

Considerations for a European animal welfare standard to evaluate adverse phenotypes in teleost fish

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Fish, in particular genetically modified zebrafish, are important model organisms for biomedical research and research into human diseases. The European Directive 2010/63/EU on the protection of animals used for scientific purposes entails that genetically altered vertebrates need to be assessed for pain, suffering, distress, or lasting harm (collectively referred to here as “adverse phenotypes”). If such phenotypes are present, maintenance of genetically altered animals is now subject to project authorization. As genetically altered fish are commonly imported into the EU and exchanged between laboratories, fish lines should be classified consistently. To this end, the German Federal Institute for Risk Assessment (BfR) organized a workshop in June 2015 to define criteria for assessing genetically induced adverse phenotypes in fish. Here, we describe the Workshop’s considerations that guided the design of dedicated evaluation sheets. We believe that broad use of these

evaluation sheets and the explanatory notes associated with them can contribute substantially to harmonizing how teleost fish phenotypes are assessed across Europe. In our view, this would protect both animal welfare and ensure progress in biomedical research.

Across Europe, welfare of laboratory animals is a growing public concern (Ormandy & Schuppli, 2014). Yet, a majority of European citizens is willing to accept animal experimentation to promote biomedical progress (http://ec.europa.eu/public_opinion/archives/ebs/ebs_340_en.pdf). The spirit of the European Directive 2010/63/EU on the protection of animals used for scientific purposes, which has been implemented by all EU member states only last year, reflects these attitudes: Recital 10 of the Directive states that the final goal is to phase out all animal testing, but it also acknowledges that animal experiments are still needed to advance research and to safeguard human, animal, and environmental health ([\[eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32010L0063&from=EN\]\(http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32010L0063&from=EN\)\). To that end, all member states agreed on a high standard of animal welfare in research and to apply the 3R principle \(replacement, reduction, and refinement\) wherever possible.](http://</p>
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The Directive now stipulates that breeding of genetically altered animals in Europe is subject to project authorization if these animals are likely to experience pain, suffering, distress, or lasting harm as a result of the genetic modification. Scientists therefore have to document whether and to what degree genetically altered animals show an adverse phenotype. This means that, on the one hand, the total number of genetically altered animals that experience adverse phenotypes is documented for the first time. On the other hand, scientists have to put more effort and resources into analyzing and documenting the effects of genetic manipulations on animal phenotypes. However, scientifically sound standards are needed on

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DOI 10.15252/embj.201694448 | Published online 22 April 2016

how to objectively, consistently, and efficiently evaluate phenotypes in order to make the resulting data reliable and the assessment feasible. At the same time, the evaluation procedures have to avoid additional distress for the animals and should not unnecessarily hamper scientific progress.

When thinking of animal in research, rodents, dog, cats, and monkeys come to mind. However, according to the European Commission's statistic report (<http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:52013DC0859&from=EN>), fish rank third behind mice and rats on the list reporting the numbers of animals used for research purposes. Moreover, while the number of mice and rats has slightly decreased during recent years, the total number of laboratory fish used in developmental biology and other areas of basic biomedical research increased by about 29% from 2008 to 2011. This trend can be particularly attributed to the fact that fish, especially zebrafish and medaka, are valuable transgenic model organisms for human diseases (Lieschke & Currie, 2007) and have a firmly established role in the pharmaceutical industry (http://www.roche.com/research_and_development/drawn_to_science/zebrafish.htm). Contributing to this increase in numbers is the fact that the use of fish instead of rodents is regarded as a refinement in the sense of the 3R, as Article 13 of the Directive requires using species with the lowest capacity to experience pain, suffering, distress, or lasting harm (<http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32010L0063&from=EN>). Moreover, using fish larvae before they start to feed independently has become an alternative method for many scientific studies, because the eleutheroembryo stages of fish do not fall within the regulations of the Directive (Strähle *et al.*, 2012). The availability of new genome editing techniques, such as CRISPR/Cas9, is likely to further increase the number of genetically modified laboratory fish in the coming years.

Putting the obligations of the Directive into practice, it becomes evident that there is a gap between the political demand to improve animal welfare and the lack of scientifically sound biomedical indicators for animal welfare. The Directive requires scientists to determine whether and to what extent animals suffer or feel pain or distress, but it does not provide objective

criteria to evaluate such conditions. A close collaboration between scientists, animal welfare officers, lawyers, and members of the executive authorities is needed to find a solution that efficiently protects animals, offers a high level of legal security, and can be implemented as efficiently as possible for the sake of ensuring scientific progress. Pursuing such an interdisciplinary approach, the BfR brought together the expertise of scientists and executive authorities in 2013 to compile workable documents to assess welfare of genetically altered mice and rats (Grune *et al.*, 2014). These documents are now used in Germany and have successfully harmonized the evaluation process of research projects that use genetically altered rodents. However, these documents cannot be easily transferred to other species: the different physiology and behavior make it necessary to define species-specific criteria for assessing animal welfare.

A similar regulatory framework for fish has so far not been developed, and, apart from guidelines for toxicity testing in fish, no feasible concepts are available to assess fish well-being and adverse phenotypes. Thus, the BfR initiated a Workshop in June 2015 to compile workable documents for the assessment of adverse phenotypes of genetically altered teleost fish (<http://www.bfr.bund.de/cm/349/severity-assessment-of-genetically-altered-fish-bony-fish-teleost-fish.pdf>). The participants were scientists with expertise in the field of physiology, pathology and behavior of laboratory fish, legal experts, and veterinary specialists for aquaculture and for laboratory animal research, for fish facility management, for pharmacology and toxicology, as well as representatives of the responsible executive authorities in Germany. They were asked to define guiding principles, criteria, and evaluation sheets to enable a scientifically and legally sound evaluation of teleost fish phenotypes for the purpose of fulfilling the EU Directive's requirements and its specific implementation in German law. With this in mind, the Workshop discussed a number of general principles that should guide the recommended screening approach to detect adverse phenotypes.

First, criteria for assessing adverse phenotypes should cover all teleost fish species used in biomedical research. Given that the new gene editing techniques are generally applicable in teleosts, the commonly used zebrafish and

medaka are not the only fish species in need of assessment. This implied that, for example, the workshop recommendations had to take into account different sizes (e.g., zebrafish vs. cichlids), behaviors (e.g., swarm vs. single swimming), physiologies (e.g., speed of larval development), and to propose criteria that live up to the requirements of assessing different species.

Second, the participants agreed that, based on the present scientific data, no final decision can be made as to the extent to which fish are able to consciously feel pain. A consensus was reached that several neuroanatomical structures exist in fish to respond to noxious events by inducing avoidance reflexes. This response, however, has to be clearly distinguished from the conscious notion of pain. Various nociceptive peripheral pathways, containing A δ - and, to a lesser extent, C-fibers, have been demonstrated in some teleost fish species, such as the rainbow trout and carp. The existence of neuroanatomical pathways from the periphery to the teleost brain is also undisputed. However, there is an ongoing debate as to whether fish are able to perceive pain at a higher level (see Rose *et al.*, 2014; Sneddon, 2015). The pallium, which, as the cerebral cortex, is necessary for conscious perception of pain in mammals, is present in fish but is not as differentiated as in humans. An amygdala, in mammals part of the limbic system and important for emotional processing of a stimulus (Neugebauer, 2015), is present in fish, but thalamo-amygdalar pathways that may transmit sensory stimuli are structured differently as studied so far (Mueller, 2012), and thalamo-cortico-amygdalar pathway-equivalent circuits have not been identified.

As there are around 30,000 different teleost fish species, which differ profoundly in their anatomy, physiology, and behavior, it also is clear that evidence derived in one species cannot be easily transferred to another. Furthermore, it would be difficult to objectively measure pain, as the behavioral responses to a noxious stimulus might differ between species owing to their different physiological and behavioral baseline (see Sneddon, 2015). For these reasons, the Workshop participants decided that for assessing adverse phenotypes in teleost fish, the focus should lie on the occurrence of "lasting harm" as an objectively measurable parameter, and secondly on the occurrence

of suffering and distress, which is less easy to ascertain.

Third, the participants agreed that some criteria, which are important for the assessment of welfare in rodents, cannot be implemented in the same form for fish. For example, a reproduction rate standardized across breeding facilities can be a valuable indicator for well-being in mice and rats, but it is difficult to use this criterion for fish. Whereas the survival rate of mice from birth till weaning is about 80% in captivity, this rate is much more variable in fish, which use a fundamentally different reproduction strategy that is much more dependent on external influences: In zebrafish, for instance, hundreds of externally developing larvae are left to their own device or even cannibalized by their parents if not separated. Moreover, the speed of development and growth of fish highly depends on external factors such as water temperature, and quality and amount of food (see Singleman & Holtzman, 2014). Hence, phenotypic assessment needs to be done against an “internal standard” of a given facility. As a consequence, institutions maintaining genetically altered fishes should be aware of the “normal” development and behavior of the respective wild-type under their specific environmental conditions, and changes in genetically modified fish lines should be related to these wild-type parameters.

Finally, the specific physiology of fish species results in somewhat different

expectations for the frequency of genetically caused adverse phenotypes. For one, considering the “lasting nature of harm”, one has to be aware that fish have powerful regenerative properties and can replace damaged tissues of the heart, fins, spinal cord, brain, and many sensory organs during adult life, which can compensate for genetically caused impairments.

In addition, as a prime organism for *in vivo* imaging due to its initial transparency, genetically altered zebrafish are often generated for observational experiments. This means that many fish lines only carry insertions of reporter genes in their genomes, coding, for instance, for fluorescent proteins or transactivators to control expression of such marker proteins. These insertions are experimentally required to be phenotypically inert. Typically, new fish lines are screened at embryonic or early larval stage before independent feeding, that is, to a time point where the Directive does not yet apply, and are excluded from further breeding. Indeed, researchers of the Workshop reported that such reporter lines—as well as the vast majority of heterozygous mutations identified in large-scale genetic screens (Driever *et al*, 1996)—are less likely to show evidence of harm. It can therefore be expected that, compared to rodents, a smaller fraction of new fish lines with an adverse phenotype will be detected, necessitating an evaluation approach that is tailored to this high-throughput/low-gain setting.

Based on these principles, three specific recommendations were adopted and guided the design of the evaluation sheets. First, genetically altered fish should be assessed at two stages, initially as larvae at the time point of independent feeding—when they first enter the Directive’s legal realm—and again as adult, sexually mature individuals. Zebrafish larvae are known to feed independently only at 120 hours post-fertilization (hpf). However, the time point for larvae to feed independently generally depends on various factors: nearly complete yolk consumption, free active swimming, the morphology of the digestive organs, and the ability to incorporate food (see Strähle *et al*, 2012).

Sexual maturity can be defined by fully differentiated testes or gonads and the production of viable gametes as well as the occurrence of secondary sexual characteristics. Most zebrafish reach sexual maturity at a standard length of 18 mm (see Parichy *et al*, 2009). From that period onwards, zebrafish just grow in size, but remain anatomically stable. Since aging processes start around 18 months (Gerhard *et al*, 2002), the Workshop participants recommended that sexually mature zebrafish are assessed at a time point between 90 and 120 days post-fertilization (dpf).

As different fish species vary highly in their speed of development, the two time points need to be determined individually for each teleost species. In addition, being poikilothermic animals, fish development also

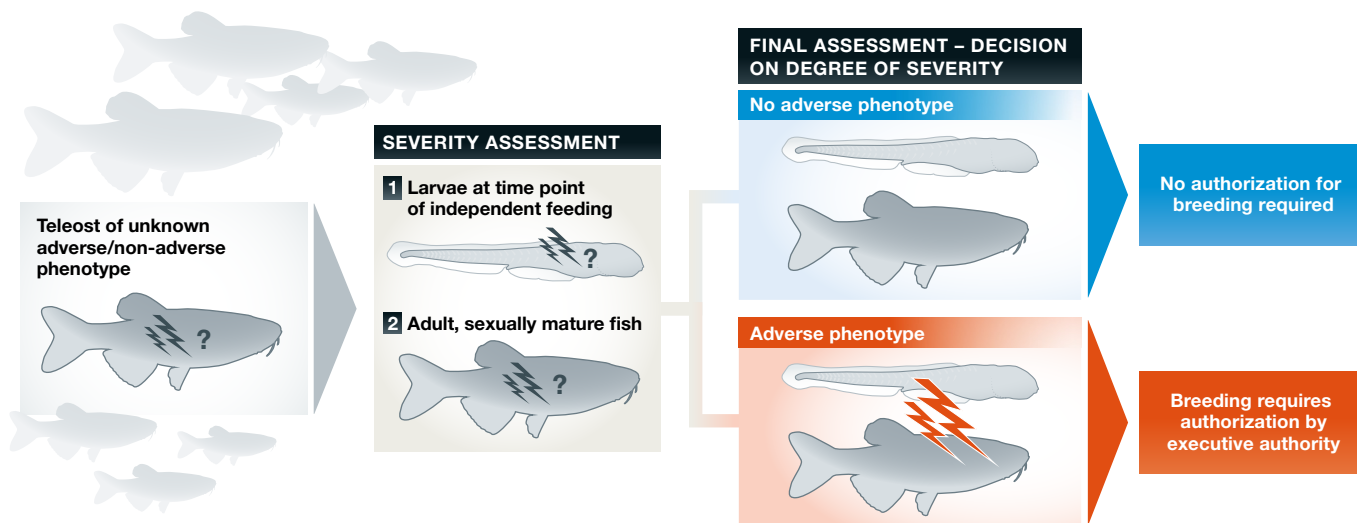


Figure 1. Evaluation process for genetically altered teleost fish with an unknown non-adverse/adverse phenotype.

Breeding of genetically altered animals needs to be authorized by the executive authority until a non-adverse phenotype has been confirmed.

depends, in addition to water temperature, on several environmental factors such as feed and stocking density. This implies that the two developmental stages have to be determined for each species at each facility.

Second, in accordance with the 3R principle, no additional fishes should be bred for the assessment, and the animals should be assessed by observation in their tanks undisturbed (i.e., by adspersion only) to avoid any additional stress. As larvae cannot be permanently marked, different animals can be used for assessment at the two different time points.

Third, a representative number of fish should be observed, which again will depend on the fish species. Keeping the number of fish progeny in mind, which differs highly from rodents, the number has to be as practical as possible for the person carrying out the observation. At the same time, the number has to provide enough data to reliably predict whether an adverse phenotype can be expected in the genetically altered fish line. As it is impossible to assess all larvae of a clutch at a glance, the participants decided to analyze a minimum of 10 larvae at the same time. Experience shows that under standard breeding conditions, larvae from one clutch can display abnormalities that cannot specifically be traced back to the genetic manipulation. Thus, it is recommended that 20 larvae from at least two clutches are analyzed as among 20 genetically modified larvae, there is a 94% probability to detect an adverse phenotype twice even if only one quarter of the larvae is affected. Based on the recommendation of the EU working document on genetically altered animals (http://ec.europa.eu/environment/chemicals/lab_animals/pdf/corrigendum.pdf), it is suggested that at least 7 adult and sexually mature fish should be assessed whether no sex-specific differences can be expected.

Taken together, this means that any fish line of any given genotype with unclear phenotype will need to be scored twice following a scheme as outlined in Fig 1. Given the large number of newly generated fish lines and that various people with different backgrounds (scientists, animal caretakers, and animal welfare officers) will be involved in the assessment, the participants tried to keep the evaluation sheets and their explanatory notes as informative and simple

as possible. The Workshop drafted three forms for the phenotypic assessment of genetically altered teleost fish: two forms for evaluating the two time points with age-matched criteria, and a summary sheet for the executive authorities. More information in the form of an explanatory note is now available in German and English on the website of the BfR (<http://www.bfr.bund.de/cm/349/severity-assessment-of-genetically-altered-fish-bony-fish-teleost-fish.pdf>).

Overall, the BfR workshops to generate consensus recommendations and evaluation sheets for animals ranging from fish to rodents show that only an interinstitutional and interdisciplinary approach focused on finding practical solutions for implementing the EU Directive can ensure a high level of acceptance within the scientific community and executive authorities. We believe that the general example of the process outlined here, as well as the specific results obtained with regard to evaluating harm in fish, might be of value across the EU. Obviously, the specific recommendations can only reflect the present state of scientific knowledge and need to be regularly adapted to new findings—ideally again using an interdisciplinary approach. To improve the welfare of laboratory fish on a scientific basis requires more fundamental research on the neurobiology, physiology, and behavior of such animals. Only with such objective information at hand, and in a continuous dialogue with the public who demands the dual assurance of animal welfare and biomedical progress, science can proceed within a well-ordered legal framework that is trusted by citizens who demand regulation and oversight, the executive bodies that execute the law, and the scientists who are subjected to it.

Conflict of interest

MB is employed by Tecniplast Germany. All other authors declare that they have no conflict of interest.

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