, Artemia and flake food (Tetramin; Tetra GmbH, Melle, Germany) and hatched artemia (Great Salt Lake Artemia Cysts; Sanders Brime Shrimp Company, USA) distributed by spray bottles. Inspection of welfare conditions is performed daily by the animal keepers and regularly by scientists and veterinarians. Age of fish is exceptionally up to three years according to aging studies. For maintenance colonies, the age of the fish should not exceed 18 months. In the same area we are housing a small colony of medaka (*Oryzia latipes*)

*Hygienic monitoring*

Hygienic monitoring is performed by a quarterly examination of pooled samples consisting of colony fish and fish showing clinical signs of illness. These fish are frozen and sent to a specialized laboratory for PCR analysis for selected pathogens [14]. If reasonable, diseased fish are separated from the colony for observation and/or sent to diagnostic laboratories together with filtered water samples for further examination (e.g. PCR or histopathology). Occasionally, *Pseudomonas neutrophilia, Mycobacxterium spp.,* *Mycobacterium abscessus, Mycobacterium chelonae, Myxidium streisingeri* and zebrafish picornavirus 1were confirmed. These pathogens are tolerated within our facility.

*Fish strains and lines*

Wild-type zebrafish and genetic altered lines are on common backgrounds as *e.g.* AB, WIK or Tuepfel Long-fin (TL). Common depigmentated strains like *casper* and *crystal* and their founder strains as *roy, alb* and *nacre* are also available in the colony. Genetically altered fish used for research in our facility are mostly transgenic reporter lines. New genetically altered fish lines with so far unknown severity are generated by an experimental animal proposal for generation, breeding and housing approved by the Government of Upper Bavaria.

*Inspection and scoring of abnormal phenotypes*

For welfare assessment of adult fish, we followed the parameters given in the recommendation no. 001/2015 by the German National Committee (TierSchG), as they are: (1) body structure (changes in length, body composition, altered flexion, swelling/tumor), (2) fins/scales/skin/gills (alterations, reddening, black pigmentation and other changes of skin color, altered gills, ulcerations) and (3) behavior (circling, swimming on the ground or surface, altered feeding, aggression). We also considered further parameters, as e.g. abnormalities of the head and eyes.

Based on the severity classifications described above, we developed a score sheet for general health assessment (Table 1). For each parameter a score is given with score 0 = none (physiologic condition), score 1 = mild, score 2 = moderate, score 3 = severe pain, distress or fear, and with score 4 = humane endpoint. Severity assessment must be made for single and for cumulative scores. Measures to be taken when a certain score is assigned are also indicated. It is specified how many days a burden can be tolerated. We generally are using this monitoring system also for our medaka colony.

**Table 1: Score Sheet for assessment of welfare parameters in zebrafish**

|  |  |  |
| --- | --- | --- |
|  | **Parameter** | **Score** |
| **Size, body condition,****development** | Normal, according age and gender (BCS 3) | **0** |
| Slight reduction of body weight (BCS2) and/or size  | **1** |
| Strong body weight reduction („*skinny*“, BCS1)  | **3** |
| **Abdomen** | Symmetric, according gender; swollen abdomen after feeding or pregnancy | **0** |
| Eggbound female | **2** |
| Asymmetric swelling, tumor suspicion | **3** |
| Abdominal dropsy, ascites | **4** |
| **Vertebral column** | Normal; slight flexion/kink with normal swimming and body condition in old fish (>1 year) | **0** |
| Minor flexion (usually with normal swimming and feeding) | **1** |
| Moderate flexion (usually with minor impaired swimming and feeding) | **2** |
| Strong flexion or lateral kink (usually with abnormal swimming and/or emaciation) | **3** |
| **Head and eyes** | Physiologic appearance | **0** |
| Brachygnathia/prognathia  | **1** |
| Abnormal head, short head | **3** |
| Blindness of one eye  | **1** |
| Both eyes blind  | **3** |
| Exophthalmos | **4** |
| **Skin, scales** | Regular pigmentation, no abnormal coverings; slight depigmentation of old fish without further abnormalities | **0** |
| Abnormal coverings or mucus; mycosis; scratching, itch | **3** |
| Depigmentation dark or grey-pink dots; hemorrhage | **3** |
| **Fins** | All fins appearing normal | **0** |
| Shortened/abnormal fin(s)  | **1** |
| missing tail fin | **2** |
| **Wounds**  | Small superficial wound with injuries of mucous layer | **1** |
| Several small or single large wound, ulcers | **3** |
| **Gill and gill covers,****breathing** | Gill covers normal, normal breathing frequency | **0** |
| Protruding gill covers or missing gill cover on one side | **1** |
| Both gill covers missing | **3** |
| Increasing movement of gill covers/breathing with additional signs of illness; swollen gills  | **3** |
| **Swimming behavior** | Normal, calm and steady within swarm | **0** |
| Minor impairment of swimming and/or separated from swarm and/or signs of stress  | **1** |
| Aggression, chasing of other fish in small groups | **1** |
| Uncoordinated swimming behavior, problems with hunting and food intake | **3** |
| Mainly at surface or at tank bottom | **3** |
| Circling, trundling; trembling; vertical or horizontal tilted; sinking when not actively swimming | **4** |
| **Further break-up criterion** | Prolaps of anus or intestine  | **4** |
| Deep wound; necrotic tissue; tumor |
| Lying on tank bottom |

***Legend Table 1: Measures***

***Single score***

***Score 0 = no pain, distress and suffering***

***Score 1 = minor severity.***

* *Abnormal behavior/swimming behavior due to stress in single fish or as a dominance problem in small groups: add 2-3 other fish. If there is no improvement 5 days after measures judged as score 2.*
* *Small single wound: if possibly due to aggression/bites observe if healing occurs within 5 days.*
* *In case of a decreased size/developmental delay: weekly assessment and documentation of overall appearance: accepted as long as all other parameters remain normal.*
* *In case of one-sided blindness, regular scoring and documentation whether for emaciation and/or abnormal swimming.*
* *In case of missing or protruding gill cover on one side: weekly scoring and documentation for signs of emaciation and/or additional signs of disease.*

*.****Score 2 = moderate severity.***

* *Inform project leader and/or veterinarian*
* *Eggbound female: gently squeeze eggs; improve husbandry (e.g. no single housing; keep in mixed groups); kill old fish if possible (> 18 months); in case of no improvement judged as score 3.*

***Score 3 = severe pain.***

* *Inform project leader that fish must be killed (organ withdrawal within 1 hour possible).*

***Score 4 = break-up criterion, humane endpoint****:*

* *fish is to be killed immediately.*

***Sum scores***

***Score 2-3 = moderate severity****:*

* *Optimize husbandry (e.g. increased feeding, divide the group in case of large groups, add fish as companions in small groups); daily scoring. If 2 days after measures no improvement takes place, the fish is to be killed.*

***Score ≥4 =******severe pain:***

* *Inform project leader that fish must be killed (organ withdrawal within 1 hour possible).*

*Radiographs (X-rays)*

Adult fish were fixed in 4% PFA for 10 days, washed with water and radiographed using a MultiFocus digital radiography system (Faxitron) under 2x zoom and using the settings: 45 kv, 5 seconds of exposure and 0.46. A total of 32 fish were X-rayed. Spinal phenotype of each fish was annotated (Fig. 3)

**Results**

Welfare assessment of laboratory fish is routinely performed by daily inspection by the animal keepers as part of their daily work schedule in the facility, weekly by the scientists using the fish in experimental animal proposals and further, regularly also by veterinarians. Once an abnormal welfare parameter is found, measures must be taken according to the specific score sheet of an experimental animal proposal and to the document on final severity assessment for fish of genetically altered lines. We evaluated the findings of 60 routine inspections of our two zebrafish facilities performed by veterinarians in the years 2017-2020. The stocking rates within these two facilities were between 12.000 and 18.000 fish at that time. We found a total number of 834 abnormalities (Fig. 1). The described phenotypes were observed independent from the genotype of fish. Mutant zebrafish lines in our facilities where in most cases reporter lines and did not include mutations inducing special phenotypes of fish.

**Fig. 1 Frequency of abnormalities found within 3 years of animal welfare inspections.**

*Legend Fig 1. A total of 834 abnormalities were found in 12.000-18.000 fish kept in two animal husbandry facilities at that time. The abnormalities were independent from any targeted mutation or genetic background.*

*Body structure: body composition and curvatures of vertebral column*

Zebrafish grow constantly during life, even after having reached adulthood. In laboratory zebrafish, a well-conditioned body composition is important for the success of breeding and wellbeing. The body condition score (BCS) is helpful for determining if a fish is obese or emaciated (“skinny”) [15]. According to the body scores, BCS 3 fish is defined as the shape of a well-conditioned fish. Emaciation (BCS 1) is a sign of developmental delay or disease, and thus suggested as a severe phenotype (Fig. 2 A). Obese fish (BCS 5) are not classified as diseased, but should be an indicator for a change of feeding amount or frequency. Obese fish are to be distinguished from fish with ascites or female fish that are egg-bound. If the body weight and/or size are slightly reduced without any other impairments or the fish shows a minor vertebral flexion with normal swimming and feeding behavior, we consider the burden to be of mild severity.

Zebrafish may show dorsal and lateral vertebral curvatures or kinks of the body axis, defined as kyphosis (dorsal convex flexion), lordosis (ventral flexion) and scoliosis (lateral flexion with vertebral torsion) due to ageing, diseases and genetics. Vertebral curvatures increasingly appear in aged zebrafish, as previously reported [16]. On the other hand, infection by *Pseudoloma neutrophilia* often cause spinal curvature which is accompanied by body emaciation [17]. Mutations in genes associated with scoliosis may lead to abnormal development of the vertebral column and phenotypic changes in the zebrafish spine, as detected in *ptk7* [18] and *dstyk* [19] zebrafish mutants. In our retrospective survey, we isolated an increasing number of fish which vertebral column phenotypes were independent of genetic background (Fig. 3). We observed morphological defects of the vertebral column appearing as described above for ageing and genetic modifications [16: 18-19], however we did not correlate these abnormalities with any underlying mutations so far. A total of 32 fish displaying variable degrees of spinal curvatures were analyzed in more detail through radiographs (X-rays) (Fig. 3). We detected mild curvature varying in positions along the vertebral column: anterior (kyphosis), transitional (between ribs and posterior vertebral column), posterior (lordosis) areas, and scoliosis in one fish. Multiple spinal curvatures (more than 2) within the same fish were found in two fish. Fusions were frequently observed in proximity with the tail fin (21/32). Two fish displayed severe fusions throughout the vertebral column and dramatic shortening.

For spinal curvatures due to ageing of fish under a general good condition (no swimming impairment; normal body condition), we suggest classifying the abnormality as “without any severity”. In case of spinal curvatures, with reduced swimming activity within the swarm, without reduced body condition, we suggest a “mild” degree of the severity. We recommend monitoring these fish every one to two weeks, depending on the severity of the curvature, since the phenotype may further deteriorate with ageing and lead to impaired swimming and hunting behavior. We recommend classifying strong curvatures observed in parallel with obviously reduced swimming activity and/or body composition and/or isolation from the swarm as a “severe” phenotype. About 4.3% of fish with abnormalities showed dorsal or lateral curvature as a single phenotype in evaluation of our welfare data (Fig. 1). Another 4.9% of fish were found with additional phenotypes, such as reduced body condition or body size, head or other abnormalities, swollen abdomen, tumor, depigmentation, or hemorrhage.

**Fig 2. Examples of fish with clinical signs of illness/abnormalities.**

*Legend Fig 2.* ***A) Body condition****. Male AB fish presenting the “skinny” phenotype with strong emaciation. Severe single phenotype (score 3).* ***B) Vertebral Column****. Longfin male zebrafish with moderate dorsal curvature of the vertebral column usually with minor impaired swimming and hunting, assessed as score 2 (moderate severity). In addition missing of one gill cover, assessed as score 1 (minor severity). Sum score 3 defining an overall moderate phenotype.* ***C)******Wound****. Female fish with large wound on lateral abdomen assessed as severe burden (single score 3).* ***D)******Blindness.*** *AB zebrafish showing blindness visible by an opaque cornea and mild exophthalmos visible on both eyes (single score 3).* ***E) Skin.*** *Female fish with abnormal coverings or mucus and subcutaneous hemorrhage (single score 3).* ***F) Abdomen.*** *Fish with swollen abdomen appearing like ascites by symmetric swelling. In transparent fish it can be distinguished if a swelling is due to ascites or tumor or if female fish are egg-bound (single score 3).*

**Fig 3.** **A-D Radiographs to show examples of fish with vertebral column abnormalities identified after visual inspection**

*Legend Fig. 3:* ***A)*** *Normal vertebral curvature.* ***B)*** *Mild kyphosis.* ***C)*** *Moderate kyphosis, lordosis and scoliosis* ***(C’)****.* ***D)*** *Multiple vertebral fusions. Dashed lines indicate areas of kyphosis (yellow), lordosis (magenta) and scoliosis (green). Yellow arrows indicate vertebral fusions. C’ dorsal view of the vertebral column to show scoliosis in C. Scale bars = 1mm.*

*Morphological defects of head, mouth and eyes*

Single fish can be found with head and mouth abnormalities due to unknown reasons or to spontaneous mutations. Severity assessment depends in first line on the fish ability to feed normally, visible by general body condition appearance. If the malformation affects the food intake, the score factor should be “severe”, thus following the suggestions of the ZFIN organization [10].

Visual impairment or blindness are occasional findings in aged zebrafish and can be due to tumors, corneal ulcers, trauma following aggression, infections, genetics or are idiopathic from unknown reason. The cornea often appears milky or cloudy (Fig. 2 D). Exophthalmos is a special finding often associated with infectious diseases or tumors. Visual impairment will strongly impede hunting and feeding of fish. For social interaction, shoaling, mating and hierarchy the recognition of color and pigmentation expression is crucial in fish [20-21]. Blind fish in most of the cases can be detected by finding them isolated from the swarm, often swimming mainly at the bottom of the tank or along the panes. This finding is frequently accompanied by emaciation. We therefore suggest to classify impaired vision of one eye as a “mild” to “moderate” phenotype. Depending on further abnormalities, such as emaciation or abnormal swimming (sum scores), this may be considered a “severe” phenotype, as well as for total fish blindness.

*Fins, scales and gills*

Adult zebrafish have five different types of fins - dorsal, pectoral, pelvic, anal and tail fins. Variable length of the fins is a common feature in several genetic background strains used around the world. Degeneration, total loss or reduced fin sizes may derive from molecular reasons, and are observed already at early developmental stages, as described for all fin types. Fin appearance can further be changed by infection as by *Flavobacterium collumnare* [22] or by injuries. Differences in fin lengths are also found in a high number of mutant lines carrying genetic alterations [23-26]. Fin size has been shown to influence swimming capability and speed in different zebrafish lines [27]. Abnormalities as frayed, shortened or totally missing fins may be an occasional finding. Depending on the kind of fins that are abnormal or missing, the severity classification may range from “mild” (shortened or abnormal fins without visible effect on swimming and hunting behavior) to “severe” (strong impairment of swimming behavior, emaciation due to impaired hunting/feeding behavior). Due to the impaired activity, we suggest to classify the total loss or a strong shortened tail fin as a “moderate” severity because swimming ability will always be impaired. In fish that already show reduced body composition by problems of hunting and food intake, a severe phenotype is suggested.

Fish opercula serve as bony protections of the gills, used for respiration and feeding mechanisms. The operculum contributes to gill ventilation via pressure changes by the so-called buccal and opercula cavities buccal pressure pump. Thus, gill covers are actively contributing to a constant water flow through the gills and to sufficient oxygen uptake [28-29]. Interestingly, during our three years documenting anomalies, the lack of one or both opercula was the most frequent single abnormality found (*N* = 219) (Fig.1). In some cases the operculum appeared shortened or abnormal but still present. Since oxygen uptake always will be decreased we suggest assigning the loss of one operculum as “mild” (Fig. 2 B) and the loss of both as a “severe” harm of the fish.

*Skin, scales, mucous layer and pigmentation*

The mucous layer protects the fish from osmotic stress and infectious agents. Thus, an intact skin and mucous layer are of high impact on health and wellbeing of fish (Fig. 2 E). In spite of the rapid regeneration of several tissues in fish including the skin, even small wounds such as bites from aggressive fish or injuries caused by handling, lead to a damage of the mucous layer. Therefore, we consider these small injuries to be at least a “mild” impairment of welfare. Recovery should be monitored carefully in the following days. All skin lesions found as ulcers, tumors or necrosis are suggested as “severe” phenotypes (Fig. 2 C). These fish should not be used for any experiments.

Zebrafish pigmentation and color relevantly influence social behavior [20; 21]. Brightening of pigmentation of single zebrafish is known as a possible indicator of anxiety, stress, environmental changes or following treatments. Paling may be a general sign of disease and darkening an indicator for dominance of a fish [13]. Thus, individual changes of color patterns give relevant additional information on wellbeing of fish and should be considered in the health assessment. Small skin abnormalities as red/hemorrhagic, grey-pink, dark or white dots may indicate infections by *Mycobacteriosis spp.* [30-32]*, Ichthyophthirius multifiliis* [33] or *Edwardsiella ictaluri* [34]*.* Dark dots may further be due to melanoma. Some research facilities accept infections of *Mycobacterium spp.* (with exception of *M. marinum*) or infections of *Pseudoloma neurophilia* since hygienic eradication is difficult. But diseased fish should always be removed and tested for infections. Test results and mortality rate in the facility should be documented for the overview on the spread of an infection. In a three years survey we found a total number of 30 fish with different skin abnormalities (Fig. 1).

*Behavior and swimming behavior*

Activity in the swarm and individual swimming behavior are the most relevant parameters for assessment of general health condition of a single fish. Thus, knowledge of relevant physiological behavioral patterns of zebrafish strains and genetic backgrounds are essential for the assessment of wellbeing. Zebrafish show circadian rhythm of sleeping and resting activities [35]. Wild-type strains of zebrafish are well known to differ in their behavior even at early stages of development [36]. Individual behavior can be observed as e.g. isolation, aggression or specific sexual behavior. Certain swimming patterns as *zigzag* swimming are signs of stress or anxiety. Impaired wellbeing will influence swarming behavior and the fish position in the tank, isolated from the swarm [12-13]. In case of fish aggression, it can be helpful to provide environment or divide the swarm. However, very small groups should also be avoided. A frequent used depigmented zebrafish line is the *nacre* mutant in which the *mitf-a* gene is mutated. Deficit in visual-motor activity was recently described for *nacre* fish under special conditions [37]. This should be considered when assessing fish of this common background if abnormal swimming patterns are observed.

Severity assessment of impaired swimming behavior depends on the type and degree of the abnormality. Strong swimming impairment with abnormal movements, such as circling, slowness, vertical and horizontal tilted body axis are suggested as a “severe” abnormality. Like other teleost fish, zebrafish possess gas-filled swim bladders for buoyancy and position in the water [38]. Defects of the swim bladder may become obvious when the fish have problems with vertical movements in the water. Occasionally, fish are found with an upright vertical position in the water – we observed this frequently in fish on the *casper* background. We consider this to be a termination criterion and these fish are immediately euthanized in our facility. During the evaluation of our data (Fig. 1), a total of 243 fish showed abnormalities of swimming behavior either as a single phenotype (*N* = 64) or as a sum score with other abnormalities as curvatures (*N* = 87), abnormal tail fin (*N* = 24) or other phenotypes (*N* = 68).

*Swellings and tumors of the abdomen*

Swollen abdomen with or without protruding scales was an occasional finding in our zebrafish facilities. Abdominal tumors and “abdominal dropsy” (edema with protruding scales, associated with ascites/peritonitis) are “severe” phenotypes, often seen following infections like *Mycobacteriosis* [39] (Fig. 2 F). If abdominal swelling is caused by a tumor or ascites, we recommend to classify this as a severe phenotype. Spawning disability (egg-bound fish) can be due to poor environmental or housing conditions. Zebrafish show group spawning behavior meaning that female fish need grouped housing of both sexes to enable physiologic spawning. Even after a short period of isolation, it can occur that female fish are no longer able to spawn and are getting egg-bound [40]. We suggest classifying spawning disability as a “moderate” severity. The eggs can be carefully squeezed manually (we recommend short anesthesia as a refinement to avoid pain and stress). Swarm fish are social animals and single housing must be avoided according to the Directive 2010/63/EU. However, under specific situations it may be necessary to isolate a fish from the swarm, e.g. until the result of genotyping is available. These fish should be housed in groups with visually distinguishable companions. We found 92 fish in our three years study with swollen abdomen due to tumors, spawning disability or ascites (Fig. 1).

**Discussion**

According to the 3Rs, pain, suffering, distress and fear of animals used in animal experiments must be avoided as far as possible. Wellbeing of all vertebrates used for research has highest priority. Documentation of the performance of regular health control and the measures following an observed abnormality enables the follow-up for every individual animal. Fish of an approved experimental animal proposal must be evaluated regularly according to specific score sheets approved by the authorities. This does not apply to fish not belonging to an experimental animal proposal, but of course these fish must also be inspected daily for their welfare. So far, only limited information is available on severity assessment of abnormal welfare terms of laboratory fish. The assessment of individual abnormalities may be subjective and could vary between the different persons performing the controls. Standardization of severity classification of common clinical findings is helpful to all researchers and animal keepers working with fish. To make the assessment of welfare parameters in fish evident, objective and easily determined, we have developed a score sheet to be used by animal keepers, scientists and veterinarians. In contrast to other laboratory animal species such as rodents, the possibilities for treatment of sick fish used for experimental research are very limited. This is also to prevent hygienic outbreaks in large fish facilities. In most cases, fish showing any clinical signs of illness or an infection will be euthanized and used for diagnostic purposes. Nevertheless, a standardized definition of abnormalities, the respective severity degrees and documentation of all abnormalities and of unusual accumulation of fish found dead are helpful for early detection of any environmental, technical and hygienic problems [41].

The ZFIN web page provides a summary of welfare terms including a detailed severity classification as “mild”, “moderate” or “severe” of single phenotypes [10]. This is very helpful for a general assessment of wellbeing in fish and for defining phenotypes and severity scores for fish of a genetically altered line. The ZFIN provides a graded severity classification e.g. for wounds, tumors, fin alterations, body composition or edema. However, for routine health control in fish facilities, it often may be irrelevant if a penetrating wound is less than 10% (classified by ZFIN as mild severity) or more than 20% of the body (moderate severity), because both fish most probably must be euthanized due to absence of therapeutic options. We therefore developed a score sheet for zebrafish with a less detailed classification of single abnormalities dedicated for daily welfare inspection.

Species-specific knowledge on physiological characteristics is essential for investigations of welfare of laboratory fish. Zebrafish typically show swarming behavior [42]. Activities in the tank are following a diurnal rhythm, also influenced by environmental conditions [43]. For welfare analysis, body size and composition of an individual has to be compared to other age-matched individuals of the swarm as zebrafish grow throughout life. Body composition strongly depends on the amount of feeding, swarm size, gender and age of fish and, above all, also on proper early development.

The ability of adult and larval fish to feel pain is still under controversial scientific and ethical discussion [44-49]. On one hand the debate is based on the anatomical and neurological conditions in fish. On the other hand this is discussed by the results obtained by specific nociceptive tests performed with or without using analgesia, resulting in avoidance behavior in the latter case [50-52]. Adding of analgesic substances ameliorated aversive reaction to acetic agents already in 5 dpf larvae making pain perception in fish more than likely [53]. Regardless of these different views, each researcher working with fish in Europe must meet the requirements of the Directive 2010/63/EU on reduction of pain, suffering and fear in all species used for animal experiments according to the 3Rs.

Spinal curvatures may be due to developmental, environmental, nutritional or genetic issues. Vertebral abnormalities may occur following treatments and in toxicology studies, or found as idiopathic single cases due to environmental or even unknown reasons [54-55]. Analyzing a number of fish found with curvatures of the vertebral column by radiographs confirmed the severity of the abnormalities that are not always immediately recognizable in their severe manifestations on the external inspection. This underlines the importance of paying special attention to this phenotype in terms of animal welfare.

We observed a relatively high number of fish (26.3% of all abnormalities) with losses of one or both gill covers. We could not find similar data on the frequency of these abnormalities in the literature. Thus, it remains an open question why quite a lot of fish showed this finding. Occasionally, the gill covers appeared only shortened or malformed. Young fish with this problem often remained smaller than other fish of the same age within the swarm. Adult zebrafish with opercular anomalies often showed additional, eventually secondary abnormalities such as depigmentation or poor body condition indicating an overall impairment of wellbeing. Some researchers assign the abnormality as an inbreeding depression in the breeding colony (personal communication). Since these were mostly individual cases in our facilities, inbreeding was probably not the cause. Developmental mutations responsible for craniofacial abnormalities including the gill apparatus have been described already in early larvae [56] and relevant genes are already described for opercular development in zebrafish and in other fish species [57-58]. We assume that unilateral loss of a gill cover may be due to aggression, biting and other injuries e.g. caused by catching with nets. However, in case that both opercula are missing and if most fish of a line are affected this probably may be due to molecular reasons. Some reports can be found on opercula deformity or losses for species held in marine hatcheries and aquaculture. Mechanical trauma or malnutrition (HUFA-n3, vitamin C) have been suggested as underlying reasons leading to irregular mineralization in aquaculture large fish species [59]. It remains an open question whether similar reasons are also responsible for this phenotype in zebrafish. To our knowledge, abnormalities like injured, malformed or missing opercula are sometimes accepted in other zebrafish facilities. On the ZFIN welfare terms this finding so far is not assigned to any severity classification at all. Considering that sufficient water flow through the gills and oxygen uptake is depends also on the movement of the opercula and further, that respiratory frequency is an important behavioral parameter for assessment of pain and stress [60], this estimation is suggested for reconsideration. Monitoring of anesthesia is no longer possible without observation of the gill covers. According to welfare terms, we are of the opinion that the loss of any anatomical functional structure cannot be accepted as normal, especially when physiologic processes such as respiration may be affected. However, in the available literature no study was found on the investigation of the influence of this abnormality on wellbeing of fish. We observed that euthanasia of these fish was a special challenge when using immersions of MS-222 overdose. Zebrafish with missing gill covers needed a very long time (>15 minutes, data not shown) until absence of any reaction on tactile stimuli could be stated. Since MS-222 is mainly ingested through the gills and to a lesser extent *via* the skin [61] obviously no sufficient amount of anesthetic could be absorbed in these fish because the opercula cavities buccal pressure pump was mechanically disturbed.

Craniofacial phenotypes are clinically defined as e.g. brachygnathia (short maxilla), brachycephalia (short head, short maxilla and mandible) or lockjaw. Numerous genes have been identified as relevant for the development of these craniofacial bony structures and abnormalities [62-64]. We decided to assign craniofacial abnormalities as a severe harm as far as general condition and body weight are also impaired.

Fleisch and Neuhaus reviewed visual acuity in zebrafish and showed that already young larvae obtain a highly developed functional visual system as shown by analysis of optokinetic and optomotor response [65]. Unlike other vertebrates, teleost fish are capable of spontaneous regeneration and restoring of vision following injury [66]. Adult fish show an even broader range of optical spectrum than human cones with a range from ≈360 nm ultraviolet (UV-) sensitive cones, to short wavelength (blue opsin, ≈415 nm) single cones, middle wavelength (green opsin, ≈480 nm) and long wavelength (red opsin, ≈570 nm) cones [67]. The guidelines of the Working Group of Berlin Animal Welfare Officers suggest the classification of complete blindness in rodents in case of normal behavior as a mild severity [7]. Rodents have more possibilities for orientation than fish that rely to a higher degree on an unimpaired vision for hunting and feeding. We therefore suggest total blindness in fish as a severe abnormality.

It was reported that water temperature at rearing is influencing swimming activity of adult zebrafish [68]. In first line swimming performance will depend on anatomical and neurological structures. Depending on the speed, the different fin types are used to varying degrees for locomotion. Fast swimming is achieved primarily through the movements of the tail fins [27; 69-70]. In our facility, we occasionally found fish with abnormal tail fins appearing as described for the spontaneous *ntl* mutant [70]. Plaut et al. described how fin size affected significantly swimming performance and activity in three different zebrafish lines including fish carrying the no-tail mutation [71]. Concerning fin alterations, severity classification in our facility is based on swimming performance of the fish. Fish with a missing tail fin will always show abnormal swimming patterns and are often found additionally with reduced body size.

 In summary, we are herewith presenting for the first time a recommendation for routine daily visual inspection of laboratory zebrafish and the assessment of abnormalities of welfare terms. We have created a score sheet that supports all persons performing welfare inspections to easily evaluate fish on their wellbeing. Animal caretakers, veterinarians and scientists use this score sheet in our institution for objective assessment of any disease, pain or stress in adult fish. The measures to be taken were specified in order to save the fish from suffering and to improve animal welfare. This score sheet can also be used to evaluate newly generated lines and it can serve as an example for other research institutions working with zebrafish to develop similar documents. Our evaluation on the frequency of abnormalities may help other institutions to pay special attention to these parameters found in our survey, as they may be generally a genotype-independent frequent finding in zebrafish.

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