INTERNATIONAL ZEBRAFISH AND MEDAKA COURSE

IZMC

European Zebrafish Resource Centre (EZRC)
Karlsruhe Institute of Technology (KIT)
Heidelberg University

Theoretical Part

EZRC

SEVENTH FRAMEWORK PROGRAMME

ZF-HEALTH
Zebrafish Regulomics for Human Health

Karlsruhe Institute of Technology

UNIVERSITÄT HEIDELBERG
ZUKUNFT SEIT 1386

International Zebrafish/Medaka Course (IZMC)

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Synopsis

Fish models have increasingly gained importance in biomedical research during the last few decades. Particularly two teleost species, zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*), have become essential model systems for analyzing gene function in vertebrates. Their suitability for forward genetics especially their small size, the transparency of the embryos and the extra-uterine development made them ideal systems for systematic studies of developmental processes. In the meantime these fish have also provided many disease models that allow the study of pathophysiological processes in real time and at high cellular resolution in the intact embryo.

The Directive 2010/63/EU of the European Parliament and of the Council of Europe demands that persons performing animal experimentation should be adequately trained. Today in Germany, a plethora of courses which follow the FELASA B concept is offered by universities and other research institutions. These courses mainly address research with mice and rats; however, work with fish is not covered. In order to train technicians, students, graduates, postgraduates and principal investigators in the use of zebrafish or medaka for biomedical research, the European Zebrafish Resource Center at the Karlsruhe Institute of Technology offers a course teaching all relevant topics.

The course is held either in English or in German depending on requests of the participants. The theory element of this course is presented using online material accessible for participants via the ICMZ website. Theory topics include – amongst others –legal regulations and guidelines, ethical aspects of animal experimentation (3R, alternative concepts), biology, husbandry, breeding and feeding of zebrafish and medaka, as well as health monitoring and diseases. Other fish models in toxicology and biomedical research will be discussed. The practical elements of the course will include training in the handling, husbandry, breeding, anesthesia, fin clipping and euthanasia of zebrafish and medaka using the facilities of the EZRC and the associated KIT Institute of Toxicology and Genetics.

The course will be completed with a multiple choice examination. Those who have passed the course and examination successfully will receive a certificate confirming their specific knowledge of zebrafish and medaka as well as their skills to work with these animals meeting the legal requirements.

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1. History and Ethics of Animal Experimentation

(Almut Köhler, Jürgen Weiss)

The development of animal experimentation as a model for humans has largely taken place in parallel with the development of medical science itself. The fundamentals of western medicine originate from ancient Greece. Indeed Greek Philosophers were amongst the first to perform vivisection[1] for scientific purposes.

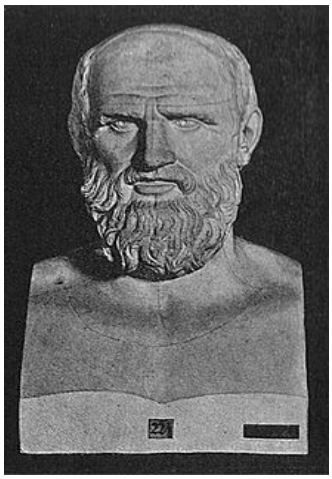


Fig. 1: Hippocrates of Kos (460 – 370 before Christ), father of "scientific medicine"

Some examples are described in the first medical compendium, the "Corpus Hippocraticum", a collection of classical medical texts compiled between the 5th and 2nd century B.C. Generally the Corpus is attributed to Hippocrates of Kos (**Fig. 1**), but it is likely he only contributed a few parts. At that time medical science was primarily descriptive and focused on anatomy.

Galen of Pergamon lived in the 2nd Century A.D. and was – next to Hippocrates – the most important physician of the classical world. As a physician, anatomist and physiologist, he used pigs, monkeys and dogs for his research and established the foundations of the medicine of his time and for centuries to come. The Roman culture did not actively support further development of medicine and biology and, with the spread of Christianity, experimental bioscience almost disappeared completely, at least, a thousand years.

At the same time, the so called Islamic Golden Age between the 8th and 13th century resulted in enormous progress for medicine and science. The importance of science those days can be seen in the arab prefix "**al**" encountered in words such as **Al**gebra, **Al**chemie, **Al**cohol and **Al**kalies. Abu Ali ibn Sina from Chorasan in Central Asia, known as Avicenna (ca. 980 – 1037 A.D.), is the most famous representative of this era. He studied multiple disciplines, including medicine, natural sciences and philosophy. Avicenna wrote in detail about the curative effects of ethereal oils and wrote roughly 100 books, among them, the "Canon of the Medicine". The Canon remained to be the most important book about the art of healing until the 17th century.

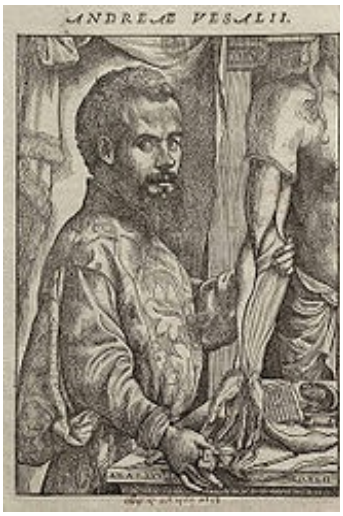


Fig. 2: Andreas Vesalius (1514-1564), founder of modern anatomy and morphological thinking.

With the Renaissance in Central Europe at the beginning of the 15th century, a resurgence of experimental biology and medicine took place, and empiricism gained importance. Vesalius (see **Fig. 2**), a Flemish anatomist, is considered to be the founder of modern anatomy and "morphological thinking" in medicine.

[1] *Operating a living organism*

With the early 17th century there was growing interest in physiological processes. For example, William Harvey (1578-1657) delivered the first description of blood circulation, and Johann C. Brunner (1653-1727) performed animal experiments to understand the function of the pancreas. Brunner's research work led to the discovery of a system of glands that neutralizes gastric acid when entering the small intestine. These glands bear his name to this day.

Charles Darwin published "The Origin of the Species" in 1859, building a base for the theory of evolution. The awareness that similarities between humans and animals were based on homology, presented a reasonable basis for the use of animals as models for humans. Homology in this context means that consistent features of various animal groups do not evolve independently from each other, but have shared origins.

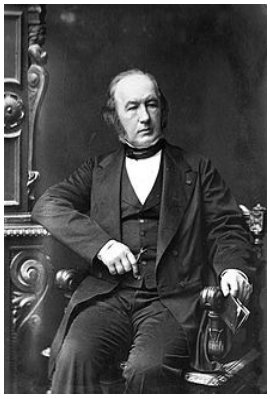


Fig. 3: Claude Bernard (1813-1878), french physician and physiologist

In 1865 Claude Bernard (**Fig. 3**) published the "Introduction to Experimental Medicine", describing methodology for the development of physiological experiments. Often called the father of experimental medicine, Bernard investigated the impact of the pancreas for digestion, the modulation of blood glucose concentration by the liver and the contraction and expansion of blood vessels triggered by the vasomotor system.

From the end of the 19th century, the development of new scientific areas such as pharmacology, toxicology, virology and immunology, as well as the emerging industries developing new pharmaceuticals, caused a rapid rise in animal experimentation. By the end of the 19th century, the predominant experimental subjects were domestic animals. Later, other animal species were introduced and, later still, animal models were developed. At the beginning of the 20th century, genetics initiated a new dynamic period. Initially, geneticists drew on a multitude of variants of mice that were kept as pets in those days. However, soon scientists began breeding the first strains of laboratory mice, e.g. DBA, C57BL/6, as well as rats (e.g. Wistar, Sprague Dawley).

In parallel with the emergence of new inbred and outbred strains of laboratory rodents, scientists also began to consider housing conditions of the animals as well as the experimental methods being employed. The suggestion was that standardization of as many parameters as possible is a key prerequisite for the reproducibility of experimental results. Experimental animal science is the scientific branch dealing with these issues.

Ethics of Animal Experimentation

Philosophy has dealt with the issue of human responsibility for non-human beings for centuries. There are a couple of basic concepts which focus on the complex issue of human-animal relations.

Classic "Anthropocentrism" takes the view that animals have no intrinsic value at all. Traditionally this was the opinion of important philosophers such as Aristotle until

Immanuel Kant and also of Christian moral theology. Anthropocentrism is based on the assumption that only humans are worthy of protection, because only they are rational beings and in the image and likeness of God. Jeremy Bentham questioned this "group egoistic" concept. Because Darwin's theory of evolution was mostly accepted by scientists by the middle of the 19th century, the assumption of biological uniqueness of humans could no longer be maintained.

Into the second half of the 17th century, animals had been still believed to be non-sensitive creatures. Philosopher R. Descartes (1596-1659) had argued that animals had neither a soul nor awareness, only humans could think and feel. The concept of utilitarianism was developed in the 18th century; in brief, it wants us to act in a way, that a maximum of happiness is achieved. Jeremy Bentham (1748-1832), a famous representative of utilitarianism, questioned the classic anthropocentrism a "group egoistic" concept. He stated that the traditional way of estimating animals as non-thinking and non-feeling creatures was no longer acceptable. Bentham defined the capacity for pain perception as crucial for how to deal with other creatures. His key question was not if a creature could think or speak, but whether it could suffer.

"Holism" is an even more extreme concept at the other end of the spectrum that demands even mountains or rivers have an independent right to exist. There are strongly metaphysical premises for holism such as the soulfulness of nature in general which are not shared by the current philosophical or religious mainstream. It is an unrealistic concept.

Nobel-prize winner Albert Schweitzer developed the concept of "Radical Biocentrism" that admits all creatures to the "moral community"[2]. This type of biocentrism prohibits the impairment of any kind of life, no matter whether it is an animal, a plant or a bacterium. Actually Schweitzer named himself a mass murderer, because he, as a physician, would necessarily have killed millions of bacteria, whenever vaccinating a person. It seems to be almost impossible to implement this concept.

The Australian philosopher Peter Singer, as well as many other animal protection activists, stand for "Pathocentrism", which concedes an inherent moral value only on animals with an intact central nervous system[3]. However, it is not made really clear why only these species. Many representatives of pathocentrism see some animals as "persons", while they deny some humans their personhood. According to Singer, great apes, whales and dolphins are definitely "persons", while other kinds of mammals may be persons too, at least with a high probability. In contrast, according to Singer, newborn babies, mentally handicapped people or comatose humans are just "members of the species *Homo sapiens*", not real persons.

Today the concept of "moderate biocentrism" represents a moral theory that appears to be more widely acceptable. The idea bestowes on all creatures a certain moral status. The intrinsic value of a creature, however, depends on its position in the "hierarchy of organisms" (Scala naturae or stairway of nature), initially described by

[2] Albert Schweitzer, Die Ehrfurcht vor dem Leben: Grundtexte aus fünf Jahrzehnten, Hrsg. v. Bähr, H.W. (1991), 6.Aufl., München

[3] *Peter Singer:* Practical Ethics*. Cambridge University Press, Cambridge 1979; 2nd edition, 1993; 3rd edition, 2011, ISBN 9780521707688*

Aristotle. From today's perspective, it would mean, for instance, that the health of a dog is more worthy of protection than the health of a rat or a beetle. In both concepts, humans maintain an exceptional position as "persons".
Considering the consequences when following one of these concepts, only the moderate biocentrism seems pragmatic and defensible for the average person. However, the estimation of an insect as "less worthy" than, for example a dog, does not mean it's life has no intrinsic value at all. Also insects have an intrinsic value and this fact has to be respected. What does that practically mean? An invasion of a house by an "army" of cockroaches should be fought in a way that the insects are killed quickly and without the possibility of pain. The risk of potential infections for the residents and/or the uninhabitability of the house represents a sufficiently reasonable ground to exterminate the insects. In contrast, a single mouse that has somehow found its way into the house, could be caught with a live trap and released outside. It is an ethical obligation to carefully evaluate such encounters and find a suitable way to solve the problem while respecting the "interests" of the animals.

According to moderate biocentrism the use of experimental animals for scientific purposes is acceptable. But only provided that the 3Rs are implemented in their full sense to avoid any unnecessary burden for the animals. However, the estimation of the use of animals for scientific purposes by the public often times is skeptical, if not dismissive or even hostile. Many people experience a mouse, a cat or a dog emotionally as a beloved pet or even a close friend. They cannot understand how a scientist can observe such an animal just as an organism, whose function or malfunction he or she wants to understand. Animal experimentation is an extremely complex issue and a non-scientist has difficulty understanding the workings of this field. That means investigators should be open to talk to laypeople about these issues. That should be done very carefully and with understanding and empathy for their concerns. It makes sense to start such educational work as early as possible. For example, volunteering to visit school classes and showing them what animal experimentation means and its value for the society.

It is, nevertheless, forseeable in the near future that the use of animals for scientific purposes will be viewed critically by the majority of the public. On the other hand, there are countless outstanding questions and unsolved problems in the field of biomedical research. Answering questions and solving the problems will require many more years before the biomedical field can conclude that animal experimentation is no longer necessary.

2. Animal Welfare, the 3R-Concept and Alternative Methods
(Almut Köhler, Jürgen Weiss)

Animal Welfare

Concern for animal care and wellbeing has existed since domestication, which occurred at least 10.000 years ago in Neolithic times. The humans' appreciation and respect for animals led to their domestication, animal agriculture and animal husbandry. The animal welfare ethic that developed in the Neolithic era obligated people to consider their animals' welfare in order to achieve their own purposes. The resulting "arrangement" was: "If we take care of the animals, the animals will take care of us". A special relationship, nowadays called human-animal bond.

How can animal welfare be defined today? Actually the standards of "good" animal welfare are under constant review and are debated, created and revised by animal welfare groups, legislators and academics worldwide. At its simplest one could say, an animal is faring well, if it is healthy and producing well. However, there are limitations to seeing animal welfare only in terms of the body. One limitation is that genetics and the environment can produce desirable physical outcomes, even though the animal's mental state is compromised. For example, a canine breed champion may have perfect conformation and be in perfect health, but it may be very anxious in its home environment. Another limitation is that some physical parameters (heart rate, plasma cortisol) are difficult to interpret, because they can be increased by both positive and negative experiences, such as the presence of a mate and the presence of a predator.

This would suggest that animal welfare includes not only the state of the animal's body, but also its feelings. Today most people would agree that animals have feelings (fear, frustration), and it has been proposed that animal welfare consists entirely in feelings and that these have evolved to protect the animal's primary needs. Thus, if an animal feels well, it is faring well. A feelings-based approach to welfare research typically measures behavioral outcomes, such as willingness to "work" (e.g. pushing open a weighted door), and behavioral signs of fear or frustration. Such research has led to the conclusion that animals have fundamental behavioral needs that they must be allowed to satisfy for humane reasons.

Another view of welfare, linked to the feelings-based approach, is that animals fare best if they can live according to their nature and perform their full range of behaviors. In this case, physical suffering, such as feeling cold, and mental suffering, such as the fear induced by being preyed upon, may be acceptable.

Summarizing various aspects of the problem, animal welfare comprises the state of the animal's body and mind, and the extent to which its nature (genetic traits manifest in breed and temperament) is satisfied. However, the aspects of welfare sometimes conflict, and this presents practical and ethical challenges.

Animal welfare, with special respect to animal experimentation, began to take a larger place in Western public policy in 19th-century Great Britain. The first organization worldwide against animal experimentation was the "Society for the Protection of Animals Liable to Vivisection (SPALV)", which was founded by Frances

Power Cobbes in 1875. The foundation was a consequence of increasing numbers of people protesting the use of more and more animals for research purposes. Claude Bernard (1813-1878), a key player in the history of physiology, became the first target of animal protection activists.

Since those days two basically different ideas about animal welfare have emerged in western societies. On the one side so-called animal activists fight animal experimentation in principle and claim to represent animal protection or welfare for themselves. On the other side animal experimentation is legally regulated down to the last detail. Investigators are obligated to observe all legal regulations, respect all aspects of animal welfare and apply the concepts of the 3Rs and of the five freedoms. In Germany each institution where animal experimentation is performed must have one (or more) Animal Welfare Officer (AWO), who advises investigators and monitors their work with animals.

With respect to animal welfare, in the scientific context, Directive 2010/63 EU determines that animals used for experimental purposes must receive appropriate care and treatment. All individuals interacting with the animals should have the appropriate training and experiences under the tutelage of a supervisor. Animals must be housed in cages which are sufficiently large and in a species specific environment. Whenever possible, animals should be housed in groups[4]. Enrichment techniques [5] should be used to encourage physical exercise, maneuvering, exploration and cognitive activities. Methods for euthanasia must limit pain, suffering and distress felt by the animals. Animals may be killed only by a person with the required skills.

The 3R-Concept and the Five Freedoms

Directive 2010/63/EU of the European Parliament and Council, as well as most national European legislations, determine that the 3Rs[6] must be considered when planning and performing animal experiments. The 3Rs refer to **R**eduction, **R**efinement and **R**eplacement.

The concept was developed by William Russel, a zoologist, psychologist and a classical scholar and the microbiologist Rex Birch. In 1954 Russel and Birch were appointed by the Universities Federation for Animal Welfare (UFAW) to inaugurate a systematic study of the ethics of laboratory techniques. In 1956, they prepared a general report to the Federation's committees. Finally, in 1959 the 3R-concept was published.

How can the 3Rs be realized? Reduction can often be achieved through careful experimental planning and statistical evaluation. Appropriate analgesia and anesthesia, the improvement of assay procedures and the development of non-invasive methods contribute to Refinement. Replacement could be achieved easily when there are legally mandated procedures. For example, according to international guidelines the lethal dose 50% (LD_{50}) test must be performed to evaluate the acute

[4] Probably inadvisable for housing adult, previously mated male mice together
[5] Difficult to meet maintaining Zebrafish and Medaka
[6] See also: < http://ec.europa.eu/environment/chemicals/lab_animals/3r/alternative_en.htm>

toxicity of a substance. The test has its critics, however because it requires huge numbers of animals and clearly involves an onerous burden on the animals. Consideration should be given if the LD_{50} can be replaced in some situations by alternative methods and to reduce the number of animals used.

The "Five Freedoms"[7] is a concept, which outlines five aspects of animal welfare under human control. Initially developed for the welfare of widely used farm animals, the concept can be applied to the maintenance and use of experimental animals. The five freedoms are:
Freedom from hunger or thirst by ready access to fresh water and a diet to maintain full health and vigor.
Freedom from discomfort by providing an appropriate environment including shelter and a comfortable resting area.
Freedom from pain, injury or disease by prevention or rapid diagnosis and treatment.
Freedom to express (most) normal behaviour by providing sufficient space, proper facilities and company of the animal's own kind.
Freedom from fear and distress by ensuring conditions and treatment to avoid mental suffering

Animal experimentation with strict observance of the 3Rs and the five freedoms is not only an ethical but also a scientific requirement. An animal, e.g. being under chronic stress because of insufficient housing conditions or improper treatment within an experiment will not yield reproducible results.

Alternative Methods

Animal experiments have a special status for human-animal relations. For that reason, the temptation in western societies is to replace animal experiments altogether with alternative methods[8]. An example often cited as an alternative is use of *in vitro* cell culture techniques.

In vitro studies typically are performed with cells or biological molecules studied outside their normal biological context; for instance proteins are examined in solutions, or cells in artificial culture medium. Examples of *in vitro* studies include isolation, growth and identification of microorganisms or of cells derived from multicellular organisms (cell or tissue culture), subcellular components (e.g. mitochondria or ribosomes), cellular or subcellular extracts (e.g. wheat germ, reticulocyte extracts), purified molecules (proteins, DNA, RNA) and pyrogenicity tests.

A primary advantage *in vitro* work is to reduce the complexity of the system under study, so that the investigator can focus on a small number of components. The primary disadvantage of *in vitro* studies is generalizing the results back to the biology of an intact system or organism with its multiply interacting parts. Investigators doing *in vitro* work must be careful to avoid over-interpretation of their results, which can sometimes lead to erroneous conclusions for organismal and systems biology.

[7] See also: < http://webarchive.nationalarchives.gov.uk/20121007104210/http:/www.fawc.org.uk/freedoms.htm>
[8] See also: < http://www.bfr.bund.de/de/center_for_alternatives_to_animal_testing__caat_-4296.html>

However, in most types of scientific investigation only the initial experimental stages can be tackled with *in vitro* methods, such as testing the response to a toxin by different cell types. A reliable assessment of the effect on the entire organism usually requires *in vivo* testing. Alternative methods probably can never fully replace *in vivo* experiments, but instead can complement them and help reduce the number of animals used.

It is the task of ZEBET[9] (Zentralstelle zur Erfassung und Bewertung von Ersatz- und Ergänzungsmethoden zum Tierversuch) at the "Bundesinstitut für Risikobewertung" (BfR) in Berlin (Germany) to evaluate alternative methods. ZEBETs counterpart at the European level is the "European Center for Validation of Alternative Methods (ECVAM)[10]" located in Ispra (Italy).

More information can be obtained by numerous search tools, such as
EURL ECVAM Search Guide (http://bookshop.europa.eu/de/the-eurl-ecvam-search-guide-pbLBN124391/),
EURL-Data Pool (http://www.eurl-pesticides-test.eu/),
Medline (https://www.dimdi.de/static/de/db/dbinfo/me66.htm),
PubMed (http://www.ncbi.nlm.nih.gov/pubmed),
Go3Rs (http://www.gopubmed.org/web/go3r/)
and others.

2.1 Recognition of pain, suffering and distress in fish
(Almut Köhler)

The German Animal Welfare Law and the EU Directive 2010/63/EU for the use of animals for scientific purposes require minimizing the level of pain, suffering, distress or lasting harm for animals. Also it is necessary that people working with laboratory animals know the signs of pain, distress or suffering and are aware of strategies to reduce them. Besides the legislative aspects, it is also preferable for experimental reasons to reduce pain, suffering and distress as much as possible. This is because the physiological and psychological reactions caused by pain can influence the results of an experiment in many ways and will potentially lead to variability in results that may be difficult to interpret.

The recognition of pain, suffering and distress in fish is not an easy task since many discussions are still on-going as to whether fish can feel pain at all. The most difficult aspect in this discussion is the definition of pain itself and how this can be adapted to animals. Pain is not only the result of an activation of nociceptors leading to a signaling that is processed in the spinal cord and brain stem areas. It also involves modulation of the signal of these levels leading to integration of enhancing and inhibiting signals. This process is not completely understood until now, but it is known from humans that the same stimulus can cause different painful emotions depending on the particular circumstances of the individual. Additionally, nociception is evaluated in higher brain areas and can lead to different pain sensations depending

[9] *http://www.bfr.bund.de/de/zebet-1433.html*
[10] *http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam*

on the individual as well as their different previous experiences. So, the same stimulus can be painful for one person and not painful for another. The difference between nociception and pain becomes obvious when people are anesthetized: the nociceptor is still activated by the surgery but no actual pain sensation takes place (see surgery). Therefore, consciousness is a prerequisite for the sensation of pain.

To assess pain in animals usually a number of criteria are considered. The similarity of anatomical structures and physiological process together with the development of avoidance behavior after an exposure to a painful stimulus and the effectiveness of analgesics in reducing this avoidance behavior are regarded as the main indicators. Due to the great diversity of fish species compared to mammals and the immense evolutionary distance separating fish and mammals, the literature is not very consistent in interpreting experimental data obtained from different fish species. Often research is done in salmonids and data from this are simply extrapolated to other species without providing any experimental evidence. In addition, the great evolutionary distance has resulted in many fundamental physiological differences between fish and mammals making it difficult to transfer human experience to the fish situation.

Pain usually is generated by stimulation of free nerve endings of a nociceptor resulting in a signaling event via mostly Aδ (fast) and C (slow) fibers to the dorsal horn of the spinal cord. There the signal is modulated and forwarded to the brain stem and the higher brain areas. In different areas of the mammalian cortex the signal is interpreted on the one hand for its quality, location and intensity. On the other hand an emotional evaluation occurs, mainly triggered by the signal from the C fibers. In the brain stem, fast reflex responses are induced. These responses are still elicited even in patients lacking cortex function while pain perception as an emotional event seems to depend on consciousness.

Mechanoreceptors and different types of nociceptors have been identified in fish but they vary in terms of composition and localization between species. Concerning signal processing, only bony fish have been studied extensively. Almost no data are available for cartilaginous fish. In bony fish, the signal is principally processed at the brain stem and contains a very low element of C fiber signals. Higher brain areas comparable with the mammalian cortex have not yet been identified in fish. However, homologous structures of the limbic system, the emotional center of the mammalian brain, have been described (based upon similarities in the neurotransmitters expressed and the firing patterns of neurons) but these seem to be connected to different brain areas in fish compared with mammals.

Since pain perception in mammals depends on consciousness, the possibility that animals such as fish experience consciousness is always a matter of great debate. Some fish species have been shown to exhibit local orientation and to be able to identify individual conspecifics. So a basal level of primary consciousness is evident. While some species develop avoidance behavior in some experimental settings others don’t. The avoidance behavior depends on the experiment and on the species investigated. Based on these observations, a kind of self-consciousness is assumed although classical tests for this have not been successfully applied in fish.

L. Sneddon investigated the effect of injection of irritating substances in the lips of rainbow trout. While some behavioral indicators were not changed, some complex behavioral patterns were observed such as rocking or rubbing of the lips against the wall of the tank. This could be reduced by a dose of morphine administered before the injection of the irritating substances. Since the dose of morphine used in this study is 15-30 times higher than the lethal dose of humans and the behavioral pattern is not observed very often in treated animals, the result of the study is not always accepted as an indicator of pain in fish.

So in summary, nociception is certainly present and this signal is relayed to the brain stem in several fish species. However, whether it is subsequently interpreted as pain by the animal is not clear and there are pros and cons for each point of view. The development of pain reception might be regarded as conferring a selective advantage during evolution as it can enhance survival of an individual. Since functionally similar but morphologically different systems may also develop in parallel in evolution, we cannot exclude pain sensation in fish.

Nevertheless, fish should be handled carefully since any kind of stress may influence the experiment, regardless of whether they feel pain in the same way as humans. Therefore, in the preparation of an experiment it is important that to the best of the researcher's abilities, all possible eventualities are foreseen and that the researcher is prepared to deal with a range of potential difficulties. If he/she recognizes that an instrument / drug is needed throughout the experiment this should be already available at the beginning of the experiment. Additionally, the level of burden for the animal generated by the experiment should be carefully considered. There are four categories of burden characterized by the EU Directive and examples are given for each of the categories in the Annex VIII of the Directive. These examples represent a basic guide for the evaluation but each project has to be critically reviewed with regards to the burden accumulated by the animal. It is important to focus on the ethical evaluation of the cost-benefit-analysis between burden of the animal and benefit of the result throughout the whole procedure. When this balance is shifting towards the burden of the animal without an acceptable gain in knowledge, then the experiment should be terminated. This represents the Principle of Humane Endpoints. In general, the idea is: "As early as possible (to stop the experiment for the animal and releasing it from burden), as late as necessary (to gain the level of knowledge justifying the burdening of the animal)." If experiments are stopped too early and the relevant knowledge is not gained this leads to the use of a higher number of animals or the burdening of the animals without gaining any benefit. The humane endpoints should be defined before the experiment as objectively as possible. Therefore, the parameters and their evaluation should be defined in advance. Sometimes, keeping Score Sheets can be quite helpful to keep the evaluation objective and to harmonize the treatment of animals throughout a project.

In fish, the possibility to detect pain, suffering and distress is limited and difficult to distinguish between the effect of the experiment and maybe "normal" illness that may occur simultaneously, but independently. There are behavioral parameters that can be observed such as changes in swimming behavior, but also physical abnormalities that can indicate a fish is not healthy. Although the cause-effect-relationship may not always be clear, humanity should be a principle of action.

3. Legal regulations for animal experimentation

3.1 Animal welfare regulations for experimentation in Germany (Almut Köhler)

3.1.1 Legal framework

On November 9, 2010, the new Directive 2010/63/EU[11] entered into force and had to be incorporated into national legislation by all Member States of the European Union. This has been done in Germany by the publication of the Third Act to Amend the Animal Protection Act on July 4, 2013 (Drittes Gesetz zur Änderung des Tierschutzgesetzes (TierSchG), BGBL I S. 2182 (Nr. 36))[12].
Furthermore, in the „Verordnung zur Umsetzung der Richtlinie 2010/63/EU des Europäischen Parlaments und des Rates vom 22. September 2010 zum Schutz der für wissenschaftliche Zwecke verwendeten Tiere“[13], a framework was defined for the implementation of the amended Animal Protection Act. It regulates the actual implementation of the TierSchG.

The Animal Protection Act (Tierschutzgesetz, TierSchG) consists of a total of 12 chapters dealing, for instance, with the sacrifice of animals for scientific purposes (third chapter, § 4 (3)), animal experiments (fifth chapter, §§ 7 – 9) and the role of the Animal Welfare Officer (Tierschutzbeauftragter (TSchB) (sixth chapter, § 10). Since the TierSchG is a more general regulation and also contains chapters for farm or pet animals, slaughterhouse etc. more detailed description about the interpretation of the TierSchG are made in the General Administrative Directive for the Execution of the Protection of Animals Act (Tierschutz-Versuchstierverordnung, TierSchVersV). For reporting the use of animals for scientific purposes a Decree on the Reporting of Laboratory Animals exists (Versuchstiermeldeverordnung, VersTierMeldV)
The actual versions of these texts can always be found on this internet source:

Regulation	Source
TierSchG	http://www.gesetze-im-internet.de/tierschg/
TierSchVersV	http://www.gesetze-im-internet.de/tierschversv/index.html
VersTierMeldV	http://www.gesetze-im-internet.de/verstiermeldv_2013/index.html

The most important regulations related to keeping, breeding and the use of animals for scientific purposes as well as the application procedures in the district of Karlsruhe are listed below. However, further changes to the application of the new Animal Welfare Act are expected in the near future.

[11] *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 (izmc.ezrc.kit.edu/documents/<DIR_2010-63-EU_en_de>)*
[12] *Drittes Gesetz zur Änderung des Tierschutzgesetzes (TierSchG), BGBL I S. 2182 (Nr. 36) (izmc.ezrc.kit.edu/documents/<Aenderung_TierSchG>)*
[13] *Verordnung zur Umsetzung der Richtlinie 2010/63/EU des Europäischen Parlaments und des Rates vom 22. September 2010 zum Schutz der für wissenschaftliche Zwecke verwendeten Tiere (izmc.ezrc.kit.edu/documents/<tierschutzversuchsvo>)*

3.1.2 Obligations of the establishment

The body or person responsible for an establishment which houses animals (vertebrates or cephalopods) for use in experiments, or acts as a source of tissues or organs intended for scientific use must ensure the day-to-day compliance with the requirements laid down in the TierSchG and the TierSchVersV. A veterinarian experienced in laboratory animal science should be available if needed. Moreover, such establishments are obliged to appoint one or more Animal Welfare Officers (TierSchB, Tierschutzbeauftragte) and must notify the competent authority of this appointment before the start of their activities. The requirements for the appointment and the rights and duties of the TierSchB are described in § 5 of the TierSchVersV. The establishment must also set up an Animal Welfare Committee (Tierschutzausschuss). The composition and tasks of the Animal Welfare Committee are defined in § 6 of the TierSchVersV.

3.1.3 *Application procedures*

In Germany, statutory applications for the permission to use animals for scientific purposes can be obtained by several routes. They are classified in the following three categories according to the specific purposes of the animal experiment (see also Table 1)

1. *Animal experiments requiring authorisation („genehmigungspflichtige Versuchsvorhaben“)*
2. *Animal experiments requiring a formal declaration („anzeigepflichtige Versuchsvorhaben“)*
3. *Euthanasia of animals for scientific purposes (“Tötung zu wissenschaftlichen Zwecken”)*

3.1.4 *Projects requiring authorisation („genehmigungspflichtige Versuchsvorhaben“)*

Anyone who plans to use animals for scientific purposes should contact the competent TierSchB at a very early stage, in order to discuss the project and to check whether the necessary conditions for its implementation are met at the establishment in question. The applicant should then contact an experienced biostatistician, in particular to obtain advice on the required number of animals and the statistical methods that need to be applied to analyze the results. If possible, a brief report of the statistician should be attached to the application. While still in draft form, the application should be forwarded to the TierSchB, thus enabling the TierSchB to provide advice if needed. The final application form must be accompanied by the following documents:

- a completed checklist for the TierSchB („Stellungnahme des TierSchB”);
- a non-technical project summary („nichttechnische Projektzusammenfassung (NTP)“);

- a reference list;
- data sheets documenting genetically modified animals, if applicable;
- the forms “Formblatt Angaben zur biometrischen Planung” or, alternatively, an expert evaluation of the statistical correctness of the application;
- the completed personal information forms („Personenbogen“);
- a list of the abbreviations used in the application.

Six copies of the application and its annexes must be submitted to the authority for transmission to the Animal Welfare and Ethics Committee as stipulated by § 15 of the TierSchG. The competent authority will inform the applicant about the decision taken on his/her application within 40 working days of receipt of all the relevant information. Stating valid reasons, the authority may extend this period by up to 15 working days.

The project authorisation is valid for a maximum of 5 years. This period cannot be extended further.

Procedure (use of the animal)	Primary purposes of the procedure	Administrative procedure	Application documents (according to Directive 2010/63/EU)	Application documents (according to the Regierungspräsidium Karlsruhe)	Project evaluation and granting
Interventions and manipulations in animals for experimental purposes	• Basic research • Research with the aim of diagnosis or to restore, correct or modify physiological conditions or functions in human beings or animals • Note: This category does include projects which use animals for creation of a new genetically altered line[1] which exhibits a clinically detectable adverse / pathological / harmful phenotype!	Application for project authorisation (Antrag auf Genehmigung eines Versuchsvorhabens an Tieren)	• the project proposal • a non-technical project summary • information on the elements set out in Annex VI, Directive 2010/63/EU[2]	1. the project proposal (application form) 2. a completed checklist for the animal protection officer (Stellungnahme des TSchB) 3. a non-technical project summary (nichttechnische Projektzusammenfassung (only applicable to "Genehmigungsanträge")) 4. a reference list (Liste der Literaturzitate) 5. data sheets for genetically altered animals[3] (Datenblatt für genetisch veränderte Tiere) 6. the form „Angaben zur biometrischen Planung" or an expert evaluation of the statistical correctness of the application (statistisches Gutachten) 7. the completed personal information forms (Personenbögen) 8. a list of the abbreviations used in the application (Abkürzungsverzeichnis) 9. 6 copies of the application form (Antragsformulars) (only applicable for "Genehmigungsanträge")) (till element 5, anonymized if necessary) including documents (2,4,5,6,8)	By the competent authority and the advisory committee for the protection of animals used for scientific purposes
Projects containing procedures classified as 'non-recovery', 'mild' or 'moderate' and not using non-human primates, that are necessary to satisfy regulatory requirements, or which use animals for production or diagnostic purposes with established methods.	• Regulatory testing e.g. Testing of the quality, effectiveness and safety of drugs (statutory safety tests) • Diagnosis of diseases, suffering or physical impairment in human beings or animals • Testing of sera, blood products, vaccines, antigens or test allergens in authorization procedures or quality control e.g. Vaccination, blood sampling or other diagnostic measures with established methods • Removal of organs or tissue in the manufacturing process, production, maintenance or amplification of substances, products or organisms or for diagnostic purposes e.g. Tissue sampling of genetically altered animals` for the purposes of genetic characterisation (e.g. fin clipping)	Simplified administrative procedure (Anzeige von Eingriffen und Behandlungen an Tieren)	• the project proposal • information on the elements set out in Annex VI, Directive 2010/63/EU[4]		By the competent authority
Interventions and manipulations in (treatment of?) animals for education and training of persons working with laboratory animals by means of established methods	• Higher education or training for the acquisition, maintenance or improvement of vocational skills				
The killing of animals solely for the use of their organs or tissues ("Euthanasia of animals for scientific purposes")	e.g. Humane and established methods for killing of animals without a clinically detectable adverse phenotype for their organs and tissues (applicable for genetically altered and not genetically altered animals)	Internal declaration (informal or by means of a form „Anmeldung der Tötung von Wirbeltieren zu wissenschaftlichen Zwecken gemäß §1, §4 Abs.3 TierSchG (4.7.2013) und TierSchVersV (12.08.2013) §2, Anlage 1, Anlage 2)")	Provide the following information: • the name and address of the user or recipient of the animals • the name and competence of the person carrying out the euthanasia • the species of animals • Methods of killing • the number of not genetically altered animals • the number of genetically altered animals • the intended purpose for which the animal/line/strain is used • the project in which the animals are used	No application is required	By the authorized person at the institution (e.g. the head of the animal facility)

3.1.5 *Project leader, substitute project leader („Leiter, Stellvertreter")*

Persons wishing to act as project leader („Leiter") or deputy project leader („Stellvertretender Leiter") of an application must have a level of education that corresponds to a university degree in veterinary medicine, human medicine, dentistry or natural sciences. They also must have the requisite competence and expertise and at least 3 years of practical experience in animal experimentation projects. Alternatively, the requisite competence, expertise and experience can be obtained by participation in a course on laboratory animal science that meets the requirements of a FELASA C course (approx. 80 h).

3.1.6 *Staff*

The names of all persons carrying out tasks in the planned project must appear in the project application and the personal information form („Personenbogen") and appropriate documentary evidence of their competence and expertise must be provided.

Staff with a university degree as specified above and with the requisite competence and expertise is always allowed to perform the tasks independently and without supervision.
Persons without a university degree require a special authorization certificate („Ausnahmegenehmigung“) to be allowed to work independently. This certificate should be requested by sending the appropriate application form accompanied by documentary evidence of the competence and expertise in the work to be performed. The authorization allows the person to perform the specified techniques independently and is not restricted to the current project. However, it is no longer valid if the person moves to another institution.
Persons without a university degree, but who have successfully completed a professional education that involved work with laboratory animals (such as staff trained to become a „Biologielaborant/in“ or „Veterinärmedizinisch-technische/r Assistent/in“) are allowed to carry out their tasks without an authorization certificate („Ausnahmegenehmigung“).

Employees who lack either a university degree or a special authorization certificate („Ausnahmegenehmigung“) are allowed to work under supervision only. A person with the appropriate experience must be present at all times to provide this supervision. If work under supervision is planned, the option „Aus-, Fort- und Weiterbildung“ („training and continued education“) must be checked in section 17 of the application form as an additional aim of the experiment. However, supervised work with animals is only possible in an ordinary project requiring authorization if it involves relatively minor procedures such as blood collection. If experience using more difficult and therefore more stressful procedures is required and/or additional animals are needed for learning this procedure, a separate statement of „training and continued education“ („Anzeige für Aus-, Fort- und Weiterbildung“) must be made.

3.1.7 *Projects requiring a formal declaration („anzeigepflichtige Versuchsvorhaben“)*

Paragraph 8a of the German Animal Welfare Act lists the different legal conditions under which a project involving vertebrates or cephalopods can only require “declaration” rather than authorization. The appropriate category must be indicated in section 16 of the relevant application form. For example, the generation of antibodies by immunization of experimental animals only requires a notification, provided that the antibodies are then used exclusively for laboratory work and not for any additional animal experiments.
The new legal framework, like the old one, classes interventions and treatments for the production, preparation, storage and amplification of substances, products or organisms performed according to established procedures, and not for experimental purposes (e.g. the generation of antibodies by immunization) as projects that only require formal notification.

The form for projects that require notification is identical to that of a project application. However, the notification does not have to be accompanied by a non-technical project summary („nichttechnische Projektzusammenfassung” (NTP)) or biostatistics expertise, nor does it have to be submitted to the Animal Welfare and Ethics Committee as stipulated by § 15 of the TierSchG. For projects involving infliction of severe pain and suffering to animals with, a notification is not acceptable. Such experiments are always subject to authorization.

The competent authority receiving a notification must acknowledge its receipt by writing to the applicant once all the required documents have been submitted successfully. Twenty working days after receipt of the request the project can be started even if the authority has not yet indicated its approval. The maximum possible duration of a project requiring only notification is 5 years and it cannot be extended.

The provisions of the new Animal Protection Act do not apply to experimental projects which have been approved before the entry into force of the amended Act, with one exception: If a new person needs to be reported to the authorities for such „old projects“ the new “Personenbogen” must be used. From January 1st, 2018, the new Animal Protection Act will apply exclusively and without exceptions in Germany.

3.1.8 *Euthanasia of animals for scientific purposes*

Euthanasia of vertebrates for scientific purposes (without any prior treatment) requires only an internal application (referred to as „Interne Tötungsanmeldung“). Most institutions provide their own application form for this purpose[14]. The applicant should complete the form and send it to the competent TierSchB for examination and to ensure that the proposed killing method is carried out in accordance with the requirements of Appendix 2 of the TierSchVersV, that the responsible staff is adequately educated, competent and continuously trained in compliance with Appendix 1, Section 2, and that all other relevant requirements are fulfilled. Regrettably, the list of killing methods set out in Appendix 2, TierSchVersV is very limited. It lacks, for example, the use of hypothermia as a method for euthanasia of zebrafish even though this is a method approved internationally and also by the Regierungspräsidium Karlsruhe.

3.1.9 *Reservation on approval of genetically altered breeds with harmful phenotypes*

According to § 11 TierSchG, an institution receives general approval to keep and breed experimental animals. However, according to the new Animal Protection Act the keeping and breeding of genetically altered animal lines requires additional approval if the individuals of these lines may be subject to pain, suffering or damage due to their genetic alteration. This separate requirement for approval applies to all genetic lines that have been generated or genetically modified by manipulation of embryos (transgenesis, homologous recombination, enzyme-mediated mutation etc.), by irradiation or by treatment with mutagenic substances. It also applies to the generation of new lines by crossing of two lines which apparently lack harmful phenotypes, but have not been sufficiently characterized, as well as for spontaneous mutations if they are to be bred selectively and harmful phenotypes are expected. The keeping and breeding of lines that develop harmful tumors due to a genetic alteration must always be subject to approval, even if the tumors appear only at a certain age.

[14] *Form“Interne Tötungsanmeldung” (izmc.ezrc.kit.edu/<Toetunganmeldung_de_en>)*

3.1.10 *Use of larvae*

According to § 14 TierSchVersV, the provisions of §§ 7 to 9 as well as §§ 15 to 43 TierSchG, including the requirement for notification or authorization, also apply to experiments conducted with larvae of vertebrates once they are capable of independent feeding. This requirement also applies to mammalian fetuses during the last third of their normal development. Consequently, zebrafish larvae during the first 5 days of development (up to 120 hours post fertilization) are not subject of these provisions.

3.1.11 *Record keeping requirement*

According to § 9 (5) TierSchG records must be kept on animal experiments that must specify the purpose of the project, the kind and number of animals used, the type and progress of the project, the names of the participating persons as well as some additional information. The record-keeping can either be done on a special form or in lab books provided that they are appropriately structured and kept up to date. Project leaders and deputy project leaders are responsible for meeting this record keeping requirement.
Records can be inspected by the authority or the Animal Welfare Officer on duty. Additionally, all data regarding a project should also be made available to the supervisor of the animal facility to ensure that the correct treatments are performed and that the wellbeing of animals is controlled. In case anybody observes burden to the animal requiring the killing or the treatment of animal and its exclusion from the project the responsible person should be informed and the proper records should be made. Criteria for this decision should be made in advance (“Principle of Humane Endpoint” (see lecture “Pain, Suffering and Distress in Fish”).

3.1.12 *Annual report on laboratory animal use*

According to the „Versuchstiermeldeverordnung“ (VersTierMeldV) the leader of a project involving laboratory animals needs to provide the following data once per calendar year: animal species, legal classification of the project (i.e. requiring notification or authorization, number of animals used in an experiment for the first time, proportion of transgenic animals, number of animals used in further experiments, source of the animals, information on how the animals were used and the level of burden observed on the animal regardless from what was expected in the application form.

3.1.13 *National Animal Welfare Committee*

Beside the institutional Animal Welfare Committees a national committee was opened in September 2015 (www.bf3r.de) as it is required by the Article 49 of the EU Directive 2010/63/EU and § 15a of the TierSchG. This committee advises the local authorities and the institutional Animal Welfare Committees regarding acquisition, breeding, housing and care of vertebrates and cephalopods or the use of them in scientific procedures. The National Committee enhances the information transfer

about state of the art research between the institutions and the National Committees of other EU member states.

3.1.14 *Directive 2010/63/EU on the Protection of Animals used for Scientific Purposes*

Introduction

Council Directive 2010/63/EU on the protection of animals used for scientific purposes replaces the "old" Council Directive 86/609/EEC[15], that had introduced legal provisions in the EU to harmonize national regulations covering the welfare of animals used for experimental and scientific purposes for the first time. Directive 86/609/EEC consisted of 27 articles plus an annex and defined the use of animals on 7 pages in a very general manner; it became applicable November 24th, 1986. Interestingly, 21 (!) years later, in 2007, the "Recommendation of the (now) EU-Commission for the maintenance and care of animals used for scientific purposes" became Appendix A (2007/526/EC) of Directive 86/609/EEC.
Directive 2010/63/EU was adopted September 22nd 2010 and took full effect January 1st 2013. The Directive is firmly based on the principle of the Three Rs, to replace, reduce and refine the use of animals used for scientific purposes. The scope is now wider and includes fetuses of mammalian species in their last trimester of development and cephalopods, as well as animals used for the purposes of basic research, higher education and training. It lays down minimum standards for housing and care, regulates the use of animals through a systematic project evaluation requiring *inter alia* assessment of pain, suffering distress and lasting harm caused to the animals. It requires regular risk-based inspections and improves transparency through measures such as publication of non-technical project summaries and retrospective assessment. The development, validation and implementation of alternative methods is promoted through measures such as establishment of a Union reference laboratory for the validation of alternative methods supported by laboratories within Member States and requiring Member States to promote alternative methods at national level.

Directive 2010/63/EU has 47 pages and is available in 24 languages, from Bulgarian to Swedish, at:
<http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32010L0063>.
The Directive starts with 56 Introductory Points and is consisting of 66 Articles in 6 Chapters. According to Article 61 of the Directive *Member States had to adopt the* Directive into their national law and publish the laws, regulations and administrative provisions necessary to comply with the Directive by November 10th 2012. They had to forthwith communicate to the Commission the text of those provisions.
For that purpose national legislators developed National Implementing Measures (NIM) regulating the use of animals for scientific purposes. In a couple of member states the implementation process resulted in only 1 NIM, such as Italy, Austria,

[15] EEC = European Economic Community was founded in 1957 by Belgium, France, Italy, Luxemburg, The Netherlands and West-Germany as a regional organization in order to bring about economic integration, including a common market and customs union

Portugal and the United Kingdom, while implementation took 10 NIM in Lithuania and 20 NIM in Sweden.

Scope of the Directive

Main objective is to set out measures designed to protect animals used for scientific purposes, especially basic or applied research, particularly for the production of medical products and also for animals used for educational purposes. The Directive applies to all live non-human vertebrate animals and also to certain invertebrates which are likely to feel pain (cuttlefish, octopus etc.). The use of non-human primates is **subject to restrictions** and the use of great apes (chimpanzees, bonobos, gorillas and orang-utans) is **forbidden.**

Fields subject to Animal Testing

- Basic research
- Translational and applied research aimed at the prevention, prophylaxis, diagnosis or treatment of human or animal diseases
- The development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feed-stuffs, etc. for any of the aims above
- Protection of the natural environment in the interests of human or animal health
- Research directed at species conservation
- Higher education or training
- Forensic investigations

Evaluation of projects involving experimentation on animals

The use of animals for experimental purposes may be authorized in cases where no satisfactory substitute method exists. Projects involving experiments on animals have to be assessed by the competent authority, if the project is in accordance with legal requirements. If that is given, the authority will grant approval.
The assessment is based on a written application demonstrating the use of animals is justified and the expected advantages outweigh the harm caused to the animals. The number of animals used in a project must be reduced to a minimum without however compromising the objectives of the project. The living conditions and methods employed in the procedures must minimize any unnecessary pain, suffering or distress of the animals. Experiments may not begin before the applicants have received the approval from the competent authority.

Animal Welfare

The Directive also contains provisions reflecting animal welfare issues: animals used for experimental purposes must receive appropriate care and treatment. All individuals interacting with the animals should have the appropriate training and practice while being observed by a supervisor. Animals must be housed in cages which are big enough for them and in an environment which is adapted to the respective species. Enrichment techniques should be used in order to encourage physical exercise, maneuvering, exploration and cognitive activities. Annex III to the

Directive is giving detailed information about cage sizes, population densities and other housing parameters for a large number of animal species. Even Zebrafinches are mentioned there, but not Zebrafish nor Medaka.
Methods of killing an experimental animal must limit the pain, suffering and distress felt by the animals - according to the Directive. Animals may be killed only by a person with the required skills. 3. Table of Annex IV of the Directive is listing the following methods of killing for fish as acceptable: overdose of an anesthetic, cerebral concussion, percussive blow to the head and electrical stunning. The overdose of anesthetic would cover the MS222-procedure, but the other ones are not suitable to kill Zebrafish or Medaka. Hypothermia is not even mentioned.

Procedures

The only authorized procedures – according to the Directive - are those which have been approved as part of an approved project. These procedures are classified to their degree of severity, based on Annex VIII to the Directive: they range from Non-recovery to Mild, Moderate and Severe. Procedures must be carried out under anesthesia, analgesia or using another suitable method. There is an exception, if anesthesia is judged to be more traumatic to the animal than the procedure itself, or if it is not appropriate otherwise. Procedures are designed to result in the death of as few animals as possible and to reduce the duration and intensity of suffering. As far as possible, the life of the animal must be spared.
Reusing an animal is a way of reducing the total number of laboratory animals. However, before reusing an animal, the severity of the cumulated procedures, the health of the animal and the opinion of the veterinarian must be taken into account. Animals kept alive shall receive appropriate care and accommodation as defined in Annex III.

Authorization

Breeders, suppliers and users and their establishments must be authorized by a competent authority and registered with it. Authorized establishments must have installations and equipment adapted to the species of animals housed and the performance of the procedures (where they are carried out). Establishments should possess records in which they register all developments in information on the animals, their origin and purpose. The records are kept for five years and made available to the public. Each dog, cat and non-human primate must have an individual identification and history file. The history file must contain relevant reproductive, veterinary and social information on the individual animal and the projects in which it has been used.

Inspections

Competent authorities must carry out regular inspections of all breeders, suppliers and users and their establishments in order to ensure compliance with the requirements of the Directive. The frequency of inspections is determined by the risks specific to each establishment. At least one third of the establishments of users are inspected each year. An appropriate proportion of the inspections shall be carried out without prior warning. Breeders, suppliers and users of non-human primates are inspected at least once a year.

Final Remark

Directive 2010/63/EU replaces Directive 86/609/EEC and is registered in the context of an EU policy which is aimed at limiting experimentation on animals as far as possible and eliminating it if it is not strictly necessary. Experimentation on animals for the production of cosmetic products, which is not covered by this Directive, has therefore been completely forbidden since 2009 (Regulation (EC) No 1223/2009).

3.2 Legal considerations for the shipping of fish, fish eggs and sperm samples intended for scientific research

(Jana Maier, Robert Geisler)

The shipping of fish as well as fish eggs and sperm through a commercial carrier is subject to a number of regulations and laws. Their aim is to prevent and contain transmittable diseases, to protect living animals during transport, and to prevent any unintended release of genetically modified organisms into the environment. The legal groundwork is provided by the following EU directives and EU regulations as well as the national laws which implement them:

Animal health protection:

- Council Directive 2006/88/EC on animal health requirements for aquaculture animals and products thereof, and on the prevention and control of certain diseases in aquatic animals[16]
- Commission Regulation (EC) No 1251/2008 on conditions and certification requirements for the placing on the market and the import into the Community of aquaculture animals and products thereof and laying down a list of vector species[17]
- Binnenmarkt-Tierseuchenschutzverordnung – BmTierSSchV – Verordnung über das innergemeinschaftliche Verbringen sowie die Einfuhr und Durchfuhr von Tieren und Waren (Internal Market Epizootic Protection Ordinance, Germany)[18]

Biological safety:

- Regulation (EC) No 1946/2003 on transboundary movements of genetically modified organisms[19]
- Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms[20]

[16] *izmc.ezrc.kit.edu/documents/<DIR_206-88-EC_en_de>*
[17] *izmc.ezrc.kit.edu/documents/<COM_REG_1251-2008_en_de>*
[18] *izmc.ezrc.kit.edu/documents/<tierseuchschbmvo_de>*
[19] *izmc.ezrc.kit.edu/documents/<REG_1946-2003_en_de>*
[20] *izmc.ezrc.kit.edu/documents/<freisetzungGVO_DIR_2001-18-EG_en_de>*

Animal protection:

- Council Regulation (EC) No 1/2005 on the protection of animals during transport and related operations[21]
- Tierschutztransportverordnung – TSchTrV – Verordnung zum Schutz von Tieren beim Transport (German, implementing the above Council Regulation)[22]
- Directive 2010/63/EU on the protection of animals used for scientific purposes[23]
- IATA Live Animals Regulations (IATA-LAR)[24]

Carriage of dangerous goods:

- The European Agreement concerning the International Carriage of Dangerous Goods by Road (ADR)[25]
- Regulations concerning the International Carriage of Dangerous Goods by Rail (RID)[26]
- IATA Dangerous Goods Regulations (IATA-DGR)[27]

Even though the declared content and purpose of shipments is the same for sperm samples, fish eggs and fish (non- hazardous, non- infectious biological specimens for research purposes only) there may be different requirements for the necessary transport documents according to what is to be sent and the country of origin and destination. We will briefly describe these requirements, specifically for import into EU member states and transport within the EU.

IMPORT: Council Directive 2006/88/EC lays out animal health requirements for aquaculture animals and products thereof as well as for their import into and transport through the member states of the European Union. According to the directive the term “aquaculture animal” refers to any aquatic animal including those kept for ornamental purposes, in all stages, including eggs and sperm. Council Directive 2006/88/EC as well as Commission Regulation 1251/2008 and BmTierSSchV specify that the import of fish, fish eggs and sperm into an EU member state and their transport within the EU requires an animal health certificate.

Consequently each shipment from a country outside the EU must be accompanied by an animal health certificate according to Commission Regulation 1251/2008 and Commission Implementing Regulation No 1012/2012, Annex IV B (“Model animal health certificate for the import into the European Community of ornamental aquatic animals intended for closed ornamental facilities”[28]). This certificate must be signed

[21] *izmc.ezrc.kit.edu/documents/<COUNC_REG_1-2005_en_de>*
[22] *izmc.ezrc.kit.edu/documents/<tierschtrvo>*
[23] *izmc.ezrc.kit.edu/documents/<DIR_2010-63-EU_en_de >* *izmc.ezrc.kit.edu/documents/<DIR_2010-63-EU_en_de >*
[24] *https://www.iata.org/publications/Pages/live-animals.aspx*
[25] *http://www.unece.org/trans/danger/publi/adr/adr2013/13contentse.html*
[26] *http://www.cit-rail.org/en/rail-transport-law/cotif/*
[27] *http://www.iata.org/publications/dgr/Pages/index.aspx*
[28] *izmc.ezrc.kit.edu/documents/<REG_COM_1012-2012_en_de>*

by the competent official inspector and must be presented as an original at the border inspection post (or veterinary office).

ALTERNATIVE: Alternatively, for animals and products that are specifically pathogen free or not susceptible to specific diseases, the certificate discussed above may be dispensed with, and the recipient can obtain a veterinary import permit instead. In Germany this must be applied for by the state ministry responsible for the respective border inspection post. The Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen is responsible for the border inspection post Köln/Bonn. To obtain the import permit the completed application form[29] must be submitted by E-Mail (einfuhr-nrw@lanuv.nrw.de) or by FAX (02361/305-59902). The import permit is valid for single (Einzelsendung) or for multiple consignments (Jahresgenehmigung). A list with names and addresses of the institutions should be appended to the application form if there are multiple consignments from different places of origin. The Hessische Ministerium für Umwelt, Energie, Landwirtschaft und Verbraucherschutz is responsible for the border inspection post Airport Frankfurt a. M. Informal E-Mail requests indicating the place of origin, the total number of fishes, fish eggs or sperm samples, species and desired authorization period should be addressed to veteinfuhr@hmuelv.hessen.de. The permit requires that each shipment must then be accompanied by a certificate of the official veterinarian or the veterinarian supervising the facility. The certificate must indicate that the production location of the fish is an aquaculture facility that is not subject to any animal-health restrictions in connection to epizootic diseases and that the fish do not show any obvious signs of diseases transmissible to fish. We recommend the use of a health certificate according to the recommendations of the Aquatic Animal Health Code of the Office of Epizootic diseases (OIE)[30].

EXPORT: Shipments to countries outside the European Union must be likewise accompanied by a certification of the official veterinarian or the veterinarian supervising the facility. Furthermore, they must be accompanied by a customs invoice which specifies their use for research[31]. The required import permit must be applied for by the recipient at the competent national authorities.

SHIPMENT WITHIN THE EU: For shipment of specific pathogen free fish, fish eggs and sperm within the EU neither an import permit nor a customs invoice is required, nevertheless we recommend that certification of the supervising veterinarian should be included in the package.

3.2.1 Additional regulations for shipment of fish (later than 5 days post fertilization)

If fish eggs arrive at the recipient later than 5 days post fertilization (dpf) or if fish of later developmental stages are sent, the Live Animal Regulations of the IATA (International Air Transport Association) as well as those of Council Directive 2010/63/EU on the protection of animals used for scientific purposes must be observed. This entails that the shipments are declared as an animal shipment and

[29] *izmc.ezrc.kit.edu/documents/<lanuv_tierseuchen>*
[30] *http://www.oie.int/index.php?id=171&L=0&htmfile=chapitre_1.5.11.htm*
[31] *izmc.ezrc.kit.edu/documents/<customsinvoice*

that the relevant requirements for packaging and declaration must be met. In addition to the certificate and customs invoice, the shipment must be accompanied by the following documents:

- Shipper's Certificate for Live Animals (form issued by the carrier)
- Transport declaration "Transporterklärung" (form issued by the carrier and required if the shipment originates in Germany, as specified by the Tierschutztransportverordnung)
- Air Waybill
- Animal Welfare import license "Tierschutzrechtliche Einfuhrgenehmigung" (required for import to Germany; for the KIT and other recipients in the district of Karlsruhe this must be applied for at the district's Amt für Veterinärwesen und Lebensmittelüberwachung[32]; informal requests should be sent by E-Mail to veterinaeramt@landratsamt-karlsruhe.de)

As with fish eggs and sperm samples, the required transport documents for export to countries outside the EU depend on the country of destination. Information on these is therefore best requested from the carrier.

3.2.2 Additional regulations for shipment of fish that must be classified as genetically modified organisms

Biosafety regulations: According to the German Genetic Engineering Act (deutsches Gentechnikgesetz GenTG) [33], EU Directive No 2001/18/EC [34] and Regulation (EC) No 1946/2003[35], a genetically modified organism (GMO) means an organism, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally through mating or natural recombination (GenTG, Section 3 (3) and Directive No 2001/18/EG Article 2 (2)). Techniques for altering the genetic material are techniques or methods involving the direct introduction of hereditary material prepared outside the organism, by for example, micro-injection (GenTG Section 3 (3a) and Directive No 2001/18/EG Annex IA Part I). Mutagenesis is not considered as a technique for altering genetic material, unless GMOs are used as donors or recipients (GenTG Section 3 (3b) and Directive No 2001/18/EG Annex IA Part II).

[32] *http://www.landkreis-karlsruhe.de/index.phtml?sNavID=1863.88&La=1*
[33] *Gentechnikgesetz in der Fassung der Bekanntmachung vom 16. Dezember 1993 (BGBl. I S. 2066), das durch Artikel 4 Absatz 14 des Gesetzes vom 7. August 2013 (BGBl. I S. 3154) geändert worden ist (izmc.ezrc.kit.edu/documents/<GenTG>)*
[34] *Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC (izmc.ezrc.kit.edu/documents/<freisetzungGVO_DIR_2001-18-EG_en_de>)*
[35] *Regulation (EC) No 1946/2003 of the European Parliament and of the Council of 15 July 2003 on transboundary movements of genetically modified organisms (izmc.ezrc.kit.edu/documents/<REG_1946-2003_en_de>)*

Following the above legal regulations, the supplying of GMOs or products containing them, to third parties is generally regarded as “placing them on the market” which requires an authorization issued by the competent authority (for Germany: the Federal Office of Consumer Protection and Food Safety (“Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (BVL)”[36]). This authorization is not necessary if the products are intended for genetic engineering operations in genetic engineering installations (referred to as “contained used”) because these employ stringent measures to limit contact with the general population and the environment and they provide a high level of safety (GenTG Section 3 (6) and 14 1a (2) and Directive No 2001/18/EG Article 2 (4)). In this case, the GMO must be labelled “This product contains genetically modified organism”, but no further documents are required (GenTG §Section 17b (2)). It is in the shipper’s own interest to ascertain from the recipient of transgenic fish that a licensed facility of the corresponding biosafety levels is available to the recipient for keeping the GMOs. Otherwise the shipment is considered as an unauthorized placing of GMOs on the market which is punishable by a fine of up to 50,000 EUR as an offense under section 38 (1) 7 and 38 (2) GenTG.

Dangerous goods regulations: GMOs which do not meet the definition of toxic or infectious substances but are capable of transferring genetic material to their offspring and surviving in case they escape into the wild are classified as Class 9 and UN 3245 and must be labeled and packed according to the regulations of ADR/RID (road/rail transport) and IATA-DGR (air transport).

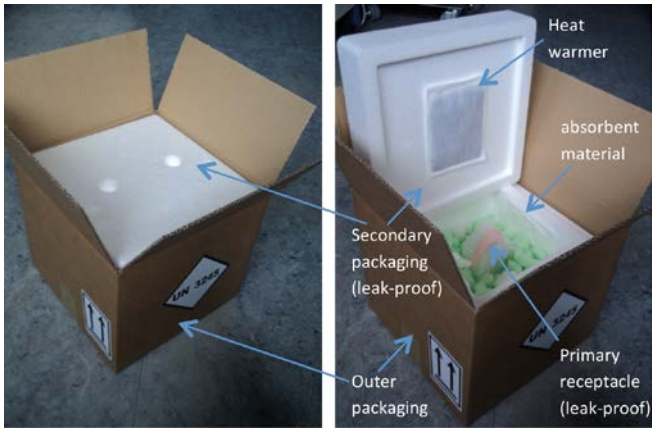


Fig. 4: Packaging for Transport

Correct Shipping name: “GENETICALLY MODIFIED ORGANISMS”

Packaging and labeling: Carriage by road or rail: Packing according to the triple packaging principle (including absorbent material between the primary and secondary containers) is required and must be compliant with Packaging Instruction P904 of the ADR[37] (Fig.4).

Air transport: Packing according to the triple packaging principle (including absorbent material between the primary and secondary containers) is required and must be compliant with Packaging Instruction 959 of the IATA[38] (Fig.4).

Labeling: The outer container must display the **UN 3245 label** (**Fig. 5**) and the **sender’s and recipient’s full name and address** BESIDE the label.

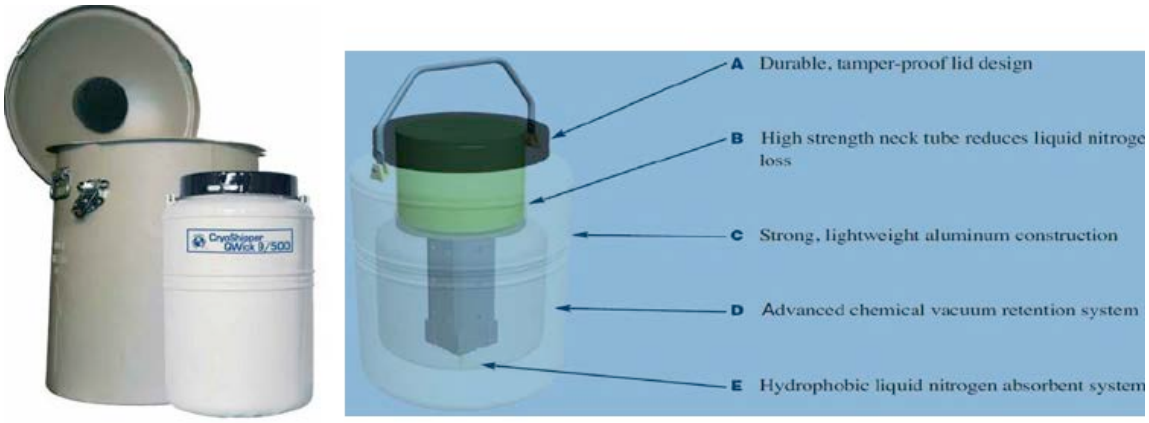
Fig. 5: UN 3245 label.
The mark shall be in the form of a square set at an angle of 45° (diamond-shaped) with minimum dimensions of 50 mm by 50 mm; the width of the line shall be at least 2 mm and the letters and numbers shall be at least 6 mm high.

[36] *The Federal Office of Consumer Protection and Food Safety (BVL) (http://www.bvl.bund.de/DE/Home/homepage_node.html)*
[37] *izmc.ezrc.kit.edu/documents/<p904_en_de>*
[38] *izmc.ezrc.kit.edu/documents/<p959_en_de>*

Specific provisions and documents: GMOs that are packed in compliance with Packaging Instructions P904 or 959 are not subject to other regulations of ADR/RID or IATA-DGR. A Shipper's Declaration for Dangerous Goods is not required.

Shipment of sperm: The shipment of frozen sperm samples requires special, safe cryogenic containers (Fig. 6) which allow transport at temperatures between -150 and -180°C (referred to as cryogenic dry shippers since they do not contain liquid nitrogen). These can be leased from the commercial carrier that conducts the shipment (e.g. World Courier, FedEx) or bought from a manufacturer (e.g. Thermo Scientific) and so consigned to a carrier that does not offer such containers (e.g. Biocare). The labeling regulations differ according to the mode of transport and are best requested from the carrier

Fig. 6: Container for shipment of sperm. Source: CryoService Limited, UK



4. Zebrafish and Medaka as experimental models

(Nicholas Foulkes)

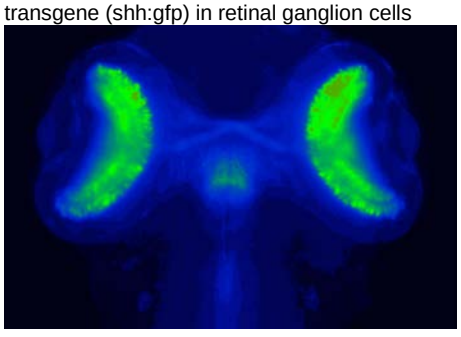
Fig. 7: Zebrafish, female

The small fresh water fishes, zebrafish (*Danio rerio*) (**Fig. 7**) and medaka (*Oryzias latipes*) (**Fig. 8**), are increasingly being used as biomedical model systems. They offer sequenced genomes and a rich repertoire of genetic, molecular and cellular manipulation tools. A unique set of properties (small size, numerous offspring, optical transparency of the embryos, amenability to genetic and chemical screens) has made them popular vertebrate animal models among biomedical researchers. The advantage of these fish models over conventional models such as mouse and rat is the possibility to carry out large-scale phenotypic screens to characterise the function of genes or the effect of small molecules. In addition to forward genetic approaches, a large tool kit of methods ranging from transgenetics to targeted knock-out of genes allows investigating gene function in the context of an entire organism, often using non-invasive imaging techniques. Zebrafish and medaka can thus contribute significantly to meeting the challenge of the post-genome sequencing era to understand gene function on a global scale. Their value as models has been further increased significantly by parallel developments in microscopy technologies, which now allows us to image intact live embryos at subcellular resolution (Fig. 9, 10).

Fig. 8: Medaka, Southern Cab strain; top: female; bottom male. Courtesy Jochen Wittbrodt

It has become clear that the potential of small fish models goes far beyond the fields of cell biology and developmental genetics, and their use has lead to innovation in almost all biomedically relevant areas. This has been recognised by scientists and funding agencies worldwide and considerable resources have been allocated to develop fish-related technologies and infrastructure [39]. Zebrafish models have been described for polycystic kidney disease, heart arrhythmias, cardiomyopathies and myopathies of the skeletal musculature, congenital heart defects, anaemia, cholesterol processing, glaucoma, cancer (especially melanoma and leukaemia), addiction, tissue regeneration, hearing disorders, ectodysplasia, epidermolysis bullosa, neural and retinal degeneration as well as infectious disease. In some cases, a novel disease gene was discovered in the zebrafish and only subsequently a human disease was linked to it, such as the iron transporter ferroprotein gene in hemochromatosis type 4 and anaemia in humans.

Fig. 9: Transgenic zebrafish expressing a transgene (shh:gfp) in retinal ganglion cells



[39] *Zebrafish – A Practical Approach, Dahm and Nüsslein-Volhard eds. OUP 2002, 2005*

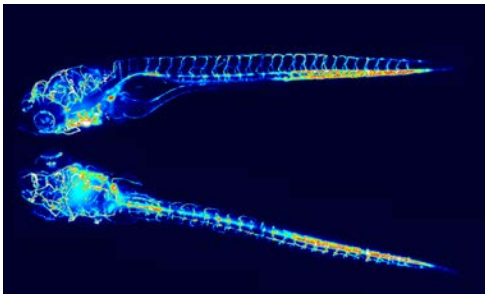


Fig. 10: *kdrl::mCherry-CAAX* transgenic zebrafish embryo marking the blood vessels. Top: dorsal view, bottom: lateral view.

The zebrafish is also attracting increasing attention from the pharmaceutical industry both with the aim of screening for drugs as well as to use the zebrafish embryo as a whole animal system for toxicological studies. The first examples have already appeared in the literature where chemicals were successfully screened for by their suppression of genetic defects[40]. The first drug candidates developed with zebrafish embryos are in the clinical testing phase[41] (Len Zon, pers. comm.). In Germany, the zebrafish embryotoxicity assay has become a DIN standard for testing the water quality of sewage plants[42]. Moreover, molecular studies suggest that the zebrafish embryo may offer a suitable model for the systematic testing of chemicals under the European REACH initiative[43] and other systematic toxicity screens that aim at understanding toxicity mechanisms[44].

Due to the evolutionary divergence of medaka and zebrafish, forward genetic approaches in medaka often complement those of zebrafish[45]. For example, due to its long history as a genetic model system a large number of pigmentation mutants are available that have provided new insight into human disease such as oculocutaneous albinism (OCA). Similarly, forward genetic approaches in medaka have provided novel insight into bone and cartilage formation, sex determination and gonadal development. Furthermore, germ line stem cells in the ovaries of medaka have been reported. Thus, medaka also represents an emerging model to study stem cell niches that may be relevant for reproductive biology.

[40] Peterson, R.T., et al., *Chemical suppression of a genetic mutation in a zebrafish model of aortic coarctation.* Nature biotechnology, 2004. **22**(5): p. 595-9.
[41] Martin, C.S., A. Moriyama, and L.I. Zon, *Hematopoietic stem cells, hematopoiesis and disease: lessons from the zebrafish model.* Genome medicine, 2011. **3**(12): p. 83.
[42] DIN (2001) German standard methods for the examination of water, waste water and sludge – Subanimal testing (group T) – Part 6: Toxicity to fish. Determination of the non-acute-poisonous effect of waste water to fish eggs by dilution limits (T 6). DIN 38415-6; German Standardization Organization.
Lammer, E., et al., *Is the fish embryo toxicity test (FET) with the zebrafish (Danio rerio) a potential alternative for the fish acute toxicity test?* Comparative biochemistry and physiology. Toxicology & pharmacology : CBP, 2009. **149**(2): p. 196-209.
[43] Yang, L., et al., *Zebrafish embryos as models for embryotoxic and teratological effects of chemicals.* Reproductive toxicology, 2009. **28**(2): p. 245-53.
[44] http://www.epa.gov/ncct/download_files/chemical_prioritization/padillaSept28presentation.pdf
[45] *Takeda, H. and Shimada, A. (2010). The art of medaka genetics and genomics: what makes them so unique?* Annu Rev Genet *44, 217–241.*

4.1 The biology of zebrafish (*Danio rerio*)

(Thomas Dickmeis)

The zebrafish is a popular fresh water aquarium fish originating from the Indian subcontinent. It is easy to breed and quite hardy, making it particularly suitable for beginner aquarists. These properties have also contributed to its increasing use as a model animal in research, as they complement its experimental advantages such as small size and transparency of the offspring and the availability of an ever increasing toolkit for genetic studies.

Morphology, anatomy and physiology

Adult zebrafish are about 3-5 cm in length and show a characteristic pattern of blue-black horizontal stripes that run along the body and the anal and tail fins (hence its name, **Fig. 7**). These black stripes are formed by melanin-containing melanophore cells. The stripes are separated from each other by xanthophores, which contain pteridin and carotenoid pigments that confer an orange-yellowish colour. A third type of pigment cells, the iridophores, contains guanine-rich platelets that provide an iridescent appearance [46]. Zebrafish exhibit sexual dimorphism, with the female characterized by a larger belly and the male by its comparative slimness and a more yellowish hue.

As a ray-finned fish, zebrafish possess the basic body plan of this vertebrate group, which is distinguished by some characteristic features such as fins supported by bony rays emerging from the base of the fin, gills on both sides of the body in a cavity covered by an operculum, and a swim bladder regulating buoyancy. An interesting aspect of neuroanatomy is the presence of an “everted” brain, the result of an eversion process during development, which results in the ventricle being spread out along the dorsal and lateral outer surface of the brain (Wullimann and Mueller, 2004). Some anatomical features of zebrafish are specific to the minnows, the systematic family to which zebrafish belong. Thus, zebrafish lack teeth in the jaw. Instead, they possess pharyngeal teeth in the back of their throat. Minnows also lack an adipose fin on the back (Nüsslein-Volhard, 2002).

The zebrafish shares many features of physiology with other vertebrates, constituting part of its appeal as a model organism for biomedical research. Thus, zebrafish have been used in studies of muscle and cardiac physiology, neural function and synaptic transmission, as well as endocrinology (Asnani and Peterson, 2014; Gibbs et al., 2013; Leung et al., 2013; Lohr and Hammerschmidt, 2011). Zebrafish have also served to study more fish-specific aspects of physiology related to its aquatic habitat, such as osmoregulation by ionocytes in the gills (Guh et al., 2015). Mechanosensory systems of the zebrafish are the lateral line organ and the ear. The lateral line is composed of a series of mechanosensory receptors (hair cells) on the head and along the body axis, which detect e.g. vibrations in the water (Nüsslein-Volhard, 2002; Ostrander, 2000). The ear is composed of an inner ear only. The hair cell containing macula organs of this ear serve both as balance organs, as they do in mammals, and additionally for hearing. As the zebrafish forms part of the Otophysi group of fish, its inner ear is linked to the swim bladder by small bones (the Weberian ossicles). This enhances hearing and makes the zebrafish one of the “hearing specialists” among the fishes (Nicolson, 2005; Whitfield, 2002). Zebrafish are capable

[46] *Parichy DM. 2003. Pigment patterns: fish in stripes and spots.* Curr Biol ***13****: R947-950.*
Rawls JF, Mellgren EM, Johnson SL. 2001. How the zebrafish gets its stripes. Dev Biol ***240****: 301-314.*

of colour vision, and in addition to cone types receptive to wavelengths of the three colours perceived also by humans (red, green, blue), they possess cones sensitive to UV light (Slijkerman et al., 2015). Also olfaction plays an important sensory role, as zebrafish smell nutrients (amino acids), kinship cues, ovulation-inducing pheromones (steroid glucuronides), and a "Schreckstoff" (alarm substance), the glycosaminoglycan chondroitin, which is released upon injury and elicits vivid escape responses (Gerlach and Lysiak, 2006; Koide et al., 2009; Mathuru et al., 2012; van den Hurk et al., 1987).

Systematics

Like most bony fishes, zebrafish are part of the teleosts, the largest subclass within the ray-finned fish or *actinopterygii*. Zebrafish are cyprinid fish, i.e. they belong to the family *Cyprinidae* (minnows or carps), which is one of the largest families of the teleosts and thus of vertebrates in general[47]. This family includes the common carp and the goldfish. Originally named *Danio rerio* by its discoverer Hamilton in 1822[48], the zebrafish was referred to as *Brachydanio rerio* in the scientific literature for many years, before it was reassigned to the *Danio* genus by more recent phylogenetic analyses including DNA sequence data[49]. Today, about 45 species are included in the genus *Danio*[50]. The name *Danio* derives from the Bengali "dhani", which refers to green rice fields.

Laboratory strains and variants

A number of different wild-type laboratory strains have been established by several research labs and have been bred in captivity for prolonged periods of time. Popular strains include the AB and Tübingen (TÜ) strains, which were also used for the first large-scale mutagenesis screens[51].

The AB strain was established by G. Streisinger in Eugene, Oregon, USA, while the TÜ strain was generated in the laboratory of C. Nüsslein-Volhard, Tübingen, Germany[52]. These strains were established from fish purchased in pet shops while another important strain, WIK[53], is derived from a wild catch in India – hence its name: "Wild-type India Kolkata" or "Wild Indian Karyotype". It has served as a highly polymorphic reference strain for the mapping of mutations generated in the TÜ background[54].

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[47] *Nelson JS. 2006.* Fishes of the World*. John Wiley and sons, New York, NY.*

[48] *Hamilton F. 1822.* An Account of the Fishes Found in the River Ganges and its Branches*. Archibald Constable and Co., Edinburgh and London.*

[49] *Mayden RL, Tang KL, Conway KW, Freyhof J, Chamberlain S, Haskins M, Schneider L, Sudkamp M, Wood RM, Agnew M et al. 2007. Phylogenetic relationships of Danio within the order Cypriniformes: a framework for comparative and evolutionary studies of a model species.* J Exp Zool B Mol Dev Evol ***308****: 642-654.*

[50] *Fang F, Douglas ME. 2003. Phylogenetic Analysis of the Asian Cyprinid Genus Danio (Teleostei, Cyprinidae).* Copeia ***2003****: 714-728.*

[51] *Driever W, Solnica-Krezel L, Schier AF, Neuhauss SC, Malicki J, Stemple DL, Stainier DY, Zwartkruis F, Abdelilah S, Rangini Z et al. 1996. A genetic screen for mutations affecting embryogenesis in zebrafish.* Development ***123****: 37-46.*

Haffter P, Granato M, Brand M, Mullins MC, Hammerschmidt M, Kane DA, Odenthal J, van Eeden FJ, Jiang YJ, Heisenberg CP et al. 1996. The identification of genes with unique and essential functions in the development of the zebrafish, Danio rerio. Development ***123****: 1-36.*

[52] *Dahm R, Geisler R, Nüsslein-Volhard C. 2006. Zebrafish (Danio rerio) Genome and Genetics. in* Encyclopedia of Molecular Cell Biology and Molecular Medicine*. Wiley-VCH Verlag GmbH & Co. KGaA.*

[53] *Rauch GJ, Granato M, Haffter P. 1997. A polymorphic zebrafish line for genetic mapping using SSLPs on high-percentage agarosegels.* Technical Tips Online ***T01208****.*

[54] *Geisler R, Rauch GJ, Geiger-Rudolph S, Albrecht A, van Bebber F, Berger A, Busch-Nentwich E, Dahm R, Dekens MP, Dooley C et al. 2007. Large-scale mapping of mutations affecting zebrafish development.* BMC Genomics ***8****: 11.*

Genome sequencing data have revealed that the different zebrafish wild-type strains show high levels of genetic diversity, both between and within the strains. For example, single nucleotide polymorphisms (SNPs) appear to be at least four-fold more frequent in zebrafish than in humans (Howe et al., 2013; LaFave et al., 2014; Parichy, 2015; Patowary et al., 2013). It is largely unknown to what extent this genetic heterogeneity contributes to variation in experimental outcomes.

In addition to the wildtype strains, fish carrying pigmentation mutations or other morphological variation are frequently used in breeding experiments. They also provide easily recognizable companions for group housing, thereby avoiding single housing of fish. An example for a pigment mutation is the recessive *leopard* mutation (*leo*), which leads to a disruption of the stripe pattern in homozygous fish[55] (**Fig. 11**). These fish were initially described as a different species called *Danio frankei*[56]. Fish that are homozygous for the *golden* mutation have strongly reduced melanin levels in their melanophores and appear yellowish[57]. The gene mutated in *golden* fish, a putative cation exchanger, has also been linked to pigmentation differences in humans[58]. The "Tübingen (or "Tüpfel") long fin" (TL) strain[59] carries both the *leo*^{t1} mutation and a dominant mutation *lof*^{dt2} ("long fin"[60]) which causes overgrowth of the fins. Finally, the *casper* mutants[61], which are double mutants for *nacre*/*mitfa*[62] and *roy* (*roy orbison*), lack all melanocytes and iridophores. Therefore, they are transparent even as adults, a feature that makes them well suited for live imaging applications.

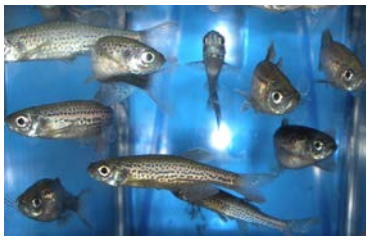

Fig. 11: The leopard *Danio*.

[55] *Kirschbaum F. 1975. Untersuchungen über das Farbmuster der Zebrabarbe Brachydanio rerio (Cyprinidae, Teleostei).* Wilhelm Roux's Archives ***177****: 129-152.*
Watanabe M, Iwashita M, Ishii M, Kurachi Y, Kawakami A, Kondo S, Okada N. 2006. Spot pattern of leopard Danio is caused by mutation in the zebrafish connexin41.8 gene. EMBO Rep ***7****: 893-897.*
[56] *Meinken H. 1963. Mitteilungen der Fischbestimmungsstelle des VDA XLII: Brachydanio frankei spec. nov., der Leopard-Danio. .* Monatsschrift für Ornithologie und Vivarienkunde ***42****: 39-43.*
Spence R, Gerlach G, Lawrence C, Smith C. 2008. The behaviour and ecology of the zebrafish, Danio rerio. Biol Rev Camb Philos Soc ***83****: 13-34.*
[57] *Lamason RL, Mohideen MA, Mest JR, Wong AC, Norton HL, Aros MC, Jurynec MJ, Mao X, Humphreville VR, Humbert JE et al. 2005. SLC24A5, a putative cation exchanger, affects pigmentation in zebrafish and humans.* Science ***310****: 1782-1786.*
Streisinger G, Walker C, Dower N, Knauber D, Singer F. 1981. Production of clones of homozygous diploid zebra fish (Brachydanio rerio). Nature ***291****: 293-296.*
[58] *Lamason RL, Mohideen MA, Mest JR, Wong AC, Norton HL, Aros MC, Jurynec MJ, Mao X, Humphreville VR, Humbert JE et al. 2005. SLC24A5, a putative cation exchanger, affects pigmentation in zebrafish and humans.* Science ***310****: 1782-1786.*
[59] *Dahm R, Geisler R, Nüsslein-Volhard C. 2006. Zebrafish (Danio rerio) Genome and Genetics. in* Encyclopedia of Molecular Cell Biology and Molecular Medicine*. Wiley-VCH Verlag GmbH & Co. KGaA.*
[60] *Iovine MK, Johnson SL. 2002. A genetic, deletion, physical, and human homology map of the long fin region on zebrafish linkage group 2.* Genomics ***79****: 756-759*
Tresnake I. 1981. the long-finned zebra Danio. Trop Fish Hobby ***29****: 43-56.*
van Eeden FJ, Granato M, Schach U, Brand M, Furutani-Seiki M, Haffter P, Hammerschmidt M, Heisenberg CP, Jiang YJ, Kane DA et al. 1996. Genetic analysis of fin formation in the zebrafish, Danio rerio. Development ***123****: 255-*
[61] *White RM, Sessa A, Burke C, Bowman T, LeBlanc J, Ceol C, Bourque C, Dovey M, Goessling W, Burns CE et al. 2008. Transparent adult zebrafish as a tool for in vivo transplantation analysis.* Cell Stem Cell ***2****: 183-189.*
[62] *Lister JA, Robertson CP, Lepage T, Johnson SL, Raible DW. 1999. nacre encodes a zebrafish microphthalmia-related protein that regulates neural-crest-derived pigment cell fate.* Development ***126****: 3757-3767.*

Habitat and ecology

Zebrafish are found all across India in a variety of habitats[63]. Common typical characteristics of their habitats are low water flow, ground covered with sand, silt or pebbles, and overhanging vegetation. Such habitats are frequently found in secondary or tertiary channels connected with the main channel of a river, or adjacent to wetlands and paddy fields. Physical and chemical parameters of these habitats vary largely: in two recent surveys, temperatures ranged from 12.3°C to 38.6°C, pH values varied from 5.9 to 9.8, and conductivities from 10 µS to 271 µS were observed[76]. These findings are consistent with the large tolerance to ambient conditions zebrafish show in captivity.

In research facilities, zebrafish are generally kept in aquaria devoid of additional structures, in order to facilitate monitoring and cleaning. Experiments involving additional tank structures attempting to mimic more "natural" environments have yielded mixed results regarding welfare benefits. For example, while some studies observed a reduction (Basquill and Grant, 1998) or no difference (Hamilton and Dill, 2002; Kistler et al., 2011) in aggressive behaviour in tanks with vegetation, others found that vegetation or the presence of (easily sanitizable) glass rod structures rather stimulated aggression (Bhat et al., 2015; Wilkes et al., 2012). Importantly, many of the proposed enrichment items may pose a risk to health management, by obstructing the view on the fish in the tank and making cleaning more difficult. Given that the social environment seems to play a more important role for normal behaviour of zebrafish (see also below, "*behaviour*"), the use of tank enrichment items may be beneficial only in special situations, e.g. when isolating single fish by co-housing them with fish of a strain with different pigmentation is not possible (Collymore et al., 2015).

Zebrafish share their habitat with a variety of other fish that may act as competitors for food, including other Cyprinids like *Puntius* barbs, other *Danio* species and especially *Esomus danricus*, which has a similar size and gape and which occupies similar positions in the water column[64]. Gut content analysis suggests that zebrafish mainly feed on allochthonous materials (i.e. not deriving from their habitat), such as red ants or other terrestrial insects that have fallen into the water, but also on aquatic insect larvae and crustaceans as well as zooplankton and phytoplankton[65]. The upward directed mouth of the zebrafish is consistent with feeding mainly at the water surface[66], as also observed in the aquarium; however, live food such as *Artemia*

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[63] *Arunachalam M, Raja M, Vijayakumar C, Malaiammal P, Mayden RL. 2013. Natural history of zebrafish (Danio rerio) in India.* Zebrafish ***10****: 1-14.*
Engeszer RE, Patterson LB, Rao AA, Parichy DM. 2007. Zebrafish in the wild: a review of natural history and new notes from the field. Zebrafish ***4****: 21-40.*
Spence R, Gerlach G, Lawrence C, Smith C. 2008. The behaviour and ecology of the zebrafish, Danio rerio. Biol Rev Camb Philos Soc ***83****: 13-34.*

[64] *Engeszer RE, Patterson LB, Rao AA, Parichy DM. 2007. Zebrafish in the wild: a review of natural history and new notes from the field.* Zebrafish ***4****: 21-40.*

[65] *Spence R, Gerlach G, Lawrence C, Smith C. 2008. The behaviour and ecology of the zebrafish, Danio rerio.* Biol Rev Camb Philos Soc ***83****: 13-34.*
Arunachalam M, Raja M, Vijayakumar C, Malaiammal P, Mayden RL. 2013. Natural history of zebrafish (Danio rerio) in India. Zebrafish ***10****: 1-14.*
McClure MM, McIntyre PB, McCune AR. 2006. Notes on the natural diet and habitat of eight danionin fishes, including the zebrafish Danio rerio. Journal of Fish Biology ***69****: 553-570.*

[66] *Gerlai R. 2013. Antipredatory behavior of zebrafish: adaptive function and a tool for translational research.* Evol Psychol ***11****: 591-605.*

larvae are eagerly chased, too, and thereby constitute a form of easily applicable tank enrichment stimulating natural prey capture behaviour of adult and larval zebrafish. Birds, e.g. herons and kingfishers, as well as larger fish species such as snakeheads (*Channa sp*.) or the needlefish *Xenentodon cancila* found in the same habitat may prey on adult zebrafish, while dragonfly larvae might hunt zebrafish larvae[67].

Behavior

Zebrafish are diurnal animals and thus show highest levels of activity during the day. At night, the frequency and duration of sleep bouts increases. Sleep bouts may last a few minutes and are often characterized by a characteristic position with drooping of the caudal fin of the fish, either close to the water surface or at the bottom of the aquarium[68]. Interestingly, mating behavior tends to be restricted to the onset of the light phase[69].

By adapting behavioral tests from rodent models to adults and even larvae, zebrafish are now also increasingly studied for behavior related to learning and reward as well as to aggression and anxiety[70]. For example, when placed into a novel tank, fish will initially dive to the bottom and remain there, before they start to explore the open tank space. Anxious fish will need a longer time for this transition. Anxious fish might also show freezing behaviour, or on the contrary exhibit longer-lasting phases of increased locomotor activity (e.g. zig-zagging rapidly on the tank bottom)(Maximino et al., 2010). Remarkably, there appear to be strain specific differences in these behavioural responses.

Zebrafish are social animals that form shoals with their conspecifics. In the wild, zebrafish have been observed to form shoals of varying sizes, from 4 to up to 300 individuals, depending on the habitat (Suriyampola et al., 2016). Also shoaling with another species, *Rasbora spp*., has been reported. Shoaling behavior is common in many teleosts and may increase the likelihood of an individual recognizing food sources and predators (“many eyes see more than two”), as well as enhancing the chances of escape during predator attacks due to confusion of the predator or dilution of the individual in the crowd[71]. Cues used for the formation of shoals in zebrafish are still ill-defined, but seem to include both visual and olfactory cues. Zebrafish prefer conspecifics over other species, kin over non-kin and familiar over non-familiar partners for shoal formation[72].

Zebrafish also display hierarchical and territorial behavior. Interestingly, a recent study found that dominant-subordinate relationships seem to be relieved with

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increasing numbers of fish (males) kept in a tank[73]. Thus, the number of attacks dropped when densities were increased from 2 to 6 males/liter and no attacks were observed when 10 individuals were kept per liter. Also whole body cortisol levels were lowest at a relatively high density (5 fish/l) in this experiment, indicating reduced stress levels. Consistent with these results, another recent cross-laboratory study examined the effect of stocking densities on reproductive performance, which equally can be influenced by stress levels. No negative effects occurred at stocking densities as high as 12 fish/liter[74]. Thus, shoaling apparently has beneficial effects regarding the interaction both with the environment and with conspecifics.

Mating

Zebrafish are group spawners, with several males and females engaging in reproductive behavior[75]. In the wild, a certain seasonality in reproduction seems to exist, with the mating season linked with the monsoon period. However, the presence of mature eggs in females captured in the wild all year round suggests that the observed seasonality may rather be linked to food availability and not reflect endogenous seasonal behavior. In line with this, zebrafish in captivity breed all year around under different photoperiods (12-14 hours of light).

While it is still poorly understood which cues determine the choice of mating partners, a typical suite of behaviors during mating has been described[76]. Thus, male and female “undulate” through the water column, and the male “chases” the female, often touching her with his snout. He then “escorts” her to a spawning site, where they “circle” each other oriented head to tail. The male then “quivers”, i.e. he oscillates his body at high frequency and low amplitude close to the female over the spawning site. He may also wrap his body around the female. Oviposition then occurs in bouts of 5 to 20 eggs.

Observations in the wild suggested that zebrafish prefer to mate along the shallow shore of their habitats, and that they prefer gravel substrate over silt, probably because eggs are better protected from predation (including cannibalism) if they fall into the gaps in the gravel substrate[77]. This behavior can be exploited for egg production in captivity. A classical method for continuous egg production from a tank consists in placing a box filled with marbles into the tank[78]. Fish then mate preferentially over the marble inset, and eggs can easily be harvested every morning after removing the marbles. Another method uses the preference for very shallow

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Sessa AK, White R, Houvras Y, Burke C, Pugach E, Baker B, Gilbert R, Thomas Look A, Zon LI. 2008. The effect of a depth gradient on the mating behavior, oviposition site preference, and embryo production in the zebrafish, Danio rerio. Zebrafish ***5****: 335-339.*

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water to increase egg production[79]. Typical mating boxes for single couple or group matings usually are composed of a tank with an inlay, the bottom of which is formed by a mesh. Eggs fall through the mesh into the lower compartment and cannot be eaten by the fish. If the inlays are now tilted so as to produce a gradient of water depth, the fish strongly prefer to mate in the shallow area, as revealed by recording the sites of oviposition. Importantly, this tilting method, also referred to as the "sex on the beach" method, significantly increased egg production compared with non-tilted inlays. Apparatus have been developed that make use of the stimulation of egg laying and mating behavior by shallow water for the production of synchronously developing eggs on a larger scale[80].

Life cycle and sex determination

One reason for the popularity of the zebrafish among developmental biologists is the rapid development of its transparent embryos. Soon after fertilization, divisions of the blastodisc sitting on the top of a large yolk cell commence, and morphogenetic movements such as epiboly, gastrulation and convergence-extension have shaped the main body axes of the embryo by about 10 hours post fertilization (hpf)[81]. Major organ systems are formed over the next 24 to 36 hours, and hatching occurs after 48 h. The hatched embryo is now referred to as an eleutheroembryo, which derives its nutrients from its yolk[82]. At 120 hpf (5 days post-fertilisation (dpf)), feeding behavior commences and the free feeding larval stage is reached. After a further 25 days (30 dpf), metamorphosis has occurred[83]. The fin folds have been transformed into the adult fin pattern, and also the pigmentation pattern is now that of the adult. This stage is called the "juvenile" stage[84]. Around the same time (23-25 dpf) the first signs of sexual differentiation become evident at a histological level in the gonads, since the juvenile gonad starts transforming from a so-called "juvenile ovary" state into a testis in males[85]. 3 months after fertilization (in the wild, this may take as long as 6 months[86]), sexual maturity is reached, and the fish has become a fully-grown adult. The maximum life span under laboratory conditions can be between 4 to 6 years depending on the strain[87].

Recent analyses involving several different laboratory strains, some of them very recently obtained from wild catches, were able to locate a sex-determining region on chromosome four(Wilson et al., 2014). This region seems to underlie a WZ/ZZ-type sex determination system, in which the heterogametic (WZ) sex is female (as

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opposed to the familiar XY-system, where the heterogametic sex is male). Most interestingly, the region was lost in several classical laboratory strains including TÜ and AB (but not in WIK), in which alternative sex determinants have replaced it fucntionally. It remains unknown what is the nature of these alternative mechanisms, whether they have a function also in wild populations, and how they may affect breeding behaviour or other aspects of biology in these laboratory strains.

4.2 Biology of Medaka (*Oryzias latipes*)

(Daniela Vallone, Felix Loosli)

Medaka (*Oryzias latipes*) is a small fresh water fish native to Japan, Taiwan, Korea and China. As a member of the family *Adrianichthyidae* in the order *Beloniformes*, medaka is more closely related to pufferfish (*Tetraodon* and *Fugu*) and stickleback than to zebrafish. Zebrafish and medaka are predicted to have separated from a last common ancestor approximately 150 mya, a distance roughly corresponding to the distance separating humans and chickens. The genus *Oryzias* contains more than 20 species that are widely distributed in Eastern and Western Asia and occupy a wide range of different habitats in tropical and temperate zones. As members of the class *Actinopterygii* (ray-finned fish) medaka has fin rays consisting of bony spines supporting the fins. These fin rays are directly attached to the basal skeletal elements. Medaka has a caudal or tail fin, a dorsal fin, a ventral or anal fin, paired pelvic fins that are considered to be homologous to the hind limbs of tetrapods and paired pectoral fins behind the head. Medaka physiology is comparable in many aspects to that of other vertebrates (Iwahashi et al 2009). For this reason, medaka is often used as a model system in toxicity tests of chemicals.

Habitat and Biology

Medaka (*Oryzias latipes)* is found in Japan, with the exception of Hokkaido Island, in small rivers, creeks and rice paddies. It can also tolerate brackish water. In Japan, medaka is divided into two distinct groups: the Northern Population of the Northern region of Honshu Island and the Southern Population of Southern Honshu, Kyushu, Shikoku and Okinawa. The divergence time between these groups is estimated to be approximately 4 mya. Recently the Northern population has been classified as an independent species based on morphological criteria (*Oryzias sakaizumii*). Due to the divergence time of about 4mya of *Oryzias latipes* and *Oryzias sakaizumii* these species show a high rate of polymorphism in the range of 1-4 % (Spivakov et al. 2014). Under laboratory conditions individuals of *Oryzias latipes* and *Oryzias sakaizumii* mate and male and female F2 hybrids resulting from these interspecies crosses are fully fertile. This is important for genetic purposes as it allows one to introduce polymorphisms into a given genetic background. This approach of crossing a Southern and a Northern inbred line is often used to genetically map a mutation or a specific phenotypic trait. The boundary separating the Northern and Southern population correlates with geographical barriers, thus the two groups are not in sympatry (their habitats do not overlap). However, introgression (gene flow) between these groups has been detected in some areas along the boundary.

Medaka has a diploid karyotype with 48 chromosomes. The haploid genome size is about 700 Mb, half the size of the zebrafish (*Danio rerio*) genome. Adults can reach a length of up to 4 cm. Medaka as many other teleost species has four types of pigment cells (chromatophores): black melanophores, white leucophores, red-yellow xanthophores and silvery iridophores. The natural pigmentation is greyish-brown. The Japanese medaka is native to a temperate zone with distinct seasons and can tolerate a wide range of temperatures (4-40^{o}C). Thus, the rate of embryonic development can be controlled by the ambient temperature. Embryos will tolerate 4^{o}C however their development is almost arrested at this temperature. The life span of medaka under constant mating conditions in the laboratory is about 12 months. This can be extended to more than 2 years under lighting conditions where the fish

do not mate in combination with a reduced temperature (i.e. 10 hours light/14 hours dark at 19^{o}C, see below).

Behaviour
Medaka is a diurnal animal with high locomoter (swimming) activity during the light phase. Feeding activity is also diurnal with no obvious preferential feeding time. Thus feeding activity is spread throughout the light phase. Medaka mate preferentially at the onset of the light phase. Since medaka are native to a temperate zone with distinct seasons, medaka are seasonal breeders and the light phase has to be significantly longer than the dark phase for mating to occur (see also below). Temperature has only a minor influence on the mating drive of medaka (Koger et al 1999). Medaka is a social animal and therefore should not be kept as isolated individuals for long periods. However medaka can tolerate periods of isolation without exhibiting signs of stress or suffering. Recently medaka has also been used for behavioral studies. To this aim, behavioral tests have been established to study the underlying genetics of these complex traits (Okuyama et al. 2014; Tsuboko et al. 2014)).

Medaka, a genetic model system
Medaka breeding is a traditional hobby in Japan dating back to the 16th and 17th centuries. Consequently, several natural pigmentation mutations have been isolated over the centuries that laid the foundation for genetic studies. In 1921, Mendelian inheritance of these traits was demonstrated, including for the first time sex-linked inheritance. Since medaka exhibits a clear sexual dimorphism, medaka sex determination has been studied in great detail, leading to the discovery of the male determining gene *DMY*.

Medaka is very tolerant of inbreeding. Over the decades, a large number of wild catches has been collected to establish laboratory strains and highly inbred strains, which in some cases have been inbred for more than 80 generations by successive brother-sister crosses and are therefore isogenic inbred strains. Currently about 14 highly inbred strains and more than 60 wild strains from both the Northern and Southern populations are available (NBRP medaka stock center at Okazaki, Japan; http://www.shigen.nig.ac.jp). Thus, medaka provides a unique repository for genomic and population genetic studies. Medaka inbred strains differ in morphological, behavioral and physiological characteristics (Takeda and Shimada 2010; Tsuboko et al. 2014). Although this differences are subtle and do not require special consideration for the husbandry of the respective strains, it is important to be aware that this may potentially influence scientific studies and should therefore be considered in the experimental design appropriately. The widespread, easily accessible habitat of medaka allows researchers to collect, analyse and evaluate new wild catches for a given purpose such as interactions of a population with their habitat.

Sourcing and transport
As described above, the medaka stock center in Okazaki provides a large number of established wild and inbred strains. With the exception of population genetic field studies, wild catches are therefore not necessary. Medaka are very hardy fish and easy to breed. In addition, the generation time for most strains that are used in research is 2 month at 28^{0}C. It is therefore easy to maintain a small colony of

medaka adults with appropriate numbers for most purposes, thus precluding shipment of adult medaka. Adult medakas tolerate shipment well, for his reason they were also selected as teleost model for space expeditions (see below). Adults should be transported in appropriate containers that protect the fish against mechanical shocks and temperature fluctuations, such as closed plastic containers protected by a styrofoam box. The container (2 liter for up to 5 adults) should only be half filled with water to ensure sufficient supply with oxygen. Before transport adult medaka should not be fed for 24 hours. Otherwise feces wil affect the water quality during transport. In most cases however it is not necessary to transport adult medakas. The external fertilization and extrauterine development of the yolk-rich eggs renders them especially suited for shipment. The medaka embryo is mechanically well protected by the very tough chorion. Furthermore, medaka hatch only after 7-10 days (at 28^0C), thus also longer shipment periods are possible with unhatched embryos.

In 1994, four medaka fish were carried aboard the Space Shuttle Columbia on a 14 day space expedition. The fish mated in space producing healthy offspring that hatched in orbit, showing that gravity is not essential for embryonic development. Thus, medaka represents the first vertebrate to have mated in space! In 2012 medaka returned to space aboard the Soyuz TMA-06M spacecraft and was housed in the International Space Station ISS. This time the main focus was on studying the influence of gravity on bone formation.

Mating and early development
Medaka are photoperiodic breeders. Thus, in the wild, the reproductive period is in summer: therefore, medaka will mate only when the daily light period is longer than the dark period. In the laboratory as in the wild medaka will mate every day under 14 hours light/10 hours dark conditions. However, continuous mating for more than two months exhausts the fish. Males and females should be separated for several weeks to prevent exhaustion and, if possible, kept under non-reproducing conditions (i.e. 10/14 illumination). As in zebrafish, medaka mate at the onset of the light period. The mating behavior of medaka consists of a fixed sequence of behavioral patterns in which the male approaches the female of choice and tries to induce mating. This sequence includes approaches to the female and swimming in circles under the female of choice. In case the female responds positively to the male ‘dance’ she will allow the male to approach and align side by side upon which the male will grasp the female with its dorsal fin. The female then releases the oocytes, which are subsequently fertilized while still being attached to the female. The female carries the eggs for several hours before releasing them. The clutch size is 10-40 eggs depending on the size and age of the female. Under laboratory conditions, a male can mate with several females every day.

The medaka egg size is about 1.2 mm. Unfertilized eggs are initially opaque and become completely transparent after fertilization. It is therefore easy to identify unfertilized eggs in a clutch. At 28^oC gastrulation starts after about 10 hours, neurulation after 20 hours and organogenesis after about 1.4 days. Medaka hatch after 7-8 days as mature, feeding larvae.

5. Husbandry, breeding and feeding

5.1 Zebrafish

(Thomas Dickmeis)

Sourcing and transport

Zebrafish can easily be maintained and bred in the laboratory. For this reason, it is generally not necessary to obtain wild catches, and the EU directive 2010/63/EU requires that zebrafish used in research be purpose bred. Transport of fish between facilities is necessary e.g. for the dissemination of mutant and transgenic lines. Simulated transport experiments have been reported to lead to increased levels of cortisol and other stress markers in the fish, both immediately after packing and upon “arrival” (Dhanasiri et al., 2013). The same study suggested that application of a nitrifying bacterial consortium during the simulated transport experiment may speed up acclimation, although further evaluation of the benefits of such a treatment is necessary. When following established practice, zebrafish seem to travel well even on long-distance flights and appear healthy upon arrival (Reed and Jennings, 2011). An often preferable alternative to transporting adult animals is the shipment of bleached embryos, which will hatch during transport. This eliminates possible welfare concerns and reduces the risk of spreading disease to receiving laboratories. (also see: 3.2 Legal considerations for the shipping of fish, fish eggs and sperm samples intended for scientific research)

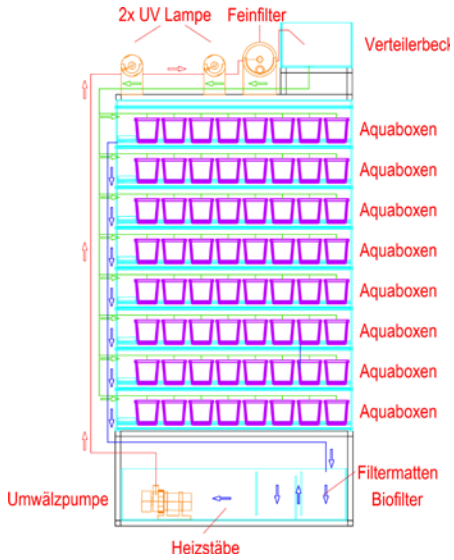

Fig. 12: Scheme of a recirculation system (courtesy Aqua Schwarz, Göttingen)

Housing and care

Fig. 13: The tank system at the European Zebrafish Resource Centre at KIT.

For research that requires a small breeding colony of fish, tank systems (including filtering illumination and heating) can be purchased in aquarium shops. A professional aquarium system is, however, recommended, if you plan to maintain larger numbers of mutants and transgenic fish. There are a number of suppliers that provide such aquarium systems. These comprise fixed installations of tanks but also flexible systems where plastic tanks are placed on shelves and linked to the water supply. All these systems operate as recirculation systems where fish water from a reservoir flows constantly into the tanks. An overflow system collects the water. Waste such as feces, ammonia and nitrite are removed by passing through a biological filter system. The treated water is then pumped back into the reservoir. Frequently, these systems also expose the water to UV light before it re-enters the reservoir in order to reduce pathogens and algal growth. Usually 5% of fresh water is added per day to dilute waste substances and to compensate for evaporation. Recirculating systems do not need specific treatments to oxygenate the

water, as the constant movement of the water and its passage over a dry filter is sufficient for oxygenation.

Depending on the local water quality, some laboratories use tap water without further processing. However, chlorine in tap water is a highly potent fish poison. In most locations, it is therefore essential to pass tap water through a charcoal filter system with an appropriate capacity in order to remove chlorine. Also, if the tap water is too rich in calcium carbonate, it should be premixed with deionized water. Pipes connected to the system should not be galvanized or made of copper, as leaking heavy metals are toxic to the fish (Reed and Jennings, 2011). The optimal water conditions are between 250 to 600 µS conductivity and pH 6.5 to 8. Alternatively, some facilities use deionized water and add a defined salt mixture to it (not FE, if FE-level is high in the tap water). Weekly monitoring of conductivity, pH, nitrite and nitrate of the water is recommended. [88]

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Zebrafish are tropical fish. The water temperature should be in the range of 26 to 29^{o}C. To maintain the water temperature, it is sufficient to heat the room. Alternatively, submerged heaters in the reservoirs can be used to control the water temperature. Usually a temperature gradient exists between the top and the bottom of tanks. Furthermore, recirculation of the water in shelf installations tends to slightly increase the temperature of the water. Therefore, regular measurements of the water temperature in different locations within the tanks are recommended.

As zebrafish are shoaling fish. usually 5 fish per liter of water are maintained but higher densities are also tolerated without signs of stress (see chapter 4.1). Zebrafish can also be kept as individuals or as isolated pairs. This can be necessary for experimental purposes, as suitable marking procedures currently do not exist (Reed and Jennings, 2011). However, if the fish are kept for too long in isolation they tend to grow fat and lose their fecundity. Group housing with differently coloured strains provides an easy means to distinguish the fish of interest while providing it with companions for shoaling.

Fig. 15: Snails ("*Planorbarius corneus*, Posthornschnecke") keep the tanks clean.

There is currently no clear evidence that tank enrichment items such as artificial plants are beneficial for general welfare (see chapter 4.1). Rather, such items may be detrimental for health management by hampering monitoring and cleaning procedures.

Fig. 14: Laying cage

Zebrafish are usually kept under a 14 hour light and 10 hour dark cycle. They spawn in captivity throughout the year. Spawning typically is initiated immediately after the lights are switched on in the morning and continues for a few hours. As zebrafish will feed on their own eggs, fertilized eggs are best obtained by transfer of fish into laying cages (**Fig. 14**). These contain an insert with a grid at the bottom through which eggs will fall into a compartment below that cannot be reached by the parents. In these cages, fish can be bred pairwise or as groups. By collecting eggs after fixed time intervals, synchronized batches of fertilized eggs can easily be obtained for 2 hours after turning on the lights in the morning. To maintain fertility, it is advised not to breed more frequently than once every 1-2 weeks for prolonged periods of time (Reed and Jennings, 2011).

Zebrafish at the EZRC are fed twice a day with flake food, pellets and living *Artemia* larvae (nauplii) hatched on-site. Zebrafish prefer food floating at the surface or in the water. However, they also eagerly chase *Artemia* larvae across the water column, and this live-food stimulated prey capture behaviour can be considered an efficient form of environmental enrichment. A general problem is overfeeding. This results in fungal growth at the bottom of the tanks. In addition, food remnants as well as faeces need to be removed regularly.

Alternatively, it is possible to add snails ("Posthornschnecke" = great ramshorn, *Planorbarius corneus)* to the tanks (**Fig. 15**). These turn over uneaten food into granular sediments that are easily siphoned out of the tank. In addition, the snails

remove algae growing on the tank walls. Snail eggs can be treated with bleach to eliminate pathogens prior to introduction into the facility. Snails can be bred in the facility in special tanks. The snails can be obtained in pet shops or from the EZRC. They can be introduced into the facility via their eggs which should be bleached to eliminate pathogens. [89]

Under the maintenance conditions given in Table 2, zebrafish reach sexual maturity in 2.5 to 3 months and can easily reach 3 or more years of age. The growth rate critically depends on the density in the tank. Also sexual differentiation is affected by the density of fish in a tank. At lower density, fish tend to develop more frequently into females whereas higher densities foster male differentiation[90]. Fish enter a phase of senescence with characteristic changes in body shape[91].

Old fish past the reproductive stage frequently act as sinks for pathogens and should be removed following the procedures described in chapter 6. [92]

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5.2 Medaka husbandry and breeding

(Daniela Vallone, Felix Loosli)

It is possible to maintain medaka for a constant supply of eggs in standard aquarium tanks obtained through a pet shop. Under these conditions, the maximal fish density is 1-2 fish per liter. For optimal conditions a recirculating system should be used, where up to 5 medaka per liter can be kept. Exceeding a density of 5 adult medaka per liter in recirculating systems may cause stress for the fish and will negatively affect growth rate and fecundity. For optimal breeding conditions, one male should be kept with four to six females in 4 to 6 liter tanks. As medaka are social animals, it should be avoided to keep single fish in isolation of long periods (several weeks). A number of companies offer standard and custom designed recirculating systems: Müller & Pfleger, Rockenhausen/Germany; AquaSchwarz, Göttingen/Germany; Techniplast, Buguggiate/Italy; AquaticHabitats, Apopka/USA. Common to these systems is a recirculation of the water combined with the use of biological filters to ensure constant water conditions and enrichment with oxygen (Fig. 19. In addition, recirculating water can be sterilized using UV irradiation bulbs. Tank sizes for medaka can vary between 3-15 liters depending on their use as either husbandry tanks for young fish (3l), as mating tanks (6l) or to keep large numbers for stock maintenance. Standard conditions for medaka systems are: 26^{o}C, pH 6.8-7.2, ammonia and nitrite < 2ppm, nitrate < 50 ppm. Medaka tolerate a wide range of water salinity, 200-600 µS/cm in the fish tanks is acceptable. Reverse osmosis can be used to obtain good quality water to which then salt (Mineral Salt) is added to provide a salinity of 0.3-0.5%. Alternatively, deionized water can be adjusted with tap water, for example 70% deionized water and 30% tap water. This results in an improved buffering capacity of the water due to the carbonate buffer (depending on the water hardness). If necessary tap water should be charcoal filtered to remove chlorine. In addition, the system water can be enriched by the addition of trace elements (such as MikroSal, 100 mg/l). At the EZRC medaka facility, tap and deionized water are mixed to 300 µS/cm conductivity, then 8 g/1000 l mineral salt and 100 mg/l trace elements are added. This results in water with a pH 7 and 300-320 µS/cm. It is important that no sudden changes in the water conditions occur. Thus, water changes should not exceed 30% of the total water. It is better to exchange smaller volumes at shorter intervals, such as 10% per week. With stable water conditions, adequate feeding and snails to reduce algal growth and ensure removal of excess food, cleaning of the tank systems (i.e. removal of algal growth from the walls and debris from the tank bottom, cleaning of the biological filters, 20% water exchange) is required every 3-4 weeks. The basic water parameters pH, conductivity, hardness, nitrite and nitrate levels should be checked every week. In most cases, medaka can be kept in a zebrafish facility and vice versa, since the requirements to water quality, light and feeding are very similar.

Fig.16: Medaka facility at EZRC

With the parameters described in Table 2, the life span of medaka is about 1 year. Medaka can also be kept under light dark cycle conditions of 10 hours light and 14 hours dark (10L:14D), but under these conditions they will not reproduce and their life span will be prolonged to about 2 years.

Medaka fish facilities generally avoid the usage of tank enrichment such as vegetation or objects. It is recognized by the medaka research community that enrichment poses a large risk for contamination of fish facilities with pathogens (especially vegetation that cannot be sterilized). Furthermore tank enrichment will obstruct cleaning and maintenance of fish tanks and therefore may be a source of extra stress for fish as it will prolong these procedures.

Feeding

The growth rate depends critically on the fish density in the tanks and also on the food supply. Medaka should be fed several times a day with an amount of food that they will eat within 5-10 minutes. Feeding in excess will affect the water quality and result in excessive growth of snails. A combination of live (brine shrimp) and dry food

Light cycle	14 h light/10 h dark for reproductive conditions	Medaka are native to temperate zones and show a photoperiodic behavior. They require long days and short nights for mating	Medaka kept at 10 h light/14 h dark will not mate. This condition can be used for stock maintenance, since the life span is prolonged under non-reproductive conditions
Water provision/ Aquaria systems	Systems with recirculating water with biofilters and UV unit	Water exchange of 20% every 3-4 weeks is sufficient. Exchange should not exceed 30% at a time	
Water quality	26^{0}C, pH 6.8-7.2, ammonia and nitrite < 2 ppm, nitrate < 50 ppm, 200-600 μS/cm	Medaka tolerate a wide range of water salinity (euryhaline species). However sudden changes should be prevented	pH, Nitrate, Nitrite and conductivity should be checked regularly (every week). It is important to prevent sudden changes in water quality
Stock density	5 adult fish per liter can be kept under recirculating water conditions.		The actual number that can be kept per liter depend on housing and filtering conditions. The density of fish given refer to the conditions described here.
Environmental enrichment	Permanent enrichment devices (plants etc) are not recommended	There is no evidence for a beneficial effect of permanent enrichment	Tank enrichment with plants, plastic grass or similar tends to cause hygienic problems
Feeding	Mixture of dry food and freshly hatched artemia	Hatchlings should be initially fed with finely ground dry food for 1 week	Adults and young fish should be fed small quantities several times (2-4) a day. Food in excess affects the water quality (fouling)
Temperature	25-28^{0}C is the accepted standard temperature to keep medaka under reproductive conditions	Medaka is an eurythermal fish that tolerates a wide temperature range (4-40^{0}C). Low temperatures slow down development but are not harmful	Medaka are native to temperate zones and therefore in the wild are exposed to a wide temperature range (4-40^{0}C)
Single housing	Acceptable		Medaka are social animals and keeping them in groups of 4 or more fish reduces stress. However, since individual fish cannot be labeled keeping single fish may be necessary
Spawning	Medaka will reliably mate every day under optimal mating conditions (14/10 h illumination, 25-28^{0}C). Lower temperature and shorter day periods will reduce the mating rate	One male can mate with 4-6 females within 30 minutes also when separated the day prior to mating.	When males and females are kept together mating around the onset of day light

Tab.2: Medaka husbandry conditions

(for example Tetra-min flakes) is optimal. We feed flakes in the morning and evening and feed with shrimps around noon. Shrimps can also be fed to hatchlings starting two weeks after hatching. Feeding medaka with live brine shrimp larvae is considered a tank enrichment as it will stimulate natural feeding and prey capture behavior.

Medaka mating, egg collection:
One male together with 1 to 4 females are placed in a suitable tank (~6 l.) under re-circulating water conditions. This can either be done the day before or on the same day as egg collection. When fertilized eggs of a specific stage are required, such as for icroinjection at the 1-2 cell stage, it is best to place the male and female fish together during the first three hours of the light period (e.g. 8-11 am when the daily light period extends from 8 am to 10 am). Medaka will mate around the onset of the light period, or within about 15-30 minutes after having been placed together.

Females carry the fertilized eggs on their body for several hours. Therefore, the females have to be caught from the tank with a small net and a metal hook (rust-free steel) is used to carefully scrape off the eggs. It is important that the female is placed back into the water as soon as possible, latest after 3-5 minutes to prevent unnecessary stress or even suffocation. The eggs stick together due to hairs on the chorion, thus forming a clump. Move the hook under the belly of the female from head to tail to avoid hurting the gills, scoop out the clump of eggs and then transfer them into a dish with embryo raising medium (ERM) (max. 30 eggs in 6cm dish or 100 eggs in 9 cm dish).

Freshly fertilized eggs are initially opaque (milky) but clear within a few minutes. The chorion hardens concomitantly and so freshly fertilized eggs are initially very soft. Clumps of eggs have to be dissociated (to ensure equal oxygen supply) by gently rolling the clump with a finger in the dish. This will rupture the hairs and dissociate the clump. Rolling eggs on a wet filter paper is more suitable for injecting embryos as more hairs are removed by this approach. Remove unfertilized (opaque) eggs and incubate the dish in a suitable incubator (standard: 28^{o}C, 14/10 illumination). Medaka hatch after 7-8 days under these conditions. They reach fertility after 8-12 weeks depending on the strain and density/feeding regime.[939495969798]

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6. Anesthesia, analgesia and euthanasia in fish
(Thomas Braunbeck)

According to § 4(1) of the current German Animal Welfare Act (BGBl. I S. 1206, 1313), vertebrates may not be killed without effective elimination of pain perception (anesthesia; for definitions, see below). Likewise, any kind of experimental intervention may not be undertaken without appropriate anesthesia (§§ 7, 7a). In cases where appropriate anesthesia is not possible, then the pain should be reduced to a minimum (analgesia). While generally a veterinarian must apply anesthesia in warm-blooded vertebrates, reptiles and amphibians, experimental interventions in fish may be carried out by scientists with appropriate education and training[99]. The implementation of humane endpoints into anesthesia and euthanasia protocols for fish is an on-going process, as is the discussion about the perception of pain, suffering and distress (See below). The pertinent scientific literature should be consulted before the decision on the experimental design of a study.

Stress: Any handling induces stress in fish. Most frequently used fish species such as zebrafish, medaka and fathead minnow are schooling fish species, and maintenance in larger groups significantly reduces the extent of fish during normal maintenance and handling. External signs of stress include ataxia and tachypnea (hyperventilation). Ataxic fish swim randomly, in short bursts, at high speed, continuously changing directions. Tachypnoeic fish show increased movements of their opercula. Changes in coloration as well as subtle modifications of posture and use of space within the water column are also typical indicators of chronic stress.

Advanced stages of anesthesia (for definition, see below) generally involve a cessation of breathing which, in turn, reduces gas transfer leading to hypoxia and respiratory acidosis (due to the reduction of blood oxygen (O_2) tension and a concomitant rise in blood CO_2). As a result of the lack of respiration, increases in blood concentrations of adrenaline and cortisol have been demonstrated in fish treated with almost any anesthetic. In most cases, prolonged maintenance of advanced stages of anesthesia without gill irrigation will ultimately result in death (see below: euthanasia).

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[99] For general reading on anesthesia in fish, see:
Carter, K.M., Woodley, C.M., Brown, R.G. (2011) A review of tricaine methanesulfonate for anesthesia of fish. Rev. Fish Biol. Fisheries 21: 51-59.
Fish, R.E., Brown, M.J., Danneman, P.J., Karas, A.Z. (2008) Anesthesia and analgesia in laboratory animals, 2nd edition. Academic Press, New York.
Iwama, G.K., Ackerman, P.A. (1994) Anaesthetics. In: Biochemistry and molecular biology of fishes, vol. 3 (Hochachka, P. Mommsen, T.; eds.). Elsevier Publ., pp. 1-15.
Neiffer, D.L., Stamper, M.A. (2009) Fish sedation, anesthesia, analgesia, and euthanasia: considerations, methods, and types of drugs. ILAR J. 50: 343-360.
Ross, L., Ross, B. (2008) Anaesthetic and sedative techniques for aquatic animals, 3rd ed. John Wiley & Sons; 240 pp.
Sneddon, L.U. (2012) Clinical anesthesia and analgesia in fish. J. Exot. Pet Med. 21: 32-43.
Stoskopf, M., Posner, L.P. (2008) Anesthesia and restraint of laboratory fish. In: Fish, R.E., Brown, M.J., Danneman, P.J., Karas, A.Z. (eds.) Anesthesia and analgesia in laboratory animals, 2nd ed. Academic Press, New York, pp. 519-534.

Pain: The question as to whether fish have the ability to centrally process pain has been the subject of discussion for more than two decades. However, fish do express nociceptors and opioid receptors (mu, delta, kappa), and β-endorphins have been found in salmon, indicating that fish may be capable of pain perception and suffering[100].

6.1 Definitions

Anesthesia (alternatively anaesthesia; from Greek αν-, an-, "without"; and αἴσθησις, aisthēsis, "sensation"), describes a condition, under which sensation (including the perception of pain) is completely blocked or temporarily taken away (narcosis). Traditionally, anesthesia is a pharmacologically induced and reversible state of amnesia, analgesia, loss of responsiveness (relaxation), loss of skeletal muscle reflexes, decreased stress response, or all of these simultaneously (triad of anesthesia). These effects can be achieved by treatment with a single drug which alone provides the correct combination of effects. Occasionally, a combination of drugs (such as hypnotics, sedatives, paralytics and analgesics) is used to achieve very specific combinations of results (balanced anesthesia). This allows animals to undergo intervention, surgery and other procedures without the distress and pain they would otherwise experience. Thus, an alternative definition is a "reversible lack of awareness", including a total lack of awareness (e.g. a general anesthetic) or a lack of awareness of only parts of the body (e.g. spinal anesthetic). The pre-existing word anesthesia was suggested by Sir Oliver Wendell Holmes in 1846 as a word to use to describe this state.

Analgesia (from Greek αν-, an-, "without"; and ἄλγος álgos „pain") is the relief from pain, usually by application of any drug used as an analgesic ("pain killer"). In contrast to an anesthetic, analgesics suppress the perception of pain without affecting awareness, sensory perception and other primary functions of the central nervous system. Likewise, analgesics do not block action potentials of afferent nerve tracts.

Amnesia (from Greek ἀμνησία, ἀ meaning "without", μνησία "memory") is a deficit in memory caused by brain damage, disease, or psychological trauma. Amnesia can also be caused temporarily by the use of various sedatives and hypnotic drugs.

[100] For general reading on anesthesia in fish, see:
Carter, K.M., Woodley, C.M., Brown, R.G. (2011) A review of tricaine methanesulfonate for anesthesia of fish. Rev. Fish Biol. Fisheries 21: 51-59.
Fish, R.E., Brown, M.J., Danneman, P.J., Karas, A.Z. (2008) Anesthesia and analgesia in laboratory animals, 2nd edition. Academic Press, New York.
Iwama, G.K., Ackerman, P.A. (1994) Anaesthetics. In: Biochemistry and molecular biology of fishes, vol. 3 (Hochachka, P. Mommsen, T.; eds.). Elsevier Publ., pp. 1-15.
Neiffer, D.L., Stamper, M.A. (2009) Fish sedation, anesthesia, analgesia, and euthanasia: considerations, methods, and types of drugs. ILAR J. 50: 343-360.
Ross, L., Ross, B. (2008) Anaesthetic and sedative techniques for aquatic animals, 3rd ed. John Wiley & Sons; 240 pp.
Sneddon, L.U. (2012) Clinical anesthesia and analgesia in fish. J. Exot. Pet Med. 21: 32-43.
Stoskopf, M., Posner, L.P. (2008) Anesthesia and restraint of laboratory fish. In: Fish, R.E., Brown, M.J., Danneman, P.J., Karas, A.Z. (eds.) Anesthesia and analgesia in laboratory animals, 2nd ed. Academic Press, New York, pp. 519-534.

Essentially, amnesia is loss of memory. The memory can be either wholly or partially lost due to the extent of damage or intervention.

Sedation is equivalent to mild anesthesia. It is convenient for small noninvasive/non painful procedures such as handling, weighing, simple marking and imaging. Given the sensitivity of dermal layers and the high production of mucus by the epidermis under conditions of stress, even normal handling of fish may require mild sedation. If transport stress is a concern, it may be minimized through a light sedation brought about by low concentrations of an anesthetic such as MS-222. For surgery, sedation may only be used in conjunction with local anesthesia and analgesia.

Euthanasia (from Greek: εὐθανασία: “good death”; εὖ, eu: “well” or “good”; θάνατος, thanatos: “death”) generally refers to the practice of intentionally ending a life in order to relieve pain and suffering. In the context of animal experimentation, euthanasia is the act of humanely putting an animal to death or allowing it to die by withholding extreme medical measures. Reasons for euthanasia include incurable (and especially painful) conditions or diseases, lack of resources to continue supporting the animal, or planned laboratory test procedures. Euthanasia methods are designed to cause minimal pain and distress. Euthanasia is distinct from animal slaughter and pest control, which are performed for purposes other than an act of mercy, although in some cases the procedure is the same. In domesticated animals, this process is commonly referred to by euphemisms such as “put down”, “put to sleep”, or “put out of its misery”.

Anesthesia versus analgesia: Since fish are likely to experience pain (see above), any procedure which involves invasive methods is certain to cause some degree of pain, and steps should be taken to alleviate any suffering that could be caused. In most cases, chemical anesthetics also have analgesic properties; thus, application of appropriate anesthesia also covers reduction of pain perception.

6.2 General considerations regarding the selection of the anesthesia procedure

The ideal anesthesia provides fully reversible and controlled loss of consciousness without causing any mortality. It should be safe for the animal and the operator. Anesthesia should cause minimal changes in physiology; homeostasis should be maintained throughout the entire procedure. It should be combined with minimal stress, pain and suffering to the animal. The reasons for anesthesia thus arise both from ethical concerns and the intention to optimize conditions for experimental manipulation of the fish.

For the ***selection of the anesthesia procedure***, the following criteria must be considered:

(1) An anesthetic should induce anesthesia in less than 3 minutes, and recovery should occur within 5 minutes of placement of the fish in clean water.

(2) The anesthetic chosen should not have toxic side effects for either the fish or the handler.

(3) The anesthetic should be biodegradable and have properties, which allow the body to clear it from the tissues following exposure.

(4) The anesthetic should have no persisting physiological, immunological or behavioral effects, which could reduce the likelihood of survival of the fish or interfere with later measurements and reduce the well-being of the fish.

(5) Cost effectiveness and availability of the anesthetic should be considered, as should characteristics such as foaming, which could reduce gas transfer into and out of the water.

(6) Since the efficacy of most anesthetics is species-specific and is influenced by parameters such as body size, the density of fish in the bath, as well as water quality (e.g., hardness, temperature, or salinity), it is imperative that preliminary tests are performed with small numbers of fish to determine the optimal dosage and exposure time.

(7) Due care should be taken to control the level of anesthesia desired, through the use of the appropriate concentration of anesthetic, and to maintain constant observation of the fish as they go through the various stages of anesthesia.

(8) According to a large body of literature, there is no ideal general protocol for anesthesia, since all methods currently practiced have been shown to cause side effects to a certain extent. The selection of the anesthesia protocol will therefore also primarily depend on the purpose of the scientific experiment, since certain side effects may not be compatible with the purpose of the experiment. The following ***general considerations*** should be addressed independently of the specific protocol used for anesthesia:

   (1) Good planning is half the job: optimize preparation of both fish, drugs, facilities and personnel.

   (2) In order to guarantee for optimal reproducibility of experimental results, all aesthesia procedures should strictly follow standard operation procedures.

   (3) If using a new anesthesia protocol or species, anesthetize a small cohort of fish and follow them through full recovery to ensure drug doses and techniques are safe, and provide sufficient anesthetic depth for the intended procedures.

   (4) Consider the health status and physiological conditions of the fish to be anesthetized for the selection of the type and the dose of the anesthetics.

   (5) Do not disturb the mucus layer of fish or amphibians. Wear non-powdered, pre-moistened gloves when handling animals. Do not apply detergents or solvents to the skin, and limit contact with abrasive materials (e.g. dry paper towels).

   (6) Minimize stress prior to anesthesia in order to reduce the likelihood of complications during subsequent anesthesia.

(7) Prior to anesthesia, large fish such as trout should not be fed for up to 48 h, depending on the fish size. This reduces fecal contamination and the risk of regurgitation.

(8) Use water taken from the original fish holding tank for transport, anesthetic and recovery chambers. If using another water source, closely duplicate the water quality parameters (i.e., chlorine, temperature, pH and ammonia) of the original holding tank.

(9) For larger fish species, maintain adequate oxygenation of holding tanks throughout induction, anesthesia, and recovery (supply oxygen via air pump and air stone, or similar device).

(10) Maintain water temperature at the species' normal optimum during both anesthesia and recovery.

(11) Allow animal to reach an appropriate level of anesthesia for the planned procedures.

(12) While performing procedures, keep the fish's skin moist and the gills submerged or regularly flushed with well-oxygenated water.

(13) Evaluate respiratory rate and gill color throughout anesthesia:

a. observe movement of the operculum (rigid flap that covers the gills), as it opens and closes to assess respiration rate;
b. observe gill color: should be dark pink to light red;
c. if respiration becomes extremely slow or stops, place the fish in anesthetic-free recovery water until respiration resumes.

(14) Post-anesthetic care: Place the fish in well oxygenated, un-medicated water in a holding tank:

a. to support and speed up recovery, create a flow of oxygenated water over the gills by (a) moving the fish back and forth in the water, or, for larger fish species, (b) opening and closing the mouth several times;
b. maintain water temperatures at the species' normal optimum throughout recovery;
c. consider the use of antibiotics in case of invasive procedures.

(15) In most legislation, blood sampling *via* puncture of the heart is only allowed in anesthetized animals, which will be killed after the procedure.

(16) When working with animals, wear appropriate Personal Protective Equipment, observe proper hygiene, and be aware of allergy, zoonosis, and injury risks.

(17) Anesthetic safety practices:

a. All anesthetics should be handled with care, and appropriate Material Safety Data Sheets (MSDS) should be consulted to ensure the safety of users.

b. Wear protective clothing, gloves and goggles when handling anesthetics. Wear gloves to handle animals exposed to anesthetics.
c. If required/possible, work inside a fume hood to prepare a concentrated stock solution by mixing an appropriate amount of anesthetics in a small volume of water. Wear gloves and use a utensil to stir until all powder is dissolved. Dilute the stock solution further as required.
d. Dispose of biodegradable anesthetic wastes by flushing down the drain to a sanitary sewer with an excess of water. Do not discard anesthetics directly into surface water, storm water conveyances or catch basins.

6.3 Stages of anesthesia

In fish, the depth/steps of anesthesia can be categorized into several levels (numbering may change with authors; Tab. 3).

Tab. 3: Stages of anesthesia in fish

Stage	Descriptor	Behavior of fish	Experimental
0	Normal	Swimming actively; reactive to external stimuli; equilibrium normal; muscle tone normal	
I.1	Light sedation	Voluntary swimming continues; slight loss of reactivity to visual and tactile stimuli; respiratory rate normal; equilibrium normal; muscle tone normal	
I.2	Deep sedation	slowing down and cessation of voluntary swimming; uncoordinated movements: total loss of reactivity to visual and tactile stimuli; slight decrease in respiratory rate; equilibrium normal; muscle tone slightly decreased; still responds to positional changes	
II.1	Light narcosis	Excitement phase may precede in increasing respiratory rate; loss of tone decreased; still responds weakly to positional changes	
II.2	Deep narcosis Tolerance stage	Ceases to respond to positional changes; decrease in respiratory rate to approximately normal; total loss of equilibrium; no efforts to right itself; muscle tone decreased; some reactivity to strong tactile and vibrational stimuli	External sampling, fin biopsies, gill biopsies
III.1	Light anesthesia	Total loss of muscle tone; responds to deep pressure; further decrease in respiratory rate	Suitable for minor surgical procedures
III.2	Chirurgical stage Surgical anesthesia	Complete loss of pain sense and reactivity, complete muscle relaxation, slow respiration and heart rate, total loss of response to stimuli (→ firmly squeeze at the base of the tail to determine response to stimuli)	Suitable for any surgical procedure
IV	Medullary collapse	Irreversible stage, medullary collapse: overdose → finally leading to death	Euthanasia

Since these stages depend on the accumulation of the anesthetic, the different levels can be observed (a) with increasing doses of the anesthetic or (b) with increasing duration of the application. The depth of the anesthesia can easily be monitored *via* gill operculum frequencies (respiratory rate). The gills should be pink to light red over the entire procedure; pale gills are indicative of hypoxemia, hypotension and/or anemia.

The recovery can be classified into the following stages:

(1) Stage I: Body immobilized but opercular movements just starting;

(2) Stage II: regular opercular movements and gross body movements beginning;

(3) Stage III: equilibrium regained and pre-anesthetic appearance.

6.4 Forms of anesthesia

Anesthesia can be induced by various treatments:

(1) inhalation;

(2) non-chemical anesthesia methods;

(3) injection of anesthetics.

Inhalation: The most common anesthetic technique in fish is adding the anesthetic agent to the water. This is similar to inhalation anesthesia in terrestrial species, as the drug is taken up through the surrounding medium, enters the arterial blood circulation, and the remainder or metabolites are excreted *via* the gills, kidney, intestinal tract and skin. Once the fish are placed in the recovery bath, the anesthetic agent will be cleared from the blood, and its effects will cease.

Inhalation is definitely the easiest, quickest, safest and cheapest way to anesthetize

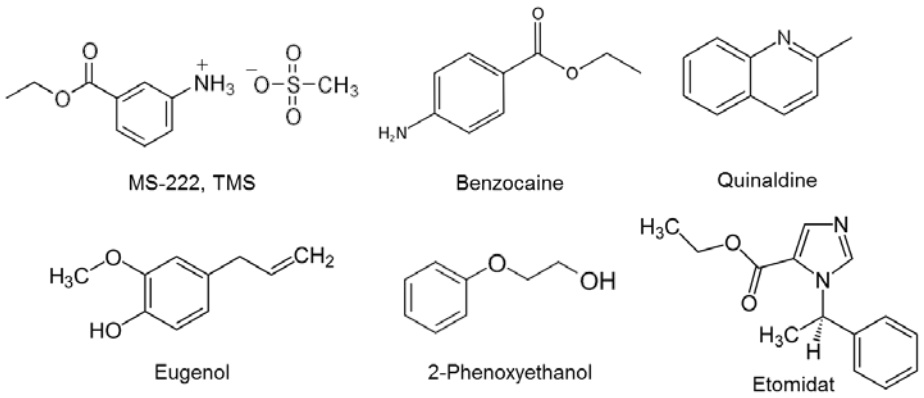

Fig.18: Chemical structure of anesthetics commonly used in fish *via* inhalation

fish. Common anesthetic agents are benzocaine, MS-222, Eugenol, 2-phenoxyethanol (bactericide) or quinaldine (**Fig. 18**). In food fish, lengthy withdrawal times are mandatory for chemical anesthesia prior to harvest, and this has led to an interest in less persistent and more natural anesthetics such as clove oil. Parameters that affect the selection of the anesthesia agent and duration of anesthesia are (a) fish species, (b) concentration (as higher as faster/longer), (c) body weight (smaller gill surface area in relation to body weight, gill permeability), and (d) water temperature (higher temperature → higher metabolism → higher oxygen demand → higher anesthetic uptake). For some species, e.g. eel (*Anguilla* spec.), inhalation anesthesia does not work well.

Benzocaine is not only used as an anesthetic in fish, but also as a local anesthetic in humans as a topical pain reliever or in cough drops. It is the active ingredient in many over-the-counter anesthetic ointments such as products for oral ulcers. It is also combined with antipyrin to form A/B Otic Drops to relieve ear pain and remove earwax. Benzocaine acts as an inhibitor of the voltage-dependent sodium channels (VDSCs) on nerve membranes (prevention of depolarization *via* blockade of sodium inflow), thus stopping the propagation of the action potential. In solution, benzocaine

is neutral (pH 7) and therefore causes less hyperactivity and initial stress reaction than buffered MS-222. However, some species of fish (e.g. eel) may retain some locomotor functions throughout lower stages of anesthesia, making benzocaine in these cases an unsuitable anesthetic for use in procedures involving surgery. Benzocaine is effective at approximately the same doses as tricaine (MS-222, 25 - 100 mg/L). Benzocaine has a fair margin of safety, although this appears to be reduced at higher temperatures. It is not safe for exposures longer than 15 minutes. Its efficacy is not affected by water hardness or pH. Benzocaine is barely soluble in water (0.4 g/L at 5 °C; 1.1 g/L at 30 °C; usually sufficient for anesthesia), but is soluble in acetone and ethanol. Benzocaine is not approved by the FDA for use in fish intended for human consumption in the U.S.

MS-222 (tricaine, metacaine, tricaine mesylate, tricaine methanesulfonate, TMS, Finquel) is a white powder commonly used for anesthesia, sedation, or euthanasia of fish. TMS is the only anesthetic licensed in the United States for fish that are intended for human consumption, but has been banned in Canada. The drug can have selective toxicity for poikilotherms due to their lower rate of metabolism in the liver. TMS is a muscle relaxant that operates by preventing action potentials (blockade of sodium and to a lesser degree potassium currents in nerve membranes). Since action potentials as well as spontaneous contraction of muscles are eliminated by MS-222, including sensory input and reflexes, no signals can be exchanged between the brain and the extremities.

MS-222 solutions should be buffered; generally, the optimum concentration used is 50 - 75 mg/L. However, the optimum may vary considerably with size and species of the fish, and other variables. MS-222 is readily soluble in water at 1250 g/L. For a review on MS-222, see [101]. Both sedation and recovery is fairly rapid. MS-222 has a good safety margin in fish. The drug is more potent in warm waters with low hardness. MS-222 is excreted in fish urine within 24 hours and tissue levels decline to near zero in the same period of time. One of the major drawbacks of MS-222 is that even when fish are deeply anesthetized, handling still increases levels of plasma cortisol, glucose and lactate concentrations as indicators of stress.

Eugenol (4-allyl-2-methoxyphenol, 4-prop-2-enyl-2-methoxyphenol, 4-allylbrenz-catechine-2-methylether, 5-allylguajacol) is the active compound of clove oil; the name is derived from the scientific name for clove, *Eugenia aromaticum* or *Eugenia caryophyllata*. Eugenol is responsible for the aroma of cloves. It is the main component in the essential oil extracted from cloves, comprising 72 – 90 % of the total. Eugenol is used in perfumes, flavorings, essential oils and in medicine and dentistry as a local antiseptic and anesthetic. Eugenol derivatives are used in formulating insect attractants and UV absorbers, analgesics, biocides and antiseptics (antibacterial, antifungal, nematocide, acaricide, insecticide, anti-termite, apifugal). For technical purposes, Eugenol and its derivatives have many uses. Especially in the U.S., clove oil is growing in popularity as an anesthetic for use in aquarium fish as well as in wild fish when sampled for research and management purposes. Readily available over-the-counter from pharmacies, it may be a humane method to

[101] Carter, K.M., Woodley, C.M., Brown, R.G. (2011) A review of tricaine methanesulfonate for anesthesia of fish. Rev. Fish Biol. Fisheries 21: 51-59.

euthanize sick and diseased fish either by direct over-dose or to induce sleep before an overdose of ethanol. Clove oil has a very high margin of safety; however, it also requires a relatively long recovery time compared to MS-222. The major advantage of clove oil is that it is inexpensive and not unpleasant to work with. Known side effects are inhibition of tumor necrosis factor, COX-1, COX-2 and cytochrome P-450; genotoxicity may arise based on activation of cytochrome P-450. However, Eugenol (or clove oil) is approved as safe for fish by the US FDA Center for Veterinary Medicine (FDA/CVM). For fish intended for human consumption, clove oil is not approved for use in the U.S., mainly because methyleugenol is a suspected carcinogen.

2-Phenoxyethanol (2-phenoxy-1-ethanol, phenyl cellosolve, phenyl glycol, monophenylglycol, ethylene glycolmonophenylether) is a bactericide commonly used as a fish anesthetic and in human dermatologic products at concentrations up to 1 %. Technical uses of phenoxyethanol range from solvents for dyes and inks, as a preservative for print media, insect repellents to fixatives in perfumes and soaps. In science, phenoxyethanol is often used in place of sodium azide in biological buffers and vaccines, because phenoxyethanol is less toxic and non-reactive with copper and lead. 2-Phenoxyethanol has a relatively large margin of safety and has been reported to produce a range of effects from light sedation to surgical anesthesia at concentrations of 100 - 600 mg/L. Concentrations of 300 - 400 mg/L are useful for short procedures, and lower concentrations of 100 to 200 mg/L are considered safe for prolonged sedation, such as during transport. 2-Phenoxyethanol is moderately soluble in water (26.7 g/l), but freely soluble in ethanol. 2-Phenoxyethanol is not approved by FDA for use on fish intended for human consumption in the US.

Quinaldine (2-methylquinoline) is a simple derivative of a heterocyclic compound quinoline. Quinaldine is used in anti-malaria drugs, in manufacturing dyes, food colorants (e.g. Quinoline Yellow WS), pharmaceuticals and pH indicators. Quinaldine sulfate is an anesthetic used in fish transportation; in some Caribbean islands, it is used to facilitate the collection of tropical fish from reefs. While it is an effective anesthetic, it is an irritant to fish, has an unpleasant odor, and is a carcinogen. Quinaldine solutions are acidic and are usually buffered with sodium bicarbonate. Induction takes 1 to 4 minutes and may cause mild muscle contractions. Recovery is usually rapid. The effective treatment concentration of quinaldine solutions varies with species, but is generally 15 - 60 mg/L. Quinaldine may not produce the deep anesthesia needed for surgery, because some reflex responsiveness is usually retained. Fish under full quinaldine anesthesia normally do not stop their gill ventilation so are not as susceptible to asphyxia from respiratory arrest as they are with MS-222. Quinaldine itself is practically insoluble in water but is soluble in acetone and ethanol; quinaldine sulfonate is readily soluble in water at 1040 g/L.
Metomidate (methyl-1-(1-phenylethyl)-1H-imidazole-5-carboxylate) has been used extensively in human medicine. It anesthetizes fish without the usual stress of an elevated heart rate. Induction is rapid (1 - 2 min), and recovery is faster than with MS-222. It anesthetizes salmonids at doses of only 2 - 6 mg/L; low doses are also effective in catfish. In salmonids, metomidate is reported to be more potent in larger, sea-water-adapted fish than in freshwater fingerlings or parr. Metomidate is not approved in the U.S. for use in food intended for human consumption; in contrast, metomidate is approved for veterinary purposes in Canada.

Apart from these commonly used anesthetics, some more exotic anesthetics such as 2-amino-4-phenylthiazole, carbon dioxide (carbonic acid; difficult to control!), sodium bicarbonate, lidocaine-sodium bicarbonate, chloral hydrate, cyanide, tobacco extracts, roteneone, etomidate, fluothane, halothane, phenthiazamine hydrobromide, propanidide and tetraethylene glycol dibutylether have successfully been used for fish sedation and narcosis. Some anesthetics are commercialized under various trade names (Table 4).

Tab. 4: Commonly used fish anesthetics commercialized under trade names

Product	Dose rate
Benzocaine	Anesthesin™, Anesthone™, Americaine™, Orthesin™, Parathesin™
Eugenol	AQUI-S™
Metomedate	Aquacal™, Marinil™, Methomidate™, Methoxynol™, Hypnomidate™, Amidate™
MS-222	Tricaine-S™, Finquel™
2-Phenoxyethanol	Phenyl cellosolve™, Phenoxethol™, Phenoxetol™, ethylene glycol monophenyl ether, and ß-hydroxyethyl phenyl ether
Propanidide	Epontol™, Sombrevin™
Quinaldine	Quinate™

There is a series of anesthetics commonly used in terrestrial vertebrates, which are not recommended for fish: these include, e.g., chloral hydrate, chlorobutanol, diethyl ether, halothane, methylpentynol, urethane.

6.5 Dosing of inhalation anesthetics

The health status and physiological conditions of the fish to be anesthetized should be considered for the selection of the type and the dose of the anesthetics.

Tab. 5: Recommendations for the dosing of inhalation anesthetics in fish; for more species-specific information, see [102] as well as [103]

Agent		Concentration	Species	Comments
Benzocaine (hydro-chloride)	Ethyl-4-aminobenzoate	25 - 200 mg/L 25 - 50 mg/L 100 mg/L 40 mg/L 55 – 85 mg/L 50 – 100 mg/L	general salmonids tilapias, catfish cod striped bass carp	Buffer solution with sodium bicarbonate to maintain neutral pH; fair margin of safety between effective and lethal doses
Carbon dioxide		200 – 1500 mg/L (1:1 CO_2 : O_2) 290 - 460 ml/min	salmonids carp	
Clove oil (Eugenol)	Aqui-S™	6 - 20 mg/L	all	very high margin of safety
	Koi Calm™	20 - 100 mg/L 25 - 120 mg/L 100 - 150 mg/L	other carp channel catfish	more soluble in hot water
	Clove oil stock solution	40 - 100 mg/L	general	
Etomidate		1 - 7 mg/L	salmonids	

[102] Iwama, G.K., Ackerman, P.A. (1994) Anaesthetics. In: Biochemistry and molecular biology of fishes, vol. 3 (Hochachka, P. Mommsen, T.; eds.). Elsevier Publ., pp. 1-15.

[103] Neiffer, D.L., Stamper, M.A. (2009) Fish sedation, anesthesia, analgesia, and euthanasia: considerations, methods, and types of drugs. ILAR J. 50: 343-360.

Agent		Concentration	Species	Comments
		2 - 7 mg/L 1.3 – 2.2 mg/L 1 mg/L	tropical species catfish striped bass	
Halothane		0.5 - 2.0 ml/L 0.4 - 0.75 ml/L	other carp	added directly to water (injection beneath surface via 25G needle) or by vaporizer
Isoflurane		0.4 - 0.75 ml/L 0.25 - 0.4 ml/L	induction maintenance	as above
Lignocaine (Xylocaine, Lidocaine)	2-(diethylamino)-N-(2,6-dimethyl phenyl) acetimide	100 – 250 mg/L 250 mg/L 350 mg/L 100 - 150 mg/L	carp, trout catfish tilapia other	do not exceed 1 – 2 mg/kg total dose
Metomidate	Methyl-1-(1-phenylethyl)-1H-imidazole-5-carbo-xylate	2 - 25 mg/L 5 - 20 mg/L 7.5 - 10 mg/L 10 – 60 mg/L 5 mg/L	general cod striped bass Atlantic halibut rainbow trout	
MS-222	Tricaine methane sulphonate (TMS) Maintenance Stock solution	25 - 50 mg/L 80 - 200 mg/L 20 - 85 mg/L 250 - 480 mg/L 150 mg/L 75 mg/L 10 mg/L 15 - 50 mg/L 1 g/L spray 1 g/L	salmonids tilapias, catfish carp Atlantic halibut striped bass cod salmonids	broad margin of safety; buffer with an equal weight of sodium bicarbonate (pH 7 – 7.5) Sedation Spray onto gills Euthanasia
Phenoxyethanol	2-Phenoxyethanol Maintenance	0.1 - 0.5 ml/L 0.4 mg/L 0.1 - 0.5 ml/L 100 - 200 mg/L	general rainbow trout carp	solution bactericidal and fungicidal
Quinaldine	Quinaldine sulfate	5 15 - 60 mg/L 15 - 40 mg/L 30 - 70 mg/L 10 - 30 mg/L 25 - 55 mg/L 15 - 70 mg/L	general salmonids catfish bluegill sunfish striped bass largemouth bass	

Tab. 6: Dose rates of major anesthetic drugs, evaluated experimentally, for a number of commonly cultured fish species (mg/L)

Anesthetic	Atlantic salmon	Rainbow trout	Common carp	Channel catfish	Nile tilapia	Striped bass
	Salmo salar	*Oncorhynchus mykiss*	*Cyprinus carpio*	*Ictalurus puntatus*	*Oreochromis niloticus*	*Morone saxatilis*
MS-222	40 - 50	40 - 60	100 - 250	50 - 250	100 - 200	100 - 150
Benzocaine	40	25 - 50	n.d.	n.d.	25 - 100	50 - 100
Quinaldine	25 - 40	n.d.	10 - 40	25 - 60	25 - 50	25 - 40
2-Phen-oxyethanol	100 - 200	100 - 200	400 - 600	n.d.	400 - 600	n.d.
Metomidate	2 - 10	5 - 6	n.d.	4 - 8	n.d.	7 - 10
Clove oil	10 - 50	40 - 120	40 - 100	100	n.d.	60

Anesthetic	Atlantic salmon	Rainbow trout	Common carp	Channel catfish	Nile tilapia	Striped bass
	Salmo salar	*Oncorhynchus mykiss*	*Cyprinus carpio*	*Ictalurus puntatus*	*Oreochromis niloticus*	*Morone saxatilis*
Aqui-S^{TM}	10 - 50	20	n.d.	20 - 60	n.d.	n.d.

n.d. – not determined

Tab. 7: Preparation of immersion anesthetic stock solutions

Product	Dose rate
Clove oil (Eugenol)	Each ml of clove oil contains approximately 1 g Eugenol. Eugenol is not completely soluble in water and should be diluted 1:10 in 95 % ethanol (mix 1 part clove oil with 9 parts 0,5% ethanol). This will create a stock solution containing 100 mg/ml.
MS-222 (Tricaine methanesulfonate)	Dissolve 1 g of MS-222 (0.5 ml scoop holds approximately 400 mg MS-222) and 1 g sodium bicarbonate ($NaHCO_3$) in 100 ml distilled water. This creates a 10 mg/ml solution. This stock solution should be labeled and dated; it can be stored in a dark container protected from light for up to 3 months.
Benzocaine	The standard approach is to prepare a stock solution in ethanol or acetone (usually 100 g/L) that will keep for more than a year when sealed in a dark bottle.

6.6 Non-chemical anesthesia

6.6.1 Hypothermia (cooling)

Among non-chemical anesthesia methods, hypothermia is a common approach to tranquilize or immobilize fish in fisheries[104]. Lower water temperatures also increase the oxygen-carrying capacity of water and reduce swimming activity and oxygen consumption of fish. Water can be cooled by refrigeration or by adding ice. Gradual cooling is recommended, because rapid chilling can produce a thermal shock. In typical cold water fish, however, hypothermia is only effective at temperatures well below zero (-2.0 to -4.5 °C), which can only be achieved by dissolving salt (NaCl) at concentrations ranging from 25 to 90 µg/L and recirculating the solutions through a thermostatically controlled chiller. The use of dry ice could result in hypercapnic (high CO_2) and acidic conditions in the water, if it is placed directly in the water. As a consequence, and since sedative effects may not be induced, if acclimation temperatures are lower than 10 °C, hypothermia can only be recommended for typical warm water species[105] .
When fish are immobilized by lowering the water temperature, the safety margin is frequently quite small, and deaths occur if the temperature is lowered too far or too quickly. Thus, the rate of cooling should be controlled carefully and the required temperature should be maintained. As a rule of thumb, the water temperature should not be reduced more than 1 °C every 15 minutes. Hypothermia only results in a slow, mild anesthesia, which is characterized by an absence of motion, reduced power of exertion and diminished nerve sensitivity[106]. For this reason, this method is often

[104] Hovda, J., Linley, T.J. (2000) The potential application of hypothermia for anesthesia in adult Pacific salmon. N. Am. J. Aquacult. 62: 67-72.
[105] Summerfelt R.C. & Smith L.S. (1990) Anesthesia, surgery and related techniques. In: Methods for Fish Biology (eds. C.B. Schreck & P.B. Moyle), pp. 213-272. Bethesda MD: American Fisheries Society.
[106] Bell G. (1987) An outline of anesthetic and anesthesia for salmonids, a guide for fish culturists in British Columbia. 16 pp. Can. Techn. Rep. Fish. Aquat. Sci. No. 1534.

used in combination with chemical anesthesia to reduce the amount of anesthetic required[107] , reduce oxygen consumption, and thereby extend the time fish can be anesthetized. As a method of anesthesia, hypothermia is not deep enough for any type of lengthy surgery[108] . It has also to be noted that hypothermia is *per se* not combined with the effects of analgesia[109].

In contrast, rapid chilling induces a lethal shock presumably due to disruption of osmoregulatory functions[110]. Hypothermia may also be the method of choice in situations where anesthetics were contraindicated as well as for larvae of certain species, which have been reported to be quite tolerant even to very high doses of chemical anesthetics (e.g. Massee et al.[111]). In these larvae, when the gills are not yet active, the uptake of chemical anesthetics is restricted to the skin and, consequently, very low. For the purpose of euthanasia, the use of cold shock as a method of immobilization, immediately followed by cranial maceration *via* an in-sink garbage disposal, is also supported by Nickum et al.[112].

6.6.2 Electro-anesthesia

Another alternative to chemical anesthesia is the use of electricity. Electrofishing is a common method for capturing juvenile and adult fish in fisheries management as well as biomonitoring[113]. Alternating current (AC), square waves in the form of chopped direct current (DC) and pulsating forms of AC and DC (rectified current) have been used in electro-fishing for many years. Direct current can cause anodotaxis (movement towards the anode pole), electronarcosis (stunning) and electrotetany (tetanic muscle contractions), whereas alternating current causes only electronarcosis and tetany. The purpose of electro-anesthesia is to induce electronarcosis, while avoiding severe muscle tetany, which can result in spinal injuries. The response of the fish to electricity depends on the intensity of the electric field and the duration of the shock. Others factors such as water conductivity, temperature, fish size and species can also affect the efficacy of electro-anesthesia. Electro-anesthesia is not effective in seawater, because seawater is more conductive than fish. In freshwater, the fish are more conductive than the water and so the easiest route for an electric current is to pass through the fish.
Fish subjected to low-voltage DC (e.g. 12 V, 30 - 150 mA) become immobile; however, this is effective only while the fish are in the electrical field. If the current stops, the fish will escape almost immediately. This procedure only immobilizes and does not produce true anesthesia. AC current produces a short-term anesthesia and

[107] Erikson, U., Hultmann, L., Steen, J.E. (2006) Live chilling of Atlantic salmon (*Salmo salar*) combined with mild carbon dioxide anesthesia. I. Establishing a method for large-scale processing of farmed fish. Aquaculture 252: 183-198.
[108] Iwama, G.K., Ackerman, P.A. (1994) Anaesthetics. In: Biochemistry and molecular biology of fishes, vol. 3 (Hochachka, P. Mommsen, T.; eds.). Elsevier Publ., pp. 1-15.
[109] Matthews, M., Varga, Z.M. (2012) Anesthesia and euthanasia in zebrafish. ILAR J. 53: 192-204
[110] Ross, L., Ross, B. (2008) Anaesthetic and sedative techniques for aquatic animals, 3rd ed. John Wiley & Sons; 240 pp.
[111] Massee, K.C., Rust, M.B., Hardy, R.W., Stickney, R.R. (1995) The effectiveness of tricaine, quinaldine sulfate and metomidate as anesthetics for larval fish. Aquaculture 134: 351-359.
[112] Nickum et al. (2004) In: American Fisheries Society, American Institute of Fishery Research Biologists, American Society of Ichthyologists and Herpetologists. 2004. Guidelines for the Use of Fishes in Research.
[113] Reynolds, J. (1996) Electrofishing. In: Fisheries Techniques, 2nd ed. (eds. B. Murphy & D. Willis), pp. 221-253. Bethesda: American Fisheries Society.

turning off the supply does not negate the effects. Larger fish are affected more rapidly than smaller ones; the length of time fish remain in the anesthetized state increases with body length. At 110 to 115 volts, anesthesia was shown to last for less than 1 minute; higher voltages (220 to 240 V) are preferable and can produce loss of reactivity to touch stimuli for up to 5 minutes. For species-specific current requirements, see[114] .
In field rainbow trout, electroshocking induces immediate elevation in plasma corticoid and lactate concentrations, with persistent increases in plasma glucose and corticoids for at least 6 hours following capture, and cardiovascular changes including rhythm changes. These responses were attributed to trauma, oxygen debt, and general adaptation syndromes; hence, the use of electro-anesthesia should be regarded as an invasive and stressful procedure. In nature, electro-anesthesia has notable impact on other taxa; it induces an up to 10 fold increase in the number of macroinvertebrates drifting after shock.

When applied in an inappropriate fashion in the laboratory, electrical stimulation may produce violent muscular responses that can disfigure or kill. When used appropriately, however, there appear to be few long-term deleterious effects on fish; however, there are acute physiological perturbations and some evidence of increased susceptibility to predation after recovery from electrofishing. Of course, the safety of the operator should be of concern when using electricity.

6.7 Injection of anesthetics

Especially in larger species of fish, injection may be preferred to incubation into a solution of an anesthetic. There are two suitable sites for injection into active/free swimming fish:

(1) I.M. = intramuscular injection (if possible, into the well-vascularized red muscle; usually limited to fairly small volumes)

(2) I.P. = intraperitoneal injection into the central body cavity (usually larger volumes possible than with I.M. injection).

Since the extent of red muscle tissues strongly depends on the life-style of the fish, there is a considerable species-dependence. As a rule, I.M. injection is restricted to large, permanent swimmers such as salmon or tuna. In the field, large free-swimming fish (e.g. tuna) may only be anesthetized *via* injection of compressed air darts fired with a harpoon after netting; in captivity, such big individuals may be sedated with the aid of syringes equipped with needles for the automatically discharging jabstick.

For intraperitoneal (I.P.) injection, common anesthetics are

(1) Ketamine HCl (Detomidine; N-methyl-D-asparticacid (NMDA) receptor antagonist)

(2) Xylazine HCl (α-2 adrenergic receptor agonist)

[114] Iwama, G.K., Ackerman, P.A. (1994) Anaesthetics. In: Biochemistry and molecular biology of fishes, vol. 3 (Hochachka, P. Mommsen, T.; eds.). Elsevier Publ., pp. 1-15.

(3) Detomidine HCl (α-2 adrenergic receptor agonist)

(4) Medetomidine HCl (α-2 adrenergic receptor agonist)

(5) Propofol (effect on the γ-aminobutyricacid (GABA) receptors)

The normal procedure is (1) to weigh the fish, (2) to calculate the desired anesthetic concentration based on the weight, (3) to inject colored anesthetic I.P., (4) to wait until loss of equilibrium, (5) to make planned interventions (e.g. blood sampling, ECG, impedance etc.), and (6) to wait for recovery. Table 6 gives recommendations as to the dose of anesthetic agents used *via* injection.

Tab. 8: Recommendations for the dosing of some injection anesthetics in fish

Agent		Dose rate	Species
Ketamine hydrochloride		66 – 88 mg/kg I.M. 130 mg/kg I.M. 30 mg/kg I.M. 30 mg/kg I.M.	general rainbow trout other salmonids cichlids
Ketamine combined with Medetomidine reversed with Atipamezole		1 – 2 mg/kg I.M. 0.05 – 0.1 mg/kg I.M. 0.2 mg/kg I.M.	
Saffan	Alphaxolone Alphadolone	12 mg/kg I.M.	

Tab. 9: Recommendations for the dosing of some analgesics in fish

Agent	Dose rate	Species	Comments
Buprenorphine	0.01 - 0.1 mg/kg I.M.	rainbow trout	reduced activity
Butorphanol	0.05 - 0.1 mg/kg I.M. 0.4 mg/kg I.M.	general koi	
Carprofen	1 - 5 mg/kg I.M.	rainbow trout	depressed activity
Ketoprofen	2 mg/kg I.M.		anti-inflammatory effect
Lidocaine	0.1 - 2 mg/kg I.M.	rainbow trout zebrafish	

Tab. 10: Recommendations for the dosing of some emergency drugs in fish

Agent		Dose rate	Species	Comments
Doxapram		5 mg/kg I.V., I.P.		Respiratory depression
Epinephrine		0.2 – 0.5 ml I.M., I.C., I.V., I.P.		Cardiac arrest

I.M. – intramuscular; I.C. – intracardiac; I.V. – intravenous; I.P. – intraperitoneal (intracoelomic)

6.8 Prolonged anesthesia and monitoring of anesthesia

In case of more complicated interventions or multiple samplings, measures have not only to be taken to prevent hypoxia, but also to prevent premature recovery of the fish. In such cases, artificial ventilation has to be secured during the intervention. In larger fish, anesthesia can be monitored *via* electrocardiogram or measurement of impedance. Following recovery, various stress indicators may report the stress imposed by the anesthesia procedure; e.g. catecholamines (adrenaline,

noradrenaline) can be measured via HPLC, or steroid hormone (cortisol) concentrations can be determined *via* ELISA. As already mentioned above, virtually all methods under current use cause side effects to a certain extent (Tab. 11); for species-specific information, see [115].

Tab. 11: Selection of well-documented side effects of common fish anesthetics[116]

Anesthetic	Dose (mg/L)	Side effects	
		Initial	Secondary
MS-222	50 - 400	Tachycardia, increased respiration, hyperglycemia	Decreased cardiovascular responses; hypoglycemia; increased lactate, hematocrit and catecholamine levels; erythrocyte swelling
Benzocaine	25 - 150	Tachycardia, increased respiration, hyperglycemia	Decreased cardiovascular responses, hypoglycemia; increased lactate, hematocrit, and catecholamine levels; erythrocyte swelling; suppressed immune function
Clove Oil	4 - 150		Decreased ventilation and cardiovascular responses
Eugenol	20 - 200		Increased catecholamines and hematocrit
Metomidate	0.06 - 10		Reduced adrenal steroid production leading to reduced cortisol; reduced respiration, circulation, and pH of blood; hypoxemia
2-Phenoxyethanol	0.25 - 600		Decreased ventilation rate, heart rate, blood pressure, and blood pH; increased adrenal hormones; hyperglycemia; reduced immune function
Quinaldine	10 - 50	Tachycardia	Decreased heart rate and respiratory function
Quinaldine sulphate	5 - 100		Increased cortisol and serum immunoglobulin M; hyperglycemia

Doses are not appropriate for all species or under all conditions (e.g., temperature, body size, and physiological state must be investigated before use). When working with unfamiliar species or agents, use the lowest doses and low numbers of fish to test anesthetic efficacy.

6.9 Euthanasia

Since the purpose of euthanasia is the act of humanely putting an animal to death or allowing it to die, in the context of animal experimentation, euthanasia should be carried out proficiently and as early and as quickly as possible using appropriate techniques on relevant species of laboratory animals. Given that due to experimental conditions the well-being of experimental fish may deteriorate at any time of an experiment, personnel trained in euthanasia should permanently be available.

In any case, care should be taken that any damage, pain and suffering of the fish is restricted to the absolute minimum possible. General rules for euthanasia[117] are identical to those listed for anesthesia. Likewise, the methods for euthanasia are basically the same as for anesthesia: inhalation (incubation) or injection (usually of an

[115] Neiffer, D.L., Stamper, M.A. (2009) Fish sedation, anesthesia, analgesia, and euthanasia: considerations, methods, and types of drugs. ILAR J. 50: 343-360.
[116] Sneddon, L.U. (2012) Clinical anesthesia and analgesia in fish. J. Exot. Pet Med. 21: 32-43.
[117] AVMA − American Veterinary Medical Association (2013) Guidelines on Euthanasia of animals: 2013 edition. Available online under http://www.avma.org/issues/animal_welfare/euthanasia.pdf, as accessed January 1, 2014.

overdose of the anesthetic), prolonged exposure to hypothermia or electric current. Overdosing of immobilization drugs is an acceptable means of euthanasia[118]. The most frequent choice is the use of immersion drugs (and particularly MS-222) at five to ten times the anesthetic concentration for a particular species, although injectable agents are also effective. Maintaining the fish in the anesthetic solution for 5 to 10 minutes after cessation of opercular movement (except in some ram ventilating species) usually, but not always, ensures that a fish has expired. Alternatively, cranial concussion, spinal transection, or exsanguination, are effective methods in a deeply anesthetized fish. Only recently, however, based on the observation of avoidance reactions in zebrafish exposed to MS-222, concern was raised about the suitability of MS-22 as an agent for both sedation, anesthesia and euthanasia[119] [120] [121].

Particular care should be taken to guarantee appropriate sedation and analgesia prior to euthanasia. The choice of the appropriate protocol primarily depends on the stage and species of fish; again, selection criteria are basically identical to those for the choice of anesthesia methods. Subsequent to euthanasia, the remains should be disposed of following current regulations. As a general rule, the fish should be cremated in appropriate facilities. In the case of genetically modified fish, additional regulations have to be considered.

6.10 Anesthesia and euthanasia in zebrafish

According to the recent review on anesthesia and euthanasia in zebrafish by Matthews & Varga[122], MS-222 is the most widely used sedative and anesthetic for zebrafish. In zebrafish, the MS-222 dose response depends on age, size, and metabolic state. An increase in sensitivity to MS-222 [123] was observed in 3- to 9-day larvae, presumably due to changes in liver detoxification activity. Median lethal concentrations for 4- to 7-day zebrafish larvae suggested that key developmental changes, consistent with the maturation of gills[124] and liver[125],[126] occur at this time.

[118] AVMA − American Veterinary Medical Association (2013) Guidelines on Euthanasia of animals: 2013 edition. Available online under http://www.avma.org/issues/animal_welfare/euthanasia.pdf, as accessed January 1, 2014.
[119] Nordgreen, J., Tahamtani, F.M., Janczak, A.M., Horsberg, T.E. (2014) Behavioral effects of the commonly used fish anesthetic tricaine methanesulfonate (MS-222) on zebrafish (Danio rerio) and its relevance for the acetic acid pain test. PLoS ONE 9(3): e92116. doi:10.1371/journal.pone.0092116
[120] Readman, G.D., Owen, S.F., Murrell, J.C., Knowles, T.G. (2013) do fish perceive anesthetics as aversive? PLoS ONE 8(9): e73773. doi:10.1371/journal.pone.0073773
[121] Wong, D., von Keyserlingk, M.A:G., Richards, J.G., Weary, D.M. (2014) Conditioned place avoidance in zebrafish (Danio rerio) to three chemcials used for euthanasia and anesthesia. PLoS ONE 9(2): e88030. doi:10.1371/journal.pone.0088030
[122] Matthews, M., Varga, Z.M. (2012) Anesthesia and euthanasia in zebrafish. ILAR J. 53: 192-204
[123] Rombough, P.J. (2007) Ontogenetic changes in the toxicity and efficacy of the anaesthetic MS222 (tricaine methanesulfonate) in zebrafish (Danio rerio) larvae. Comp. Biochem. Phys. 148 A: 463-469.
[124] Shadrin, A.M., Ozernyuk, N.D. (2002) Development of the gill system in early ontogenesis of the zebrafish and nine-spine stickleback. Ontogenez. 33: 118-123.
[125] Field, H.A., Ober, E.A., Roeser, T., Stainier, D.Y. (2003) Formation of the digestive system in zebrafish. I. Liver morphogenesis. Dev. Biol. 253: 279-290.
[126] Sakaguchi, T.F., Sadler, K.C., Crosnier, C., Stainier, D.Y. (2008) Endothelial signals modulate hepatocyte apicobasal polarization in zebrafish. Curr Biol. 18: 1565-1571.

MS-222 has been widely used for zebrafish sedation (0.01 mg/ml; [127]), anesthesia (0.168 mg/ml [128]) and euthanasia (0.2 - 0.3 mg/ml[129]).

In direct comparison with MS-222, Eugenol appears to combine several advantages for zebrafish, such as availability, low cost, and lower dosages needed to induce anesthesia. Specific stages or full anesthesia are induced less rapidly at comparably lower doses of Eugenol, but recovery takes longer than with MS-222[130]. A 2 – 5 mg/L dosage concentration is recommended to sedate adult zebrafish, and 60 - 100 mg/L is used for immersion anesthesia[131] . In addition to a wider safety margin, Eugenol also appears to mitigate the effects of crowding and handling stress in fathead minnows (*Pimephales promelas*) based on plasma cortisol level analysis and neutrophil function, whereas MS-222 did not appear to prevent this stress response[132,133].
Although there is evidence for the applicability of cooling (hypothermia) as an anesthetic in various species, Matthews & Varga[134] do not recommend hypothermia as a method for anesthesia. According to their argumentation, cooling is better applied as an initial method for immobilization, for which it is more effective than, e.g., an overdose of MS-222. In contrast, for euthanasia, they advocate the use of thermal shock in combination with immediate cranial maceration *via* an in-sink garbage disposal, which causes immediate destruction of all brain function and activity for adult zebrafish. However, hypothermal shock in adult zebrafish is said to be less stressful, produces death more rapidly as determined by the cessation of vital signs (opercular movement, righting equilibrium, and heartbeat), is more consistent, is easier to perform, and is safer for personnel than the use of MS-222 [135] . Histopathological analysis of adult zebrafish tissue showed no evidence of ice crystal formation[136].

For 5- to 6-day-old (early stage) larvae and 1- to 2-day-old embryos, the use of MS-222, even at very high concentrations (up to 1000 mg/L) is not an effective euthanasia method since they use cutaneous gas exchange for their oxygen demands. Instead, hypothermal shock is an effective method of euthanasia for these early-stage larvae as long as the larvae are exposed for at least 20 minutes. However, for 1- to 2-day-old embryos, even rapid chilling is not an effective

[127] Trevarrow, B. (2007) Section 7.59. In: The zebrafish book: A guide for the laboratory use of zebrafish (*Danio rerio*; Westerfield, M., ed.). Eugene, University of Oregon Press.
[128] Westerfield, M. (2007) The zebrafish book: A guide for the laboratory use of zebrafish (Danio rerio). Eugene, University of Oregon Press.
[129] Wilson, J.M., Bunte, R.M., Carty, A.J. (2009) Evaluation of rapid cooling and tricaine methanesulfonate (MS222) as methods of euthanasia in zebrafish (*Danio rerio*). JAALAS 48: 785-789.
[130] Grush, J., Noakes, D. (2004) The efficacy of clove oil as an anesthetic for the zebrafish, *Danio rerio* (Hamilton). Zebrafish 1: 46-53.
[131] Grush, J., Noakes, D. (2004) The efficacy of clove oil as an anesthetic for the zebrafish, *Danio rerio* (Hamilton). Zebrafish 1: 46-53.
[132] Palic, D., Herolt, D., Andreasen, C., Menzel, B. (2006) Anesthetic efficacy of tricaine methanesulfonate, metomidate and Eugenol: Effects on plasma cortisol concentration and neutrophil function in fathead minnows (*Pimephales promelas* Rafinesque, 1820). Aquaculture 254: 675-685.
[133] Welker, T., Lim, C., Yildirim-Aksoy, M. (2007) Effect of buffered and un-buffered tricaine methanesulfonate (MS-222) at different concentrations on the stress responses of channel catfish, *Ictalurus punctatus*. J Appl. Aquacult.
[134] Matthews, M., Varga, Z.M. (2012) Anesthesia and euthanasia in zebrafish. ILAR J. 53: 192-204.
[135] Wilson, J.M., Bunte, R.M., Carty, A.J. (2009) Evaluation of rapid cooling and tricaine methanesulfonate (MS222) as methods of euthanasia in zebrafish (*Danio rerio*). JAALAS 48: 785-789.
[136] Blessing, J.J., J.C. Marshall, and S.R. Balcombe, *Humane killing of fishes for scientific research: a comparison of two methods.* Journal of fish biology, 2010. **76**(10): p. 2571-7.

euthanasia method, taking at least 5 to 14 hours of exposure to ensure death[137]. Since 1- to 2-day-old zebrafish embryos are not rapidly and effectively euthanized by hypothermal shock, chemicals such as isopropyl alcohol have to be added after rapid immersion in ice water to hasten their death.

7. Health Monitoring and Diseases in Zebrafish (*Danio rerio*) and Medaka (*Oryzias latipes*)

(Uwe Strähle, Steffen Scholpp, Veterinarian Sandra Lechleiter)

7.1 Fish diseases[138] [139] [140]

7.1.1 Recognition of typical disease symptoms and pathogens

Behavioral changes: Sick fish are frequently noticed by changes in their behavior: They suddenly separate from the school, rest at the edge of the aquarium, at the water inlet, or at the bottom, and stop feeding. They may also scratch themselves against the bottom or objects and jump frequently.

Skin changes: Some diseases are associated with skin clouding. The normally clear mucosa becomes milky-translucent or reddens, and fine capillaries are suddenly visible. Skin inflammation may also take the form of abscesses, which may reach so deep that the red musculature below becomes visible. Alternatively, the scales may be raised and reddened. Large parasites such as carp lice and anchor worms as well as accretions like skin tumors can be recognized macroscopically.

Changes in breathing frequency: Under normal conditions a fish breathes steadily and regularly, averaging about 80 beats of the gill cover per minute. Accelerated breathing is often associated with fish positioning themselves close to the water inlet since the water there is often richest in oxygen. In certain diseases of the gill cover a single-sided clamping of the gills may be visible. This looks as if the fish is breathing with one gill only.

Changes of the eye: Sunken eyes are a sign of moribund fish that have used up all their reserves. Exophthalmus, in which the eyes protrude farther outside of the head than normal, may be a sign of high blood pressure or severe kidney diseases.

Changes in body shape: These may appear as a bending of the body axis or as the sudden appearance of lumps that affect one side of the normally symmetrical fish. Wasting is characterized by the fish becoming very slim posterior to the head and by reduction of the dorsal muscles.

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[137] Matthews, M., Varga, Z.M. (2012) Anesthesia and euthanasia in zebrafish. ILAR J. 53: 192-204.

[138] http://zebrafish.org/zirc/health/diseaseManual.php
BSAVA Manual of Ornamental Fish, Editor William H. Wildgoose -second edition- British Small Animal Veterinary Association, 2001, ISBN 0-905214-57-9
Fish Disease - Diagnosis and Treatment, Editor E. Noga -Second edition- Blackwell Publishing, 2010, ISBN 978-0-8138-0697-6

[139] Princeton University Environmental Health and Safety
(https://ehs.princeton.edu/laboratory-research/animal-research-health-and-safety/zoonotic-disease-information/zoonoses-associated-zebrafish)

[140] Oregon State University, College of Veterinary Medicine (http://vetmed.oregonstate.edu/zebrafish-health)

Changes of the fins: Fin rays may appear red and inflamed and fins can suddenly tear or become frayed. In the tail fin in particular, necrotic borders indicate that the fish body is receiving insufficient oxygen. White or coloured plaques may occur, as well as lesions that involve broken fin rays. The base of the fin, where it joins the body, can be affected by inflammation. In this case the fish will then clamp and rest the affected fin. Parasite infestations and discomfort due to low temperatures or infectious disease frequently cause the fish to rest at the base of the aquarium and clamp all their fins.

7.1.2 Description of the most relevant diseases

Protozoa on skin and gills, causing skin clouding

Costia, Chilodonella, Trichodina: The most common symptoms are skin cloudiness, itching that causes the fish to scratch on objects, accelerated breathing and reddening as well as occasional black spots on the skin. By microscopic examination of the skin and gills, the pathogen can be identified and then subjected to targeted treatment. The occurrence of skin clouding is typically associated with sub-optimal temperatures or poor husbandry conditions / hygiene.

***Ichthyophthirius multifiliis*, causing white spot disease (ich):** With the unaided eye white spots can most easily be seen on the fin. In most cases this observation indicates white spot disease, caused by the ciliate *Ichthyophthirius multifiliis*. It causes strong itching, breathing difficulties and fin clamping, all of which are general symptoms of skin and gill inflammation. Microscopic examination of a skin smear can help to secure the diagnosis. The ciliate is very large (up to 1 mm) and moves with slow rotational motions through the epithelium of the skin and gills. The largest stages display a pathognomonic, horseshoe-shaped nucleus. Their development involves a cyst stage with intracellular nuclear division and also swarmers released by the cyst, which swim actively through open water and infect the fish. There they bore into the mucosa and grow to their final size. *Ichthyophthirius* is considered a pathogen in the strict sense and must be treated for at least 5 days due to its developmental cycle. Treatment by bathing can reach only the swarmer stage. UV light also kills this stage very effectively in open water and is therefore useful as prophylaxis to prevent infection in a recirculating system.

Velvet disease: *Piscinoodinium pillulare* is a dinoflagellate that multiplies rapidly and is considered a pathogen for many fish species. The skin and gills are infected by dinospores which adhere and feed on the epithelium. In contrast to “ich”, the pathogen lives on and not in the mucosa. However as with ich, there is a reproductive stage outside the fish and an actively moving, infectious stage in the water. The whole life cycle takes two weeks at typical aquarium temperatures.
The symptoms are velvet-like changes of the skin, lethargy, rapid breathing and rapid death.

The diagnosis is by microscopy: Oodinium is an oval unicellular organism that does not move and appears mostly opaque in the light microscope. The infection of the gills is associated with a conspicuous epithelial hyperplasia, while the skin mucosa can appear eroded and bloody. Since Oodinium is a dinoflagellate, only medications that contain copper provide an effective treatment.

Protozoa in the inner organs

Microsporidia: Microsporidia are sporozoa that live intracellularly in the inner organs and undergo a very complex life cycle. Infected fish may show wasting or bending of the spine, but these symptoms are also encountered in other diseases. Studies have shown that zebrafish frequently contain *Pseudoloma neurophila*, often without showing any symptoms.

Microsporidia have a spore stage that can survive outside the body and may be infectious for other fish by being taken up into the gut with the food. Horizontal transmission through the egg is under investigation. The diagnosis can be made in native squash preparations of brain, musculature, ovary and kidney or by histology with HE stain or Gram stain (e.g. Accustain® from Sigma-Aldrich). The most elegant method is PCR which is used at the Zebrafish International Resource Center (ZIRC) and also at KIT. The life cycle includes a spore stage with oval spores that display a clearly visible vacuole at the narrow pole, with a size of approx. 5.4 x 2.7 µm. Moreover xenomes with up to 16 spores are found in the CNS. Transmission can be prevented by UV light; therefore it provides the best prophylaxis. Moreover all common measures for preventing transmission from one stock to the next apply: Sick animals that appear wasted or bent should be killed in accordance with animal protection regulations. Females used for spawning should be tested by PCR 4x per year at statistically relevant sample sizes (see 7.5.) The transmission from wild catches to lab animals can likewise be prevented by proper hygiene management and good professional practice. For egg production, wild animals must not be placed in the same system as lab fish. A low-stress environment generally helps to prevent outbreak of disease.

Metazoa

Myxosporidia: For a long time myxosporidia were regarded as unicellular organisms. However, today they are placed in the kingdom Metazoa. Many live in organ cavities like the gall bladder and the bile ducts or in the kidney, while others live within the tissue. In both cases they cause little tissue damage except when cysts appear in large numbers in vital organs such as the CNS, the gills or the heart.
The life cycle is very complicated and involves several vegetative stages which lead up to the production of multicellular myxospores. Their morphology is used for diagnosis. Additional stages live outside the host and in most cases there is an intermediate host however there are a few myxosporidia capable of direct development. In most cases the infection is asymptomatic. In histological examinations at ZIRC, xenomes of Myxidium or Zschokkella were identified in zebrafish. A Giemsa stain can render the pole capsules and spores more easily visible. On Testsimplets®, prestained slides normally used for the examination of blood, the spores are also very easily seen at 1000-fold magnification.
The infection may be introduced with natural food (oligochaetes) but is not considered worthy of treatment due to its low pathogenicity.

Nematodes: Nematodes, particularly of the genus *Capillaria*, are relatively frequent parasites of lab fish. In zebrafish, *Pseudocapillaria tomentosa* has been identified. These can be transmitted and introduced through an intermediate host like *Tubifex tubifex*, but a direct infection by uptake of eggs is also possible. The adult worms can also live in the body cavity of zebrafish. A mass infestation of the gut can cause

chronic wasting and also an increased incidence of neoplasias has been reported in zebrafish cultured in the presence of *Capillaria*.

The diagnosis can be made by detection of the typical eggs (lemon shaped with two polar capsules) in fecal samples or, in the course of dissection, in squash preparations of the gut or its contents. The worms are not visible with the unaided eye.
Treatment is possible with various antihelmintics. Since the infection can escalate in recirculating systems and a large number of eggs may be present in the system, several courses of treatment should be made and additional hygienic measures should be taken such as cleaning of filters and frequent rem
oval of feces by aspiration.

Bacterial pathogens:

***Flavobacterium columnare*:** Columnaris bacteria are among the most dangerous bacterial pathogens, particularly in recirculating systems. Almost behaving like hospital-acquired pathogens, they quickly develop drug resistance and can be highly pathogenic, in particular when rearing juvenile fish. Feed residues and any other kind of proteins are their basic food source, therefore good hygiene and appropriate feeding are important prophylactic measures. Water quality is also important, in particular high ammonia levels should be avoided. This is a particular risk of infection during transport and for this reason animals should not be packed too densely.
Columnaris bacteria can cause gill inflammation and hyperplasia, resulting in rapid breathing. In most cases infected fish rest near the surface. Lesions and atrophy of the fins or cotton-like white plaques can also occur. The bacteria are visible in native preparations as gliding rods, which form conglomerates within a few minutes. They are easily seen in the internal organs at 400-fold magnification, in particular in squash preparations of the spleen. Treatment is usually carried out with Chloramine-T for several days.

Fish tuberculosis: Fish tuberculosis is caused by atypical mycobacteria. These bacteria are common in water and soil worldwide and are present in a dormant state in nearly all wild catches, often without causing disease symptoms. The bacteria are encapsulated in granulomas and can be induced to reproduce by any form of stress, causing an acute disease that often affects only individual animals. Among the most important species are *Mycobacterium marinum* and *M. fortuitum*. Fish tuberculosis is the only zoonosis associated with breeding zebrafish or medaka. In humans it causes chronic skin inflammation in the area of the hands or the arms, where the animal caretaker often has small lesions that form an entry portal for the acid-resistant bacteria. Symptoms in the diseased fish include raised scales, bending of the spine, skin abscesses or chronic wasting, depending on which organ system is affected. When examining fish, the spleen, the head kidney and the gonads should be studied carefully or preserved for histology. A tentative diagnosis can be made through the detection of characteristic granulomas in which onion-like layers of mesenchyme surround a central, yellowish necrosis. The pathogen is detected by culture performed in specialized labs, as acid-resistant rods visible with Ziehl-Neelson staining or by PCR.

7.1.3 Treatment

Treatment should only be undertaken after a precise diagnosis of the disease. As an alternative, the culling of stocks may have to be considered, e.g. to prevent contamination of entire facilities, or if therapy does not appear to be promising. Since most of the medications are only available on prescription, a veterinarian should be consulted, who must decide on dosage, method and duration of the treatment.

Available methods for maintaining appropriate health status

a. General treatment procedures

Pathogen	**Treatment**
Ichthyophthirius multifiliis (white spot disease)	Parasite baths with malachite green oxalate and/or formaldehyde
Trichodina	Parasite baths with malachite green oxalate and/or formaldehyde
Costia	Parasite baths with malachite green oxalate and/or formaldehyde
Oodinium	Copper-containing preparations from pet shops
Flagellates in the gut	Bath or feed containing metronidazole
Coccidia Microsporidia Myxosporidia	Toltrazuril (Baycox® from Bayer), Fumagillin
Gyrodactylus Cestodes Nematodes	Parasite baths with antihelmintics or feed enriched with antiparasitcs
Carp louse (Argulus) Anchor worm (Lernea)	Remove mechanically (forceps) Local wound disinfection Parasite bath with diflubenzuron
Bacteria	Take swab sample! Bacteriological examination and antibiogram! Application after detailed analysis Stock problem/individual animal problem Feed Bath Antibacterial baths for reduction of germ load: Salt baths Peroxides Chloramine-T Acriflavine

b. Table salt as a therapeutic (iodine and fluorine free!)

Effect	Treatment schedule	Hazards
Bactericidal Fungicidal Amoebicidal Protocidal Disinfectant Increases appetite Reduces stress Improves mucus production Improves gill function Reduces nitrite toxicity Algicidal Improves osmoregulation Improves energy balance	Short-term baths: 10-20 g/Liter water for 10 to 20 minutes on three consecutive days Long-term baths/tank treatment: 1 to 3 kg/1000 Liter as a permanent bath, adjust dosage after water changes	Do not use together with zeolite or similar materials! Harmful for plants! Use sufficiently large containers with good aeration, cover if necessary

Enivronmental and housing conditions for laboratory animals

a. Available procedures for maintaining appropriate water conditions

Cause	**Measure**
First aid	Stop feeding Water analysis Sample water for germ count in a lab Clean filter/tubing Partial water change Check UV function
Water quality	Intensify partial water changes If necessary replace UV lamps Check pump performance, increase if necessary Review filter dimensions, connect additional filters Improve oxygenation
Ammonia (over 0.1 mg/l)	Immediate partial water change, identify cause, clean filter, stop feeding for 3-7 days, if necessary introduce living bacteria or enzymes for ammonia degradation or fresh zeolite
Nitrite (over 0.6 mg/l)	Immediate partial water change, if necessary increase salt (observe instructions!)
pH crash (below 5)	If necessary increase water hardness for better buffering If necessary service pH control, recalibrate pH probes
pH too high (over 9)	Prevent algae in time Shade Optimize aeration (so as to drive out less CO2) Buffer with chalk
Bacterial germ load	Partial water change Facility hygiene Increase salt Apply preparations that reduce germ load
Bacteria	Bacteriological examination and antibiogram Water treatments that reduce germ load: Salt Chloramine-T Peroxide Acriflavine Virkon S Actomar B100 If necessary antibiotic injections or feed
Parasites	Depending on diagnosis: parasite bath
Amoebae	Increase salt to 0.5% (observe instructions!)

Copper	Check inflow from water pipes and cisterns Partial water change with suitable water
Metals	Initiate water analysis for heavy metals Check metals used in the facility, V2A steel and higher are suitable Change usage of different metals in the same part (due to danger of electrolysis)
Ozone and its reaction products in water	Switch off systems and service if necessary If ozone was the cause the fish will look better within a few hours

b. Disease prophylaxis

Disease prophylaxis comprises all measures that prevent an excessive build-up of pathogens in the system, or that prevent new pathogens from being introduced from outside and distributed in the facility.

To this end it is necessary to engage in good practice when caring for filters, water and tanks. In particular the following basic rules should be observed:

UV burners must be regularly checked and if necessary descaled. The predetermined service lifetime (usually 6 months to 1 year) should not be exceeded.

Depending on fish density a water change of 5-10% per day with suitable tap water should be aimed for.

Routine times for filter servicing and tank cleaning should be established and documented.

Dead fish must be removed from the tanks as soon as possible.

Take care of healthy tanks first, sick fish last.

Do not use natural food/live food.

Always remove residual food as soon as possible.

Always feed before handling tanks, or after thoroughly cleaning and disinfecting hands.

Wash nets, beakers and other equipment for catching fish thoroughly after every use and dry them (e.g. on a radiator).

The bleaching of eggs is a central and extremely important requirement for maintaining fish lines. In particular when outcrosses are necessary and new parents are introduced, the bleached eggs should be transferred to fresh tanks and should not be brought into contact with their parents anymore.

Always place new fish in quarantine and keep them separate from the other animals. Plan a quarantine of at least 3 weeks.

Strictly separate all fish rooms and water circuits with respect to equipment and care. When moving from one circuit to another, washing hands is an important prophylactic measure.

7.1.5 Sample numbers for health monitoring [141]

In order to sample a fish stock of 10 to 100 animals at a certainty level of 95%, at least 10 animals per tank unit should be examined. A tank unit consists of all tanks that share a water circuit for filtration. Higher sample numbers can greatly increase the significance. In some cases, depending on how the test methodology is validated, pools of samples may be examined.

[141] Amtliche Methodensammlung: Stand Mai 2013 / Hrsg.: Friedrich-Loeffler-Institut, Bundesforschungsinstitut für Tiergesundheit; Redakt.: T. Homeier-Bachmann, H. Kubitza. - Wusterhausen : Friedrich-Loeffler-Institut, Bundesforschungsinstitut für Tiergesundheit, 2013. - 514 S.

7.2 List of potential human health hazards associated with contact with zebrafish and Medaka.

Zoonosis describes disease of animals transmissible to humans. The transmission of diseases from fish to humans is low. There are, however, a number of substances that are found in aquarium water that have the potential to be transmitted to humans. In general, humans contract fish-transmitted disease through ingestion of infected fish tissues or aquarium water, or by contamination of injured skin. An important feature of many bacterial and protozoal organisms is their opportunistic nature. The development of disease in the human host often requires a preexisting state of a compromised immune system.

The following is a list of known and potential fish-transmitted zoonoses.

Mycobacterium
Organisms in the genus Mycobacterium are nonmotile, acid-fast rods. Two species, and *Mycobacterium marinum* and *M. fortuitum*, are recognized as pathogens of tropical fish (see chapter above). Humans are typically infected by contamination of injured skin with aquarium water or fish contact. A localized granulomatous nodule may form at the site of infection, most commonly on hands or fingers. The granulomas usually appear approximately several weeks after exposure to the organism. They initially appear as reddish bumps (papules) that slowly enlarge into purplish nodules. The infection can spread to nearby lymph nodes. More disseminated forms of the disease are likely in immunocompromised individuals. It is possible for these species of mycobacterium to cause some degree of positive reaction to the tuberculin skin test.

Aeromonas spp. are facultative anaerobic, gram-negative rods commonly isolated in tropical fresh water and is considered part of the normal intestinal microflora of healthy fish. Humans infected with *Aeromonas* may show a variety of clinical signs, but the two most common syndromes are gastroenteritis and localized wound infections. Again, infections are more common and serious in the immunocompromised individual.

Other Bacteria and Protozoa: Below is a list of additional zoonotic organisms that have been documented in fish or aquarium water. Human infections are typically acquired through ingestion of contaminated water resulting in gastroenteritis symptoms or from wound contamination.

Gram-negative Organisms: *Plesiomonas shigelloides, Pseudomonas fluorescens, Escherichia coli, Salmonella spp., Klebsiella spp., Edwardsiella tarda*
Gram-positive Organisms*: Streptococcus, Staphylcocccus, Clostridium, Erysipelothrix, Nocardia*

Protozoa: *Cryptosporidium*
Allergic Reactions to Zebrafish
Human sensitivity to fish proteins in the laboratory setting is rare. It remains possible, however, to become sensitized to fish proteins through inhalation or skin contact.

Procedure to prevent infection

• *Wash your hands.* The single most effective preventative measure that can be taken is thorough, regular hand washing. Wash hands and arms after handling fish and aquarium water. Never smoke, drink or eat in the animal rooms or before washing your hands.

• *Wear gloves*. If you are in a situation in which you will spend a significant amount of time with your hands immersed in water or if you have any cuts or abrasions on your hands or arms, you should wear sturdy, impervious gloves.

• *Seek medical attention*. If you are injured on the job, promptly report the accident to your supervisor even if it seems relatively minor. Minor cuts and abrasions should be immediately cleaned with antibacterial soap and then protected from exposure to fish and aquarium water. For more serious injuries or if there is any question, students should report to their supervisor and designated health and safety officer.

• *Tell your physician you work with fish*. Whenever you are ill, even if you're not certain that the illness is work-related, always mention to your physician that you work with fish. Many zoonotic diseases have flu-like symptoms and would not normally be suspected. Your physician needs this information to make an accurate diagnosis. Questions regarding personal human health should be answered by your physician.

8 Statistical analysis[142]

(Markus Reischl)

The German animal welfare act (TierSchG) of 2013 determines in § 7 (1)1.b), that the number of animals used in experiments must be restricted to the indispensable level. The “Tierschutzversuchstierverordnung (TierSchVersV)”, that applies this legislation, specifies that the required number of animals as well as the way that this number has been calculated must be declared in a „Tierversuchsantrag“. Thus, the questions in section 1.4.2 of the application form must be answered and the form „Angaben zur biometrischen Planung“ must be completed and included with the application. Authorities as well as the ethics committee attach considerable importance to a comprehensive description of these calculations and a perfectly competent biometric justification for the number of animals used in experiments.
For comprehensive studies, it is therefore strongly recommended to contact an experienced biometrician in the early stages of preparing the application in order to get his/her advice. If possible, a competent written assessment prepared by the biometrician should be included with the application.

For simple studies only comparing mean values, the calculations should be based upon the following basic principles: An experiment consists of a set of experimental-groups (including a control group), each consisting of single experimental runs. The output of a single experimental run typically represents a continuous variable x, e.g.. intensity of fluorescence, number of cells, area of fluorescence etc.. Then, the required number of animals for the experimental-group can be calculated based on the knowledge of former experiments based on this variable. Therefore, it is essential to have a negative control group (which showed no effect, e.g.. no increased fluorescence can be seen in a larva) and a group, which is assumed to exhibit an effect (e.g.. an increase in fluorescence can be seen and the fluorescent area is measured). Mean values (m) and standard deviations (s) of all samples x_i of the negative control group and the effect-group then need to be calculated. Having n measured outcomes, they are given by

$$m = \frac{1n}{} \sum_{i=1}^{n} x_i$$

$$s = \sqrt{\frac{1}{n-1} \sum_{i=1}^{n} (x_i - m)^2}$$

(a function that can be more easily calculated by EXCEL using the commands MEAN and STDEV).

[142] ERDFELDER, E.; BUCHNER, A.; FAUL, F.; BRANDT, M.: Allgemeine Psychologie und Deduktivistische Methodologie, Kap. GPOWER: Teststärkeanalysen leicht gemacht., S. 148–166. Vandenhoeck & Ruprecht, 2004. MAYR, S.; ERDFELDER, E.; BUCHNER, A.; FAUL, F.: A short tutorial of GPower. Tutorials in Quantitative Methods for Psychology 3 (2007), S. 51–59.

Thus, the control group delivers a mean value m_c and a standard deviation s_c, and the effect group delivers a mean value m_e and a standard deviation s_e.

> ***Example:*** *An experiment measures the effect of compounds on the expression of a reporter transgene. A negative control experiment using DMSO was repeated 11 times and delivered the following relative expression levels of the reporter transgene: 0; 2; 2; 1; 0; 1; 1; 3; 0; 0; 1. An experiment with a compound delivered an effect and showed the following relative expression levels: 2; 4; 2; 3; 4; 3; 2; 3; 3; 5; 2.*
>
> *The mean value of the control experiment is* $m_c = 1$ *and can be calculated in EXCEL by*
>
> *MEAN(0; 2; 2; 1; 0; 1; 1; 3; 0; 0; 1)*
>
> *The standard-deviation is* $s_c = 1$ *and is calculated by*
>
> *STDEV(0; 2; 2; 1; 0; 1; 1; 3; 0; 0; 1).*
>
> *The experiment with effect delivers equivalently* $m_e = 3$ *and* $s_e = 1$.

If the calculated standard-deviations are equal ($s_e = s_c = s$), then the number of required samples needed to deliver significant results is given by

$$n \approx \left(\frac{2\left(z_{1-\beta} + z_{1-\alpha}\right)s}{m_c - m_e} \right)^2 .$$

where
$z_{1-\alpha}$ and $z_{1-\beta}$ are statistical parameters and are determined from statistical tables (distribution function of the standard normal distribution).
$z_{1-\alpha}$ is dependent on the significance level α and therefore on the quality of the statistical outcome (namely the percentage of false positives). $z_{1-\beta}$ is dependent on the power β, defining the number of false-negatives. α and β are chosen arbitrarily with values between 0 and 1, the lower they are, the better the statistical adequacy will be.
If no deeper statistical analysis is needed, standard parameters for the biometric justification of animal numbers may be used: Therefore, α is set to 0.05 resulting in $z_{1-\alpha}$=1.65, β is set to 0.2 then $z_{1-\beta}$ is 0.84. Any other value of α and β can be used by applying the EXCEL- command NORM.INV($1 - value$; 0; 1), see example below.

> *Example: From the former experiment, we calculated* $m_c = 1$, $m_e = 3$ *and* ($s_e = s_c = s = 1$). *In the upcoming experiment we want to assure an error rate of false positives of maximal 1% (=signifcance level* α *= 0.01), and an error rate of false negatives of maximal 10% (=power* β *= 0.1), thus we obtain*
>
> $z_{1-\alpha} = z_{0.99}$ = NORM.INV($1 - 0{,}01$; 0; 1) = NORM.INV($0{,}99$; 0; 1) = *2.32*
>
> $z_{1-\beta} = z_{0.9}$ = NORM.INV($1 - 0{,}1$; 0; 1) = NORM.INV($0{,}9$; 0; 1) = *1.28.*
>
> *Inserting these values into the formula above, the number of samples needs to be*

$$n \approx \left(\frac{2 \cdot (1.28 + 2.32) \cdot 1}{3 - 1}\right)^2 \approx 12.96.$$

Thus, the experiment needs to contain12.96 samples, each group at least 12.96/2 = 6.48 samples. Rounding up, the number of samples required is 7.

This calculation procedure is contained in software-packages such as GPower (see Fig.19 and literature below[143]). The software also delivers estimates for more complicated cases. Often a parameter called effect-size d needs to be inserted as input parameter. It is given by

$$d = \frac{m_c - m_e}{s},$$

Example: In our case $d = \frac{3-1}{1} = 2.$

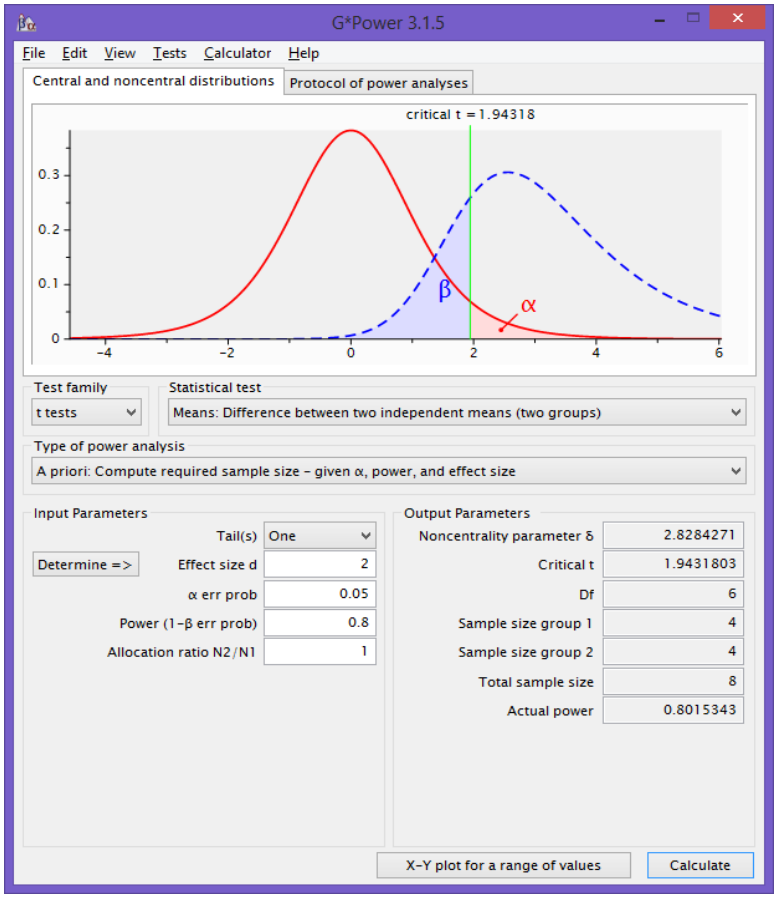

Fig. 19: Example of MS Excel statistics sheet.

[143] ERDFELDER, E.; BUCHNER, A.; FAUL, F.; BRANDT, M.: Allgemeine Psychologie und Deduktivistische Methodologie, Kap. GPOWER: Teststärkeanalysen leicht gemacht., S. 148–166. Vandenhoeck & Ruprecht, 2004. MAYR, S.; ERDFELDER, E.; BUCHNER, A.; FAUL, F.: A short tutorial of GPower. Tutorials in Quantitative Methods for Psychology 3 (2007), S. 51–59.

9. Abbreviations

AD	Anno Domini, (German: n. Chr.)
ADR	The European Agreement concerning the International Carriage of Dangerous Goods by Road
BC	Before Christ (German: v. Chr.)
BGBl	Bundesgesetzblattes, das öffentliche Verkündungsblatt der Bundesrepublik Deutschland (The Federal Law Gazette)
BioP	Regulation (EU) No 528/2012 of the European parliament and of the council of 22 May 2012 concerning the making available on the market and use of biocidal products (EU 2012).
BmTierSSchV	Binnenmarkt-Tierseuchenschutzverordnung – Verordnung über das innergemeinschaftliche Verbringen sowie die Einfuhr und Durchfuhr von Tieren und Waren (Internal Market Epizootic Protection Ordinance, Germany)
BVL	Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (The Federal Office of Consumer Protection and Food Safety, Germany) fulfils many tasks in the area of food safety and protects the economic interests of consumers across national boundaries
CLP-Regulation	The euopean regulation on classification, labeling and packaging of substances and mixtures (CLP-Regulation (EC) No 1272/2008)
CNS	central nervous system
dpf	days post fertilization (dpf)
ECVAM	European Centre for the Validation of Alternative Methods (ECVAM), hosted by the Joint Research Centre of the European Commission, which coordinated the validation of alternative approaches to animal testing in the European Union since 1991.
EDSP	US-EPA Endocrine disrupter screening program (US EPA 2011)
ELISA	Enzyme Linked Immunosorbent Assay
EU	European Union (Europäische Union)
EZRC	European Zebrafish Resource Centre (Europäisches Zebrafisch-Ressourcenzentrum)
FA	Commission Regulation (EC) No 429/2008 of 25 April 2008 on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the preparation and the presentation of applications and the assessment and the authorization of feed additives (EU 2008)
FDA	U.S. Food and drug administration
FDA/CVM	the US FDA Center for Veterinary Medicine
FELASA	Federation of European Laboratory Animal Science Associations
FET	Fish Embryo Test
FFDCA	Federal Food, Drug, and Cosmetic Act (US congress 2002)
FIFRA	US Federal Insecticide, Fungicide and Rodenticide Act (McElroy et al. 2011)
GenTG	Gesetz zur Regelung der Gentechnik (Gentechnikgesetz) (The German law on biotechnology)

GMO	genetically modified organism (genetisch veränderter Organismus (GVO))
HE stain	Hematoxylin and eosin stain
HMP	Directive 2001/83/EC on the Community code relating to medicinal products for human use (EU, 2001) and corresponding guidelines for environmental risk assessment of the European Medicines Agency (EMA/CHMP 2006)
HOCNF	OSPAR Guidelines for completing the harmonised offshore chemical notification format (OSPAR 2012)
hpf	hours post fertilization
HPLC	High Performance Liquid Chromatography
i.c.	intracardiac
i.m.	intramuscular
i.p.	intraperitoneal
i.v.	intravenous
IATA	International Air Transport Association (IATA) - an international trade association for the world's airlines representing 84% of total air traffic (240 airlines)
IATA-DGR	IATA Dangerous Goods Regulations (DGR)
IATA-LAR	IATA Live Animals Regulations (LAR)
IZMC	International Zebrafish/Medaka Course
LD50	lethal dose, 50% - the dose of a substance required to kill half the members of a tested population after a specified test duration
lof mutant	long fin mutant
MS-222	tricaine, metacaine, tricaine mesylate, tricaine methanesulfonate, TMS, Finquel
MSDS	Material Safety Data Sheets
mya	millions of years ago
n.a.	no information available
n.r.	not required by any regulation
NTP	nichttechnische Projektzusammenfassung (non-technical project summary)
OCA	oculocutaneous albinism
OIE	International Office of Epizootic Diseases (OIE), the international organisation designated under the Agreement on the Application of Sanitary and Phytosanitary Measures in application of GATT 1994 which is responsible for the establishment of international animal health rules for trade in animals and animal products.
PCR	Polymerase Chain Reaction
PPP	Commission Regulation (EU) No 283/2013 of 1 March 2013 setting out the data requirements for active substances, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market (EU 2012, 2013)
3Rs	Classification of the humane experimental techniques as replacement, reduction, and refinement by Russell and Burch in 1959 - now commonly known as the 3Rs
REACH	Regulation EC (No) 1907/2006 on the Registration, Evaluation, Authorization and Restriction of Chemicals. (EU 2006)

RID	Regulations concerning the International Carriage of Dangerous Goods by Rail
TierSchB	Tierschutzbeauftragter (Animal Welfare Officer)
TierSchG	Tierschutzgesetz (The German Animal Protection Act)
TierSchVersV	Tierschutzversuchstierverordnung (German, implementing the German Animal Protection Act (Tierschutzgesetz))
TL	Tübingen (or Tüpfel) long fin
TSCA	Toxic Substances Control Act (GAO 2007)
TSchTrV	Tierschutztransportverordnung (German, implementing the Council Regulation (EC) No 2010/63/EU on the protection of animals during transport and related operations)
TÜ	Tübingen (or Tuebingen, TU) genetic background
US EPA OCSPP	United States Environmental Protection Agency, Office of Chemical Safety and Pollution Prevention
VersTierMeldV	Versuchstiermeldeverordnung (German, implementing the COMMISSION IMPLEMENTING DECISION of 14 November 2012 establishing a common format for the submission of the information pursuant to Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes (2012/707/EU))
VMP	Directive 2004/28/EC amending Directive 2001/82/EC on the Community code relating to veterinary medicinal products (Regulation EC 726/2004) (EU, 2004) and corresponding guidelines for environmental risk assessment of committee for medicinal products for veterinary use (EMA/CVMP 2008)
WHO	World Health Organization
ZEBET	Zentralstelle zur Erfassung und Bewertung von Ersatz- und Ergänzungsmethoden zum Tierversuch am Bundesinstitut für Risikobewertung (BfR) (Centre for the Documentation and Evaluation of Alternatives to Animal Experiments at the The Federal Institute for Risk Assessment (BfR), Germany)
ZIRC	Zebrafish International Resource Center

10 Fish in toxicology and ecotoxicology
(Thomas Braunbeck)

According to § 1 of the current German Animal Welfare Act (BGBl. I S. 1206, 1313), vertebrates, especially warm-blooded vertebrates, may not be killed without sensible justification. The elucidation of the adverse effects of chemicals on fish and/or the environment is regarded as sufficient justification, and thus, experimentation with fish has now become an integral approach in toxicology and ecotoxicology. Therefore, in terms of animal rights, the (eco)toxicologist is in the paradoxical situation of exposing fish to pain, harm and suffering in an attempt to avoid such adverse effects in the wild.

10.1 Fish tests in waste water disposal

Worldwide, by far most fish are “used” for the routine testing of waste water, and in some countries the number of fish being used for this purpose is not even recorded. In Germany, the routine acute fish test according to the German Waste water Charge Act has been introduced as a tool to assess the potential toxicity of whole effluents for fish. Amongst other parameters, the toxicity of effluents to fish was used to calculate the “charge” for the contamination of the environment. Thus, the Wastewater Charge Act was the first national regulation which linked the definition of a charge to a potential environmental threat. Originally, according to DIN 38412, part 20 (DIN 1981), the test was conducted with 10 fish (usually golden ide, *Leuciscus idus melanotus*; see below) per effluent dilution over an exposure period of 48 or (later) 96 h. For animal rights´ reasons, the number was finally reduced to three, and this test was finally only used for the confirmation of pre-existing knowledge about the toxicity of a certain effluent.

In 2003, this acute fish test for the assessment of waste water toxicity was replaced by the so-called “fish egg test” (DIN 2001), which exposes freshly fertilized eggs of zebrafish (*Danio rerio*), to a series of effluent dilutions over a period of 48 h. As endpoints of acute lethality, coagulation of the embryo, lack of somite formation, lack of heart beat and non-detachment of the tail bud are used (Table 12; Figs. 20-22). Although this method does not completely avoid the use of living animals, the exposure to the potentially harmful test solutions is restricted to a developmental stage which is likely to be less sensitive to pain, distress and suffering. In terms of animal protection the test thus serves as a refinement test or a relative replacement of an animal test in the terminology of the British Home Office regulations.

Table 12: Endpoints recorded for the routine testing of sewage waters according to the German standard DIN 38415, part 6 (DIN 2001;[144]).

[144] Braunbeck, T., Böttcher, M., Hollert, H., Kosmehl, T., Lammer, E., Leist, E., Rudolf, M. and Seitz, N. (2005) Towards an alternative for the acute fish LC_50 test in chemical assessment: The fish embryo toxicity test goes multi-species − an update. ALTEX 22: 87-102.

	4h	8h	12h	16h	24h	36h	48h
Egg coagulation	+	+	+	+	+	+	+
Development of somites				+	+	+	+
Tail detachment from yolk					+	+	+
Presence of heart beat						+	+

In the US, effluent testing has only been established recently and uses typical cold water fish such as rainbow trout (*Oncorhynchus mykiss*; see below) or fathead minnow (*Pimephales promelas*; see below).

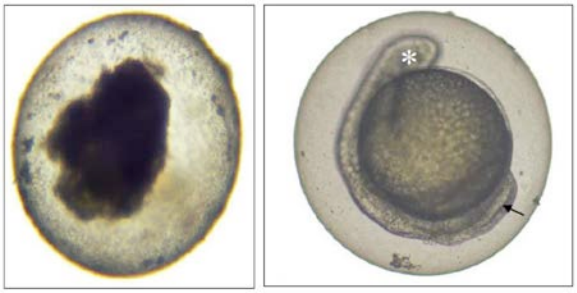

Fig. 20: (left): Coagulation of the embryo: Under bright field illumination, coagulated zebrafish embryos show a variety of non-transparent inclusions[1].(right): Non-detachment of the tail bud in lateral view (→; 96 hrs old zebrafish embryo). Note also the lack of the eye bud (*;[1]).

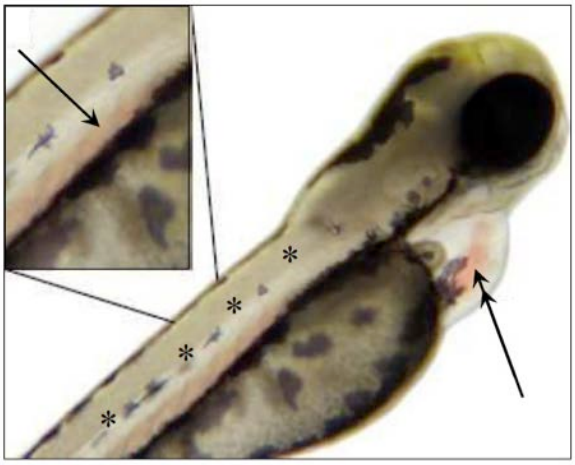

Fig. 21: Lack of heartbeat is indicated by non-convulsion of the heart (double arrow). Immobility of blood cells in, e.g., the aorta abdominalis (→ in insert) is not an indicator for lack of heartbeat. Note also the lack of somite formation in this embryo (*, homogenous rather than segmental appearance of muscular tissues). The observation time to record an absence of heartbeat should be at least of one minute with a minimum magnification of 80× ([1]).

10.2 Fish tests in chemical testing: acute toxicity testing with adult fish and the Fish Embryo Test as an alternative method

In contrast to effluent testing, which is completely subject to national regulations, regulations for the registration of chemicals have been placed under the umbrella of central EU regulations since 2006, when REACH came into force (EC 2006). As a major step forward, if compared to previous national chemical regulation, REACH not only applies to chemicals newly introduced to the market, but to all industrial chemicals handled within the European Community. In contrast to previous testing strategies, this implies that *all* chemicals have to be tested. Since the use of fish-based tests is central to a large number of regulations, a significant increase of toxicity experiments involving fish has to be expected. Indeed, according to an estimation by Hartung & Rovida[145], complying with REACH may use 20 times more

[145] Hartung, T. and Rovida, C. (2009) Chemical regulators have overreached. Nature 460: 1080-1081. Knacker, T., Boettcher, M., Rufli, H., Frische, T., Stolzenberg, H.C., Teigeler, M., Zok, S., Braunbeck, T. and Schäfers, C. (2010) Environmental effect assessment for sexual-endocrine disrupting chemicals − fish testing strategy. Integr. Environ. Assess. Manag. 6: 653-662.

animals and cost 6 times as much as previously estimated, since regulatory toxicology has no alternative high-throughput methods that do not involve animal testing (Fig. 22).

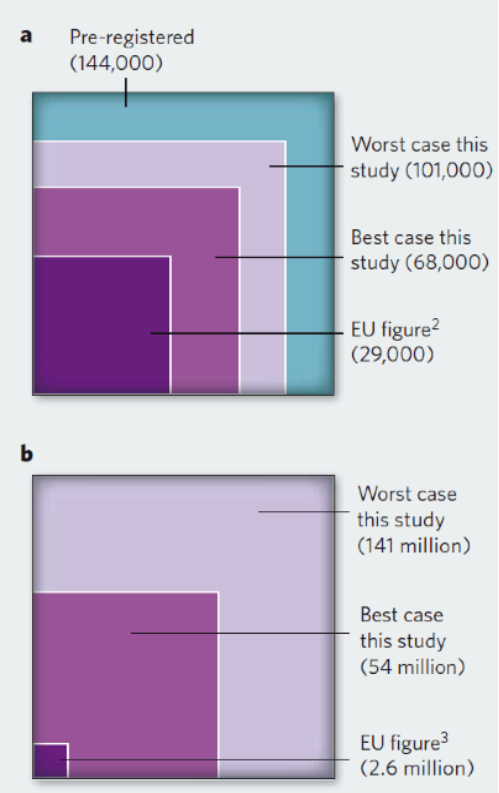

Fig. 22: Estimates for the numbers of chemicals (a) and of animals (b) expected to be needed for compliance with REACH legislation. The best-case estimates of 1 in 68,000 substances and 54 million animals are far above the official EU estimates (from:[1], as based on[1]). By far, the highest numbers of tests are required for reproductive toxicology

However, as already outlined in the EU White Paper for a future Chemicals Policy (2001) and confirmed in REACH (EU 2006), alternative test methods have to be applied wherever possible. With the adoption of the Fish Embryo Test by the Working Group of the National Coordinators for the OECD Test Guideline Program in 2013, the first alternative test method in ecotoxicology has been established ([146], Fig. 23). Although not explicitly mentioned as an alternative to the conventional acute fish toxicity test according to OECD TG 203[147] within the OECD Fish Testing Framework[148], the Fish Embryo Test (FET) is recognized as an alternative test method for the determination of the acute toxicity of chemicals for fish. Specifically, there is an excellent correlation between the results of the FET and the conventional acute fish toxicity test ([149], [150] Fig. 24). If applied in its extended version, the FET test might even serve as an alternative for prolonged fish tests (e.g.[151,152]).

[146] OECD (2013) OECD Guidelines for the Testing of Chemicals. Section 2: Effects on Biotic Systems Test No. 236: Fish embryo acute toxicity (FET) test.. Paris, France: Organization for Economic Co-operation and Development.
[147] OECD (1992a) OECD Guidelines for the Testing of Chemicals. Section 2: Effects on Biotic Systems Test No. 203: Acute Toxicity for Fish. Paris, France: Organization for Economic Cooperation and Development.
OECD (1992b) OECD Guidelines for the Testing of Chemicals. Section 2: Effects on Biotic Systems Test No. 210: Fish, Early-Life Stage Toxicity Test. Paris, France: Organization for Economic Cooperation and Development.
[148] OECD (2012) OECD Guidelines for the Testing of Chemicals. Section 2: Effects on Biotic Systems Test No. 305. Bioaccumulation in Fish: Aqueous and Dietary Exposure. Paris, France: Organization for Economic Cooperation and Development.
[149] Lammer, E., Carr, G.J., Wendler, K., Rawlings, J.M., Belanger, S.E. and Braunbeck, T. (2009) Is the fish embryo test (FET) with the zebrafish (*Danio rerio*) a potential alternative for the acute toxicity test? Comparative Biochemistry and Physiology, Part C 149: 196-209.
[150] Belanger, S.E., Rawlings, J.M. and Carr, G.J. (2013) Use of fish embryo toxicity tests for the prediction of acute fish toxicity to chemcials. Environ. Toxicol. Chem. 32: 1768-1783.
[151] OECD (1992b) OECD Guidelines for the Testing of Chemicals. Section 2: Effects on Biotic Systems Test No. 210: Fish, Early-Life Stage Toxicity Test. Paris, France: Organization for Economic Cooperation and Development.
[152] OECD (1998) OECD Guidelines for the Testing of Chemicals. Section 2: Effects on Biotic Systems Test No. 212: Fish, Short-term Toxicity Test on Embryo and Sac-Fry Stages. Paris, France: Organization for Economic Cooperation and Development.

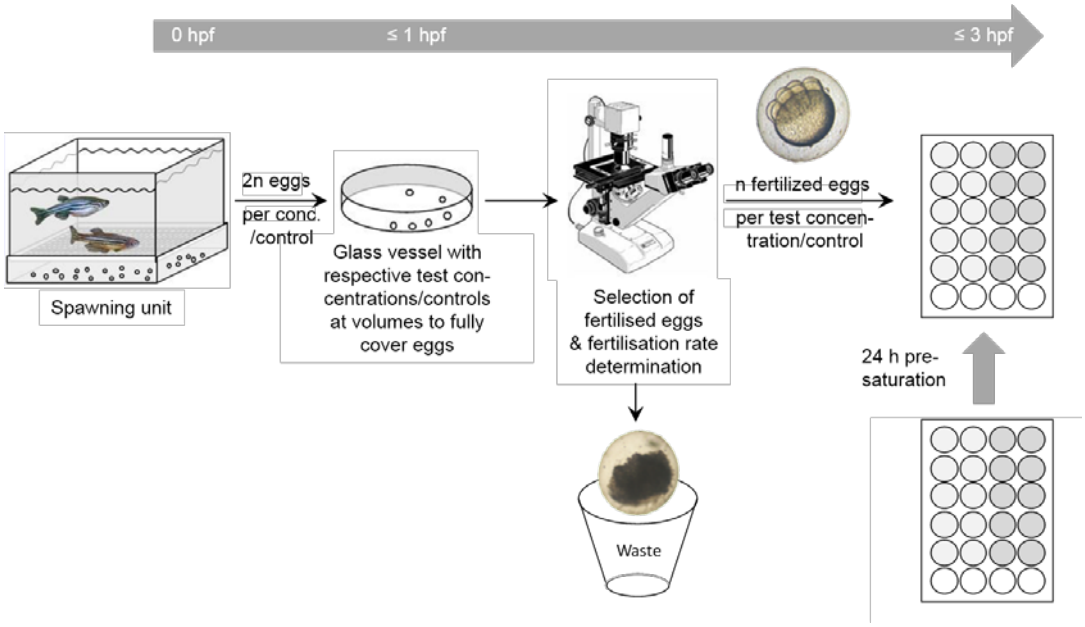

Fig. 23: Scheme of the FET test procedure (from left to right): collection of the eggs, pre-exposure of the eggs immediately after fertilization in crystallization dishes, selection of fertilized eggs with an inverted microscope or binocular and distribution of the fertilized eggs into prepared 24-well microtiter plates (Original by[1], as adapted for[1]).

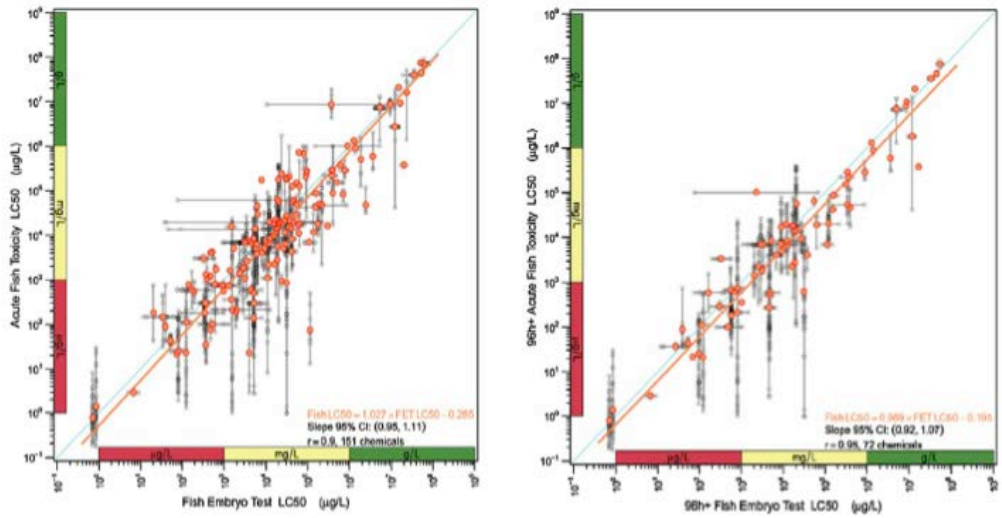

Fig. 24: Comparison of the fish embryo test (FET) versus acute fish toxicity test for 151 (left) and 72 compounds (right; data for all species are included). Open circles indicate individual test data; solid circles are geometric means of FET-acute fish toxicity results for each chemical. As is evident from the comparison between the two blots, addition of new chemicals will not significantly influence the correlation. LC_{50} = median lethal concentration[1].

However in the case of effluent testing, there are discussions among national and international regulators whether the FET should be regarded as a replacement method in the sense of the 3R by Russell & Burch[153] or whether it should only be seen as a relative or even full refinement. Nevertheless, the FET is in full compliance

[153] Russell, W.M.S. and Burch, R.L. (1959) The principles of humane experimental techniques. Methuen, London, UK, 238 pp.

with the new EU regulation for the protection of animals used for scientific purposes[154]. It is terminated after 96 h and thus exclusively uses developmental stages, which are not feeding independently and so are not regarded as protected stages in the EU[155] regulation[156]. Most importantly, the EU[157] regulation also brings to a close older discussion as to whether the critical step in fish development requiring regulatory protection should be the moment of hatching or the onset of independent feeding (Fig. 25).

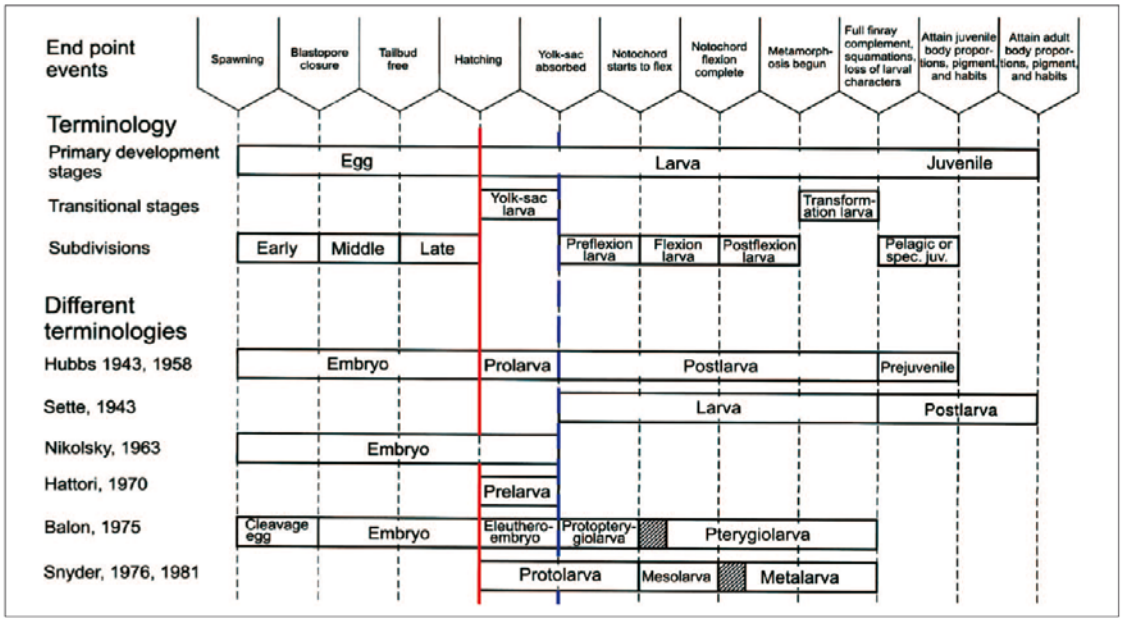

Fig. 25: Overview of different terminologies for a fish "embryo" and a fish "larva" (redrawn from Kendall et al., 1984). Whereas some older views defined the transition from embryo to larva at the point of hatching (solid red lines), the alternative view taking the absorption of the yolk sac as the critical step defining the larval stage (broken blue line) was adopted by the new EU regulation for the protection of animals used for scientific purposes[1].

As already outlined by Braunbeck et al.[158], the principle of the FET as an alternative to acute fish toxicity testing can also be applied to fish species other than the zebrafish, namely the fathead minnow and the Japanese medaka (*Oryzias latipes*), the sensitivity of which proved similar to that of zebrafish (Table 13).

[154] EU (2010) 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. Off. Journal of the EU L 276: 33-79.
[155] EU (2010) 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. Off. Journal of the EU L 276: 33-79.
[156] Strähle, U., Scholz, S., Geisler, R., Greiner, P., Hollert, H., Rastegar, S., Schumacher, A., Selderslaghs, I., Weiss, C., Witters, H. and Braunbeck, T. (2011) Zebrafish embryos as an alternative to animal experiments − a commentary on the definition of the onset of protected life stages in animal welfare regulations. Reprod Toxicol. 33: 245-153.
[157] EU (2010) 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. Off. Journal of the EU L 276: 33-79.
[158] Braunbeck, T., Böttcher, M., Hollert, H., Kosmehl, T., Lammer, E., Leist, E., Rudolf, M. and Seitz, N. (2005) Towards an alternative for the acute fish LC_50 test in chemical assessment: The fish embryo toxicity test goes multi-species − an update. ALTEX 22: 87-102.

Table 13: LC_{50} data for selected reference compounds from embryo tests with zebrafish (*Danio rerio*), Japanese medaka (*Oryzias latipes*) and fathead minnow (*Pimephales promelas*[1]).

	Sodium dodecyl-sulfate (mg/l)	n	Copper-sulfate (mg/l)	n	3,4-Dichloro-aniline (mg/l)	n	2,4-Dinitro-phenol (mg/l)	n
Zebrafish (*Danio rerio*)								
24 h	3,96 ± 0,33	3	0,25 ± 0,08	4	3,28 ± 0,32	5	1,91 ± 0,33	4
48 h	4,68 ± 0,76	6	0,26 ± 0,07	6	3,44 ± 0,32	5	1,48 ± 0,19	4
Japanese medaka (*Oryzias latipes*)								
30 h	33,08 ± 3,54	7	2,95 ± 0,41	7	24,10 ± 11,88	6	6,42 ± 1,44	5
78 h	32,92 ± 2,79	7	2,22 ± 0,25	7	21,8 ± 2,97	7	5,24 ± 0,71	5
7 days	32,1 ± 3,08	7	1,60 ± 0,30	6	15,63 ± 1,07	7	5,42 ± 0,88	5
10 days	33,1 ± 1,41	3	1,13 ± 0,38	3	12,40 ± 3,25	3	4,53 ± 0,23	3
14 days	31,08 ± 2,97	4	0,68 ± 0,04	5	4,55 ± 1,01	5	3,88 ± 0,69	3
Fathead minnow (*Pimephales promelas*)								
28 h	22,68 ± 4,13	6	1,83 ± 0,29	5	17.1 ± 0,62	4	0,82 ± 0,35	6
56 h	18,82 ± 1,99	6	1,46 ± 0,48	5	8,08 ± 2,57	5	0,41 ± 0,10	4
3 days	15,37 ± 5,39	6	0,90 ± 0,01	5	5,93 ± 4,13	4	0,40 ± 0,15	4
4 days	13,83 ± 4,79	6	0,82 ± 0,08	5	4,35 ± 2,10	4	0,31 ± 0,09	4

10.3 Legal framework for fish tests other than acute toxicity tests

In the European Union (EU), chemicals are assessed under different legislative instruments (by substance type) with respect to their risks to humans and the environment (Table 15). Most industrial chemicals are regulated by the European Union regulation on chemicals and their safe use[159] commonly known as REACH. Specific regulations exist for plant protection products[160,161], biocidal products[162,163] medicinal products for human and veterinary use[164;165] and feed additives[166,167]. The

[159] EU (2006) Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC. Official Journal of the European Union L 136, p. 3-280.

[160] EU (2009) Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing council directives 79/117/EEC and 91/414/EEC. OJ European Union. L309, 1-50.

[161] EU (2013) Commission Regulation (EU) No 283/2013 of 1 March 2013 setting out the data requirements for active substances, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market. OJ European Union. L93, 1-84.

[162] EU (1998) Directive 98/8/EC of the European Parliament and of the Council of 16 February 1998 concerning the placing of biocidal products on the market. OJ European Union. L123, 1-63.

[163] EU (2012) Regulation (EU) No 528/2012 of the European Parliament and of the Council of 22 May 2012 concerning the making available on the market and use of biocidal products. OJ European Union. L761, 1-123.

[164] EMA/CHMP (2006) Guideline on the environmental risk assessment of medicinal products for human use. EMEA/CHMP/SWP/4447/00. European Medicines Agency. Available at www.ema.europe.eu.

[165] EMA/CVMP (2008) Revised guideline on environmental impact assessment for veterinary medicinal products in support of the VICH guidelines GL6 AND GL 38. EMEA/CVMP/ERA/418282/2005-Rev.1. European Medicines Agency. Available at www.ema.europe.eu.

[166] EU (2003) Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. OJ European Union. L268, 29-43.

[167] EU (2008) Commission Regulation (EC) No 429/2008 of 25 April 2008 on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the preparation and the presentation of applications and the assessment and the authorization of feed additives. OJ European Union. L133, 1-65.

environmental concerns raised by substances used in cosmetic products are also considered within REACH[168].

The testing schemes and guidelines used in these different European regulations are strongly driven by international activities, mainly by the OECD, aiming at the harmonization of testing and regulations[169]. The OECD provides harmonized test guidelines as a basis for the mutual acceptance of data principle, avoiding duplicative testing and costs by international sharing of the hazard assessment burden. Furthermore, the WHO, through the Global Harmonization System (UN 2011), has impacted on European regulation and animal testing via the CLP Directive (directive on classification, labeling and packaging of substances and mixtures[170]), which is closely interlinked with REACH and the regulation of plant protection and biocidal products. In all the different regulations, the recommended testing procedures refer to OECD testing guidelines (Table 14).

Testing using fish is required to provide data on short-term and long-term aquatic toxicity. Additional studies may be triggered to assess bioaccumulation in fish (dependent on hydrophobicity, persistence and toxicity) and for identifying endocrine-disrupting chemicals (formal regulatory definitions for these compounds and triggers for testing are yet to be defined). A general strategy to integrate acute toxicity, chronic toxicity, bioaccumulation and endocrine disruption endpoints for fish was developed by OECD under the Fish Toxicity Testing Framework Program[171]. This OECD initiative includes scope for where alternative assays are or could be implemented.
For further information, see[172].

[168] EU (2009) Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006. OJ European Union. L353, 1-1355.
[169] Koëter, H.B.W.M., 2003. Mutual acceptance of data: harmonized test methods and quality assurance of data—the process explained. Toxicol. Lett. 140, 11-20.
[170] OECD (2012) Fish Toxicity Testing Framework. OECD Series on Testing and Assessment No. 171; ENV/JM/MONO(2012)16, 174 pp.
[171] OECD (2012) Fish Toxicity Testing Framework. OECD Series on Testing and Assessment No. 171; ENV/JM/MONO(2012)16, 174 pp.
[172] Scholz, S., Sela, E., Blaha, L., Braunbeck, T., Galay-Burgos, M., García-Franco, M., Guinea, J., Klüver, N., Schirmer, K., Tanneberger, K., Tobor-Kapłon, M., Witters, H., Belanger, S., Benfenati, E., Creton, S., Cronin, M.T.D., Eggen, R.I.L., Embry, M., Ekman, D., Gourmelon, A., Halder, M., Hardy, B., Hartung, T., Hubesch, B., Jungmann, D., Lampi, M.A., Lee, L., Marc Léonard, M., Küster, E., Lillicrap, A., Luckenbach, T., Murk, A.J., Navas, J.M., Peijnenburg, W., Repetto, G., Salinas, E., Schüürmann, G., Spielmann, H., Tollefsen, K.E., Walter-Rohde, S., Whale, G., Wheeler, J.R., Winter, M.J. (2013) A European perspective on alternatives to animal testing for environmental hazard identification and risk assessment. Regul. Toxicol. Parmacol. 67: 506-530.

Table 14: Summary of European Union (EU) regulatory frameworks that require animal testing for environmental hazard and risk assessment. For comparison and in order to indicate that similar frameworks exist outside the EU, the appropriate regulatory framework of the United States of America (USA) has been included. Requirements for the conduct of the specific tests were taken from the appropriate pieces of legislation and/or the OECD fish toxicity testing framework[173].

Test type / OECD guideline (or US EPA if appropriate)	**Regulatory framework**		**No. of animals needed for testing of one compound**
	Europe	**USA**	
ACUTE TOXICITY			
OECD 203 and OCSPP 850.1075:Fish Acute toxicity test (US EPA 1996; OECD 1992; US EPA 2002)	REACH. *Required for compounds produced or imported >10 tons/year (conditional waiving is possible).* PPP. *Mandatory rainbow trout* (threshold approach) BioP. *Mandatory for one freshwater species (and marine species if relevant; threshold approach).* VMP. *Mandatory for Tier A.* FA. Required if PEC in surface water ≥ 0.1 µg/L or in sediments ≥ 10 µg/kg. HOCNF. Mandatory if not tested already under REACH. A limit test can be used instead of a full acute fish toxicity test. <u>Effluent testing:</u> Sweden (Naturvårdsverket, 2010) (OSPAR 2000) Denmark, France, Ireland, Norway (OSPAR 2000) *Generally, detailed guidance when acute fish toxicity should be assessed for effluents is missing. Acute fish tests often required if "available data and experience is insufficient"* (OSPAR 2000)	TSCA: *Conditional requirement* FIFRA: *Cold and warm water freshwater species and 1 saltwater species* FFDCA: Tier 2 testing of drugs and biologicals for refinement of assessment factors (FDA 1998) <u>Effluent testing</u> Clean Water Act (US Congress 33 U.S.C. 1251) *Specific requirements for effluent testing vary on a state and regional basis and are usually defined within discharge permits.*	42
CHRONIC TOXICITY			
OECD 210 OCSPP 850.1400: Fish Early Life-Stage test	REACH. *Required for chemicals produced or imported >100 tons/year if the chemical safety assessment indicates the need to further investigate effects on*	TTSCA. *Conditional requirement*	420

[173] OECD (2012) Fish Toxicity Testing Framework. OECD Series on Testing and Assessment No. 171; ENV/JM/MONO(2012)16, 174 pp.

Test type / OECD guideline (or US EPA if appropriate)	Regulatory framework		No. of animals needed for testing of one compound
	Europe	**USA**	
	aquatic organisms. The need may be indicated by a PEC/PNEC ratio above 1, but also by information concerning high acute to chronic ratios of structural analogues or physical-chemical parameters indicating poor water solubility or a high bioconcentration potential. PPP. *Always required if exposure of surface water is likely and the compound is stable in water (<90% loss over 24 h by hydrolysis)* BioP. *Required for PNEC (predicted no effect concentration) refinement.* HMP. *Required for Phase II Tier A.* VMP. *Required for Phase II Tier B.* Effluent testing Sweden (Naturvårdsverket, 2010): *Conditional based on the outcome of acute tests and PEC/PNEC >1 (based on available data of components).*	FIFRA. *Required for fresh-water species; conditionally required in saltwater species* Effluent testing Clean Water Act (US Congress 33 U.S.C. 1251) *Specific requirements for effluent testing vary on a state and regional basis and are usually defined within discharge permits*	
OECD 215 Fish, Juvenile Growth test	REACH. *Can be performed instead of the Fish Early Life-Stage Test or Fish, Short-term Toxicity Test on Embryo and Sac-Fry Stages.* PPP. *Only required if Fish Early Life Stage Test or Fish Full Life Cycle Test are not appropriate.* BioP. *Required for PNEC refinement (if log K_{ow} < 5).*	n.r.	n.a.
OECD 212: Fish, Short-term Toxicity Test	REACH. *Can be performed instead of the Fish Early Life-Stage Test or Fish Juvenile Growth Test* Biocides: see REACH	n.r.	n.a.
Fish full life cycle test (not yet internationally standardised, Crane et al. 2010; US EPA	PPP: Conditionally required in cases where the bioconcentration factor is greater than 1000 and the elimination of the active substance during a depuration phase of 14 days is lower than 95 %, or the substance	FIFRA: conditionally required if estimated environmental concentration ≥ 0.1 x FELS NOEC or studies of other organisms indicate the reproductive physiology of fish may be affected (US EPA 1996).	n.a.

Test type / OECD guideline (or US EPA if appropriate)	Regulatory framework		No. of animals needed for testing of one compound
	Europe	USA	
1998)	is stable in water or sediment (DT90 > 100 days) (CTGB, 2013) .		
BIOCONCENTRATION			
OECD 305 and OCSPP 850.1730. Bioconcentration: Flow-through fish test	REACH. *Required for chemicals produced or imported in over 100 Ton/year and log K_{ow} ≥3* PPP. *Required for products with log K_{ow} >3 and considered stable.* BioP. *Required for anti-foulings, detergents and if log K_{ow} ≥3.* HMP. *Required if log K_{ow} is ≥ 4.5 and in Tier B if log K_{OW} ≥3.* VMP. *Required in Phase II Tier B if log K_{ow} is ≥4*. FA. *Optional for phase IIB* HOCNF. *Mandatory if log K_{ow} >3, but bivalve test can be conducted alternatively.*	TSCA. *Conditional requirement* FIFRA. *Conditional requirement*	108
ENDOCRINE DISRUPTION			
OECD 229 and OCSPP 890.1350: Fish short term reproduction assay	REACH. *Required in case of concern for endocrine disruption.* (OECD 2012b PPP. *based on concern from mammalian and other data* BioP *Required in case of concern for endocrine disruption.* (OECD 2012) HMP. *Conditional requirement*. VMP. *Conditional requirement*	TSCA, FIFRA, FFDCA: via endocrine disruptor screening program (US EPA 2011). EDSP tier 1 (US EPA 2011)	80 – 96 (European regulations) (US n.a.)

Test type / OECD guideline (or US EPA if appropriate)	**Regulatory framework**		**No. of animals needed for testing of one compound**
	Europe	**USA**	
OECD 230 Fish screening assay	REACH. *Required in case of concern for endocrine disruption.* PPP. *Required in case of concern for endocrine disruption.* BioP. *Required in case of concern for endocrine disruption.* HMP. *Conditional requirement*. VMP. *Conditional requirement*	n.r.	80-96
OECD 234 Fish sexual development test	REACH. *Conditionally* r*equired in case of concern for endocrine disruption.* PPP. optional, conditions not clearly defined (EU 2012) BioP. Probably r*equired in case of concern for endocrine disruption.* HMP. Probably r*equired in case of concern for endocrine disruption.* VMP. Probably r*equired in case of concern for endocrine disruption.*	n.r.	840 (definitive) 480 (screening mode)
Fish full life-cycle test (not yet internationally standardized, Crane et al. 2010; US EPA 1998)	PPP	FIFRA: Required but as part of chronic toxicity testing without focus on endocrine disruption (see above) EDSP tier 2 (US EPA 2011)	1776

Abbreviations:

BioP	Regulation (EU) No 528/2012 of the European parliament and of the council of 22 May 2012 concerning the making available on the market and use of biocidal products (EU 2012).
EDSP	US-EPA Endocrine disrupter screening program (US EPA 2011)
FA	Commission Regulation (EC) No 429/2008 of 25 April 2008 on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the preparation and the presentation of applications and the assessment and the authorization of feed additives (EU 2008)
FIFRA	US Federal Insecticide, Fungicide and Rodenticide Act (McElroy et al. 2011)
FFDCA	Federal Food, Drug, and Cosmetic Act (US congress 2002)
HMP	Directive 2001/83/EC on the Community code relating to medicinal products for human use (EU, 2001) and corresponding guidelines for environmental risk assessment of the European Medicines Agency (EMA/CHMP 2006)
HOCNF	OSPAR Guidelines for completing the harmonised offshore chemical notification format (OSPAR 2012)
n.a.	no information available
n.r.	not required by any regulation
PPP	Commission Regulation (EU) No 283/2013 of 1 March 2013 setting out the data requirements for active substances, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market (EU 2012, 2013)
REACH	Regulation EC (No) 1907/2006 on the Registration, Evaluation, Authorization and Restriction of Chemicals. (EU 2006)
TSCA	Toxic Substances Control Act (GAO 2007)
US EPA OCSPP	United States Environmental Protection Agency, Office of Chemical Safety and Pollution Prevention
VMP	Directive 2004/28/EC amending Directive 2001/82/EC on the Community code relating to veterinary medicinal products (Regulation EC 726/2004) (EU, 2004) and corresponding guidelines for environmental risk assessment of committee for medicinal products for veterinary use (EMA/CVMP 2008)

10.4 Fish generally used in (eco)toxicology

The species used in (eco)toxicology do not differ significantly from those used in other disciplines such as general pathology, developmental biology or physiology. In the following section, some information is given about the species most frequently used in common toxicity tests (cf. Table 3), with particular attention to the so-called small “OECD species” zebrafish, fathead minnow and Japanese medaka, since these can not only be used in short-term (acute) toxicity tests, but also in extended tests such as a full life-cycle test or a two-generation test (cf., e.g.[174]).

10.4.1 Zebrafish (Danio rerio)

For a description of zebrafish biology, see section 4.1 of this manual. The strains typically used in (eco)toxicology are wild-type ones, some of which are listed below:

- *AB* (AB)
- *AB/C32* (AB/C32)
- *AB/TL* (AB/TL)
- *AB/Tuebingen* (AB/TU)
- *C32* (C32)
- *Cologne* (KOLN)
- *Darjeeling* (DAR)
- *Ekkwill* (EKW)
- *HK/AB* (HK/AB)
- *HK/Sing* (HK/SING)
- *Hong Kong* (HK)
- *India* (IND)
- *Indonesia* (INDO)
- *Nadia* (NA)
- *RIKEN WT* (RW)
- *Singapore* (SING)
- *SJA* (SJA)
- *SJD* (SJD)
- *SJD/C32* (SJD/C32)
- *Tuebingen* (TU)
- *Tupfel long fin* (TL)
- *Tupfel long fin nacre* (TLN)
- *WIK* (WIK)
- *WIK/AB* (WIK/AB)

10.4.2 Japanese medaka (Oryzias latipes)

For a description of medaka biology, see section 4.2 of this manual.

10.4.3 Fathead minnow (Pimephales promelas)

The fathead minnow (*Pimephales promelas* Rafinesque) is a member of the large family Cyprinidae. Etymology of the minnow's name derives from the shape of the male's head (*Pimephales* − "fat head") and its color in breeding males (*promelas* – “forward” and “black”). The "fat head" during breeding is primarily attributable to the proliferation of epithelial cells along its anterior dorsum. Adult minnows have a moderately compressed body, a short, blunt snout, and a slightly subterminal mouth.

Colors are olive to brown on the upper body and silvery-white on the lower body with a dark midlateral stripe. There is a dusky blotch midway on the dorsal fin. Adults range from 40 - 100 mm total length and display strong sexual dimorphism. Breeding males acquire a large, grey fleshy growth on the nape, as well as approximately 16 white breeding tubercles on the snout (Fig. 26). Nuptial males tend to be larger than

[174] Knacker, T., Boettcher, M., Rufli, H., Frische, T., Stolzenberg, H.C., Teigeler, M., Zok, S., Braunbeck, T. and Schäfers, C. (2010) Environmental effect assessment for sexual-endocrine disrupting chemicals − fish testing strategy. Integr. Environ. Assess. Manag. 6: 653-662.

females with horny tubercles on the snout and a prominent pad of spongy rugose tissue on the nape.

The fathead minnow is a species of temperate freshwater habitats. The natural geographic range extends throughout much of North America, from central Canada south along the Rockies to Texas, and east to Virginia and the Northeastern United States. Fathead minnows typically inhabit the pools and backwaters of small creeks, but are occasionally found in larger streams, ponds, and lakes. They generally stay near the shoreline or close to weed cover. Adults are omnivorous, eating insects, algae, detritus, and micro crustaceans. Fatheads originated throughout the Midwest and upper Mississippi River drainage, west to Utah, north to Canada, and east to Maine. Popularity as a bait minnow has led to fathead introductions throughout the United States resulting from bait-bucket release or hatchery escapes.

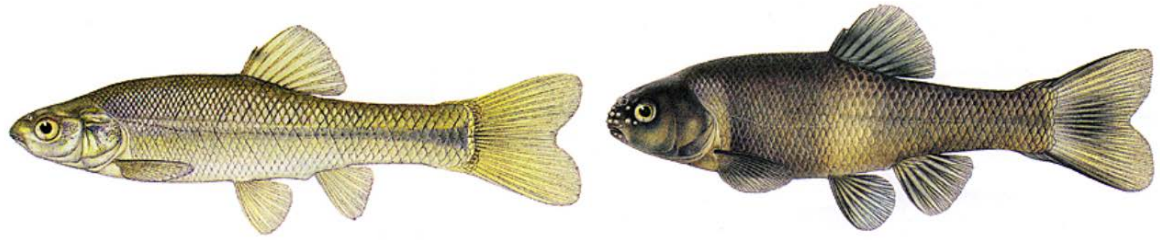

Fig. 26: Fathead minnow (*Pimephales promelas*): female (left) and male individual (right).

The male is illustrated in breeding condition with rostral tubercles. Source: http://aquaticpath.phhp.ufl.edu/fhm/intro.html.

The fathead minnow is best known for producing Schreckstoff (alarm chemicals as a distress signal). However, since it is fairly tolerant to harsh conditions, it can be found in bodies of water that may be uninhabitable to other fish, such as waste drainage sites. For this reason, it has also been studied to investigate the effects of these waste materials on aquatic life. Likewise, the fathead minnow has been the preferred fish species for the testing of chemical toxicity in the US. For this reason, the US EPA chemical toxicity database (ECOTOX) has mainly been based on data from experiments with fathead minnow.

In fathead minnow exposed to cattle feedlot effluent, males were found to be feminized and the females are defeminized. The male fathead minnows have reduced testicular testosterone synthesis, altered head morphometrics, and smaller testis size, while the females have a decreased estrogen to androgen ratio, which is a defeminized sex hormone ratio.

The effect of low pH on the fathead minnow has also been studied extensively (aquatic acidification). It has been shown that even though survival was minimally effected by extended exposure to low pH, their behavior was abnormal. They showed stress behavior, such as surface swimming and hyperactivity. In addition, there are also some deformities brought about by long exposure to low pH. In both males and females, their heads become smaller than normal. Males lose some of the brightness of their color. Females become heavy with eggs, but may not spawn, and the number of eggs per female is reduced. The eggs themselves emerge as abnormal, fragile and lacking turgidity, and the lower the pH, the less likely the eggs are to eventually hatch.

10.4.4 Rainbow trout (Oncorhynchus mykiss)

The rainbow trout (*Oncorhynchus mykiss*) is a species of salmonid native to cold-water tributaries of the Pacific Ocean in Asia and North America. The steelhead (sometimes “steelhead trout”) is an anadromous (sea-run) form of the coastal rainbow trout (*O. m. irideus*) or redband trout (*O. m. gairdneri*) that usually returns to fresh water to spawn after living two to three years in the ocean. Freshwater forms that have been introduced into the Great Lakes and migrate into tributaries to spawn are also called steelhead.
Adult freshwater stream rainbow trout average between 0.5 and 2.3 kg, while lake-dwelling and anadromous forms may reach 9.1 kg. Coloration varies widely based on subspecies, forms and habitat. Adult fish are distinguished by a broad reddish stripe along the lateral line, from gills to the tail, which is most vivid in breeding males (Fig. 27).

Fig. 27: Rainbow trout (*Oncorhynchus mykiss*). Source: species- rainbowtrout-fish.jpg.

Wild-caught and hatchery-reared forms of this species have been transplanted and introduced for food or sport in at least 45 countries and every continent except Antarctica. Introductions in locations outside their native range in the US, Southern Europe, Australia and South America have negatively impacted native fish species. Introduced populations may prey on native species, out-compete them, transmit contagious diseases (such as whirling disease), or hybridize with closely related species and subspecies, thus reducing genetic purity. Other introductions into waters previously devoid of any fish species or with severely depleted stocks of native fish have created world-class sport fisheries such as the Great Lakes.
Given the wide distribution of rainbow trout in the northern states of the US and Canada, this cold-water species has typically been used for toxicity monitoring under field conditions in these countries. However, given the difficulties of rearing them under laboratory conditions, its mere size and time to reach maturity, rainbow trout has only rarely been used for toxicity experiments under controlled laboratory conditions.

10.4.5 Sheepshead minnow (Cyprinodon variegatus)

The sheepshead minnow (*Cyprinodon variegatus variegatus*) is a euryhaline sub-species of pupfish found in salt marsh estuary environments along the Atlantic Coast and the Gulf of Mexico as well as south along the South American coastline. They prefer quiet, shallow waters and have been found in saltwater bays and estuaries, as well as coastal inland areas such as creeks, canals and ditches. Given its preference

for saltwater habitats, sheepshead minnow has extensively been used for toxicity monitoring in estuarine and marine environments in the US.
Sheepshead minnows are also referred to as variegated minnows, sheepshead killifish, sheepshead pupfish, broad killifish and chubby. Sheepshead minnow generally range in size from about 4 to 6 cm. Their distinguishing characteristics include silver, tubby bodies, one dorsal (back) and one anal fin (fin closest to tail), and no lateral line (Fig. 28). Male sheepshead minnows, on average, are larger than females. The male has a black bar along the square edge of its thick tail, while the female has an obvious dark spot on the back of the dorsal fin.

The sheepshead minnow can live in water so shallow that it may be the only species there. They can survive in water that has been deprived of oxygen by gulping air at the water's surface. Sheepshead minnows dig into sediment to hide from predators or to seek refuge from very warm or cold water. Sometimes, they hide in sea grass or algae. They travel in schools, especially when they have been frightened. Predators include red drum, spotted sea trout, Atlantic croaker, turtles and some wading birds. Sheepshead minnows are an important link in the coastal food chain. Their diet consists of plant material, algae, detritus (decomposing dead/animal matter), mosquitoes and smaller fish.

The sheepshead minnow reaches sexual maturity at three months old. In colder water, spawning occurs February through October; in warm waters, spawning can last throughout the year. Males construct nest pits in bay bottoms to attract females. When mating, males turn bright blue, fiercely defending their nests. Females can spawn several times during the spawning season at 1 - 7 day intervals, depositing between 100 and 300 eggs per spawning period. The eggs are adhesive and stick to plants, the bay bottom and to each other. Eggs incubate from 4 to 12 days, depending on water temperature. Larvae (young fish) have a total length of 4 mm when hatched and are yellowish in color. Hatching typically occurs during spring and summer. Their lifespan is unknown.

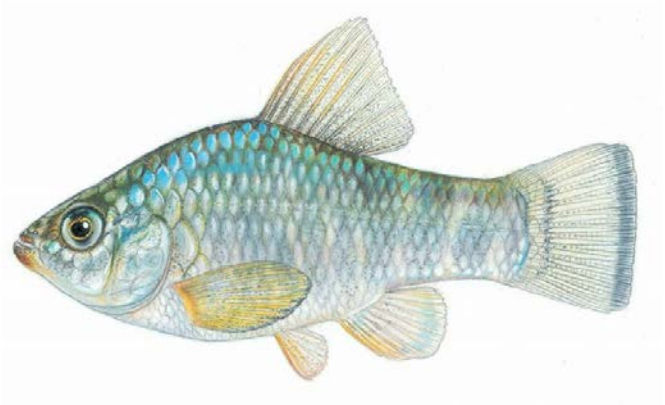

Fig. 28: Sheepshead minnow (*Cyprinodon variegatus*). Source: http://www.fishesoftexas.org/media/attachments/taxa/images/web/2701.jpg

10.4.6 Guppy (Poecilia reticulata)

The guppy (*Poecilia reticulata*), also known as million fish and rainbow fish, is one of the world's most widely distributed tropical fish, and is one of the most popular freshwater aquarium fish species. It is a member of the *Poecilidae* family and, like all other members of the family, is live-bearing. Guppies are highly adaptable and thrive in many different environmental and ecological conditions. Male guppies, which are

smaller than females, have ornamental caudal and dorsal fins, while females are duller in color. Wild guppies generally feed on a variety of food sources including benthic algae and aquatic insect larvae.

Guppies exhibit conspicuous sexual dimorphism. While wild-type females are grey in body color, males have splashes, spots, or stripes that can be any of a wide variety of colors. The size of guppies varies, but males are typically 1.5 - 3.5 cm long, while females measure 3 - 6 cm (Fig. 29). There are a variety of guppy strains produced by breeders through selective breeding, characterized by different colors, patterns, shapes, and sizes of fins, such as snakeskin and grass varieties. Many domestic strains have morphological traits that are very distinct from the wild-type antecedents. Males and females of many domestic strains usually have larger body size and are much more lavishly ornamented than their wild-type antecedents.

Guppies have 23 pairs of chromosomes, including one pair of sex chromosomes, the same number as humans. Genes that are responsible for male guppies' ornamentation are Y-chromosome-linked and heritable.

Guppies are native to Antigua and Barbuda, Barbados, Brazil, Guyana, Jamaica, the Netherlands Antilles, Trinidad and Tobago, the U.S. Virgin Islands and Venezuela. However, guppies have been introduced to many different countries on every continent except Antarctica. Sometimes this has occurred accidentally, but most often as a means of mosquito control. It was expected that the guppies would eat the mosquito larvae and help slow the spread of malaria, but in many cases, these guppies have had a negative impact on native fish populations. Field studies reveal that guppies have colonized almost every freshwater body accessible to them in their natural ranges, especially in the streams located near the coastal fringes of mainland South America. Although not typically found there, guppies also have tolerance to brackish water and have colonized some brackish habitats. They tend to be more abundant in smaller streams and pools than in large, deep, or fast flowing rivers.

Fig. 29: Wild male (upper) and female (lower) guppies (*Poecilia reticulata*). Source: http://en.wikipedia.org/wiki/Guppy.

Guppies are used as a model organism in the field of ecology, (eco)toxicology, evolution, and behavioral studies. Given their alternative breeding strategy as live-bearers, the guppy has become an interesting model in (eco)toxicology for fish not laying eggs, e.g. for bioaccumulation studies and the transfer of contaminants from one generation to another.

10.4.7 Golden ide (Leuciscus idus melanotus)

Golden ide (*Leuciscus idus melanotus*) is an ornamental form of ide. The ide (*Leuciscus idus*) or orfe is a freshwater fish of the family *Cyprinidae* found across northern Europe and Asia. It occurs in larger rivers, ponds, and lakes, typically in schools. The name is from Swedish ide, originally referring to its bright color (compare the German dialect word aitel “a kind of bright fish” and Old High German eit “funeral pyre, fire”, Fig. 30).

Fig. 30: Golden ide or orfe (*Leuciscus idus melanotus*).
Source: http://www.natur-chiemsee.de/assets/images/autogen/a_Goldorfe.jpg

The body of golden ide has a typical cyprinid shape and generally silvery appearance, while the fins are a pinkish red in varying degrees. The tail and back fin can be greyish. In older and bigger fish the body color can turn to yellow/bronze. The ide reaches a maximum length of about 60 - 80 cm though the average size is about 40 cm. Ide reaches a weight of about 4 kg. It is a predator, eating insects, crustaceans, mollusks, and small fish. In the spring, they move into rivers to spawn over gravel or vegetation; the eggs may be found sticking to stones or weeds in shallow water.

Given their natural occurrence in most German river systems, golden ide as a form typically reared in aquarium systems has formerly been used as an “indigenous species” for toxicity testing in Germany. However, since the natural distribution of golden ide is characterized by water bodies of temperate regions, the breeding behavior is very seasonal, so that golden ide is usually not available all year-round, which makes this fish species fairly unsuitable for toxicity testing. For this reason, golden ide has gradually been replaced by zebrafish, fathead minnow and medaka for routine toxicity testing, all of which can be bred throughout the year, but also spawn more regularly and are less demanding with respect to maintenance.

10.5 Diversity of fish species in alternative test methods

As outlined by Braunbeck et al.[175], the Fish Embryo Test (FET) is not restricted to the use of zebrafish embryos; rather, following minor species-specific adaptation, the test principle can easily be applied to the other so-called “OECD species”, namely the fathead minnow (*Pimephales promelas*) and the Japanese medaka (*Oryzias latipes*; Table 15).

As could be exemplified by Braunbeck et al. (2005), the variability between FETs with zebrafish, fathead minnow and medaka is not greater than the variation between the acute fish toxicity seen with different species (Fig. 31).

Table 15: Species together with maintenance, breeding and test conditions, which are demanded by the fish embryo test (FET; from[176]) for reasons of standardization[177]

	Zebra fish *(Danio rerio)*	**Fathead min-now** *(Pimephales promelas)*	**Medaka** *(Oryzias latipes)*
Origin of species	India, Burma, Malacca, Sumatra	Temperate zones of central North America	Japan, China, South Korea
Sexual dimorphism	Females: protruding belly when carrying eggs Males: more slender, orange tint between blue longitudinal stripes (particularly evident at the anal fin)	Females: more plump when carrying eggs, ovipositor Non-spawning males: black spot on dorsal fin Spawning males: black coloration of head, dorsal nuptial pad and nuptial tubercles in spawning season, black bands along body sides	Females: generally more plump, carrying sticky eggs at anal fin Males: anal fin larger, papillary processes on posterior dorsal fin rays
Feeding regime[181]	Dry food (TetraMin; Tetra, Melle, FRG) once daily; from three days before spawning, plus frozen adult brine shrimp (*Artemia spec.*) twice daily (*ad libitum*). To guarantee for optimal water quality, excess faeces was removed approx. one hour after feeding.		
Wet weight of adult fish	Females: 0.65 ± 0.13g Males: 0.5 ± 0.1g	Females: 1.5 ± 0.3g Males: 2.5 ± 0.5g	Females: 0.35 ± 0.07g Males: 0.35 ± 0.07g
Illumination[181]	Fluorescent bulbs (wide spectrum); 10-20µE/M²/s, 540-1080 lux, or 50-100 ft-c (ambient laboratory levels); 14 h light, 10 h dark		

[175] Braunbeck, T., Böttcher, M., Hollert, H., Kosmehl, T., Lammer, E., Leist, E., Rudolf, M. and Seitz, N. (2005) Towards an alternative for the acute fish LC_50 test in chemical assessment: The fish embryo toxicity test goes multi-species − an update. ALTEX 22: 87-102.

[176] Braunbeck, T., Böttcher, M., Hollert, H., Kosmehl, T., Lammer, E., Leist, E., Rudolf, M. and Seitz, N. (2005) Towards an alternative for the acute fish LC_50 test in chemical assessment: The fish embryo toxicity test goes multi-species − an update. ALTEX 22: 87-102.

[177] **Please note** that these very strict feeding, illumination, water and other maintenance conditions for medaka and zebrafish are imposed by the necessity to standardize the analysis protocol. These conditions do not reflect the spectrum of optimal maintenance conditions for Zebrafish and Medaka.
These are summarised in Tables 2 and 3.

	Zebra fish *(Danio rerio)*	**Fathead min-now** *(Pimephales promelas)*	**Medaka** *(Oryzias latipes)*
Water quality[181]	26.0 ± 0.5 °C, O_2 ≥ 8.5 mg/l, hardness: 20°dH (~ 213 mg/l $CaCO_3$), NO_3-: ≤ 48mg/l, NH_3 and NO_2-: < 0.01 mg/l, pH = 7.8 ± 0.2	16.5 ± 1.5 °C, O_2 ≥ 8.5 mg/l, hardness: 20°dH (~ 213 mg/l $CaCO_3$), NO_3-: ≤ 48mg/l, NH_3 and NO_2-: < 0.01 mg/l, pH = 7.8 ± 0.2	24.0 ± 0.5 °C, O_2 ≥ 8.5 mg/l, hardness: 20°dH (~ 213 mg/l $CaCO_3$), NO_3-: ≤ 48mg/l, NH_3 and NO_2-: < 0.01 mg/l, pH = 7.8 ± 0.2
Further water quality criteria[181]	Particulate matter < 20 mg/l, total organic carbon < 2mg/l, unionized ammonia < 1µg/l, residual chlorine < 10µg/l, total organophosphorus pesticides < 50 ng/l, total organochlorine pesticides plus polychlorinated biphenyls < 50 ng/l, total organic chlorine < 25 ng/l		
Tank size for maintenance[181]	180 l (max. 200 individuals)	180 l (max. 80 individuals)	50 l (max. 60 individuals)
Egg structure and appearance	Stable chorion, highly transparent, non-sticky, diameter ~ 0.8 mm	Chorion only hardens in multicellular stage, transparent, sticks to surfaces, diameter < 1 mm	Stable chorion with spiny hooks (adheres to anal fin of female), moderately transparent, diameter < 1 mm
Embryo development at 25 °C	18 h: Development of somites 21 h: Tail detachment 26 h: Heart-beat visible 28 h: Blood circulation 72 h: Hatching	22 h: Development of somites 25 h: Tail detachment 27 h: Heart-beat visible 30 h: Blood circulation 160 h: Hatching	28 h: Development of somites 28 h: Tail detachment 30 h: Heart-beat visible 32 h: Blood circulation 7-8 d: Hatching
Test type	Flow-through, 26 °C, 24-well plates (2 ml per cavity)	Flow-through, 25 °C, 24-well plates (2 ml per cavity)	Flow-through, 26 °C, 24-well plates (2 ml per cavity)
Major toxicological endpoints at 25 °C	24 h: Tail development, somite development 48 h: Heart-beat visible	28 h: Tail development, somite development 3 d: Blood circulation 3 d: Blood circulation 4 d: Blood circulation	30 h: Somite development 78 h: Blood circulation 7 d: Blood circulation 10 d: Blood circulation 14 d: Blood circulation
Water purification[181]	Permanent (internal filter)	Permanent (internal filter)	External (air-driven)
Male to female ratio for breeding	4:2	2:4	15:15
Breeding tanks[181]	4 l tanks equipped with steel grid bottom and plant dummy as spawning stimulant; external heating mats (cf. **Fig. 3**)	30 l tanks with black glass walls maintained at 24 °C and equipped with 2 clay tiles divided into two halves as spawning substrate	30 l tanks with black glass walls equipped with several plant dummies or *Ceratophyllum* spec. as substrate for spawning

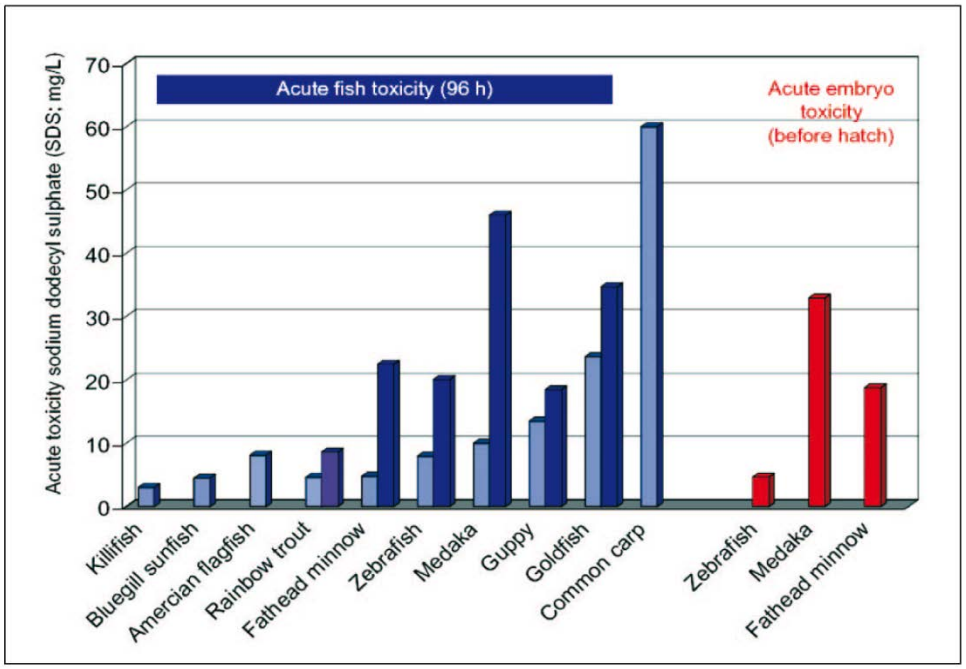

Fig. 31: Acute toxicity of sodium dodecyl sulphate (LC_{50}) to selected fish species *in vivo* as well as to embryos of zebrafish, medaka and fathead minnow *in ovo* after 48 h of exposure (red; from[178])

10.6 Alternative methods for fish toxicity tests other than those based on embryos

In contrast to fish embryos, which show an excellent correlation with adult fish in terms of their sensitivity to chemicals and effluents, fish cell cultures have frequently been studied as a potential replacement in vitro system for acute fish toxicity testing[179]. However, for the time being, fish cell lines seem less sensitive than intact fish, which make them less suitable especially for routine effluent monitoring. However, with the advent of new fish cell lines and the improvement of cell culture conditions, this problem might be overcome in the future, making fish cell lines a true alternative to conventional acute fish toxicity testing[180,181]

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11. Zebrafish embryos as models in chemical biology and chemical genetics

(Uwe Strähle, Sepand Rastegar)

Despite enormous progress in our understanding of the molecular processes of how our body functions in health and disease, the Food and Drug Administration (FDA) of the United States of America observed in the last decade a 50% decline in the registration of new pharmaceuticals. At the same time, expenditure in private and public R&D in this sector increased by 250% in the USA. In March of 2004, the (FDA) released a report entitled: „Innovation or Stagnation, Challenge and Opportunity on the Critical Path to New Medical Products". This report came to the conclusion: „Not enough applied scientific work has been done to create new tools to get fundamentally better answers about how the safety and effectiveness of new products can be demonstrated, in faster time frames, with more certainty, and at lower costs. In many cases, developers have no choice but to use the tools and concepts of the last century to assess this century's candidates". Similar conclusions were reached in Europe. Drug development has already been a major focus in FP7 with the establishment of the Joint Technology Initiative on Innovative Medicines[182]. The EU also concluded that these past efforts had not been sufficient and thus made drug development a key objective in the new research framework Horizon 2020[183].

Key issues are to develop new tools and models to improve evaluation of the effectiveness and safety of candidates at early stages of drug development. One limitation resides in the reductionist drug screening paradigms used nowadays that usually focus on isolated cells in culture, one pathway or even a single protein as targets in high-throughput assays. Although providing highest throughput, this approach is overly simplistic. Biological systems are organised in highly complex regulatory networks with an enormous compensatory potential and redundancy. In addition, the chosen target in these screens may not be efficiently druggable nor do these in vitro systems report on potential toxic side effects.

Some of the most potent drugs available to mankind were identified by whole animal exposure such as the antithrombotic drug warfarin or the heart failure drug digoxin[184]. However carrying out large scale drug screens on mammals would be ethically and financially unjustifiable. Alternatives are offered by the embryos of lower vertebrates. Pioneering work of several US laboratories have shown that zebrafish embryos can effectively be used in compound screens: The first drug candidates from such screens entered phase 1 and 2 clinical trials. For example, prostaglandin E2 increased the number of hematopoietic stem cells in the zebrafish and is now in phase 2 trials as a facilitator to re-colonise the bone marrow after transplantation in humans[185]. Dorsomorphin is an inhibitor of BMP receptors identified in morphological screens in zebrafish embryos and was the first inhibitor of the BMP pathway ever

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Rohde, S., Whale, G., Wheeler, J.R., Winter, M.J. (2013) A European perspective on alternatives to animal testing for environmental hazard identification and risk assessment. Regul. Toxicol. Parmacol. 67: 506-530.

[182] d2013_0246en01.pdf

[183] http://ec.europa.eu/research/horizon2020/index_en.cfm?pg=h2020

[184] Zon and Peterson, Nature Reviews Drug Discovery, pp 36, vol. 4, 2005

[185] North et al. Nature 2007

identified[186]. Dorsomorphin and derivatives are drug candidates to regulate iron homeostasis and to treat heterotopic ossification[187].

Zebrafish embryos are well-suited for use in chemical screens. Soluble small molecules are typically dissolved in the embryo's aquatic environment, allowing absorption into the body without the need for invasive and time-consuming injection. Zebrafish embryos can be arrayed in 96-well plates with several embryos per well, compounds can be added using pins or pipettes, and the plates can be screened either by eye or by using high-throughput automated video assays. The use of sophisticated transgenic tools combined with the transparency of the zebrafish embryo permit a depth of analysis, in an intact vertebrate that is unmatched by any other model. In the context of drug screening, zebrafish in particular offer monitoring of complex cell behaviour and physiological parameters in a living, intact organism in real time. For example, the behaviour of stem cells or immune cells can be analysed in the normal tissue context without interference from an artificial in vitro culture environment. These assays on whole animal systems have the advantage that they probe not only for an effect on cell behaviour but also for the most druggable components of the entire pathway or network under consideration. In addition, complex toxicity parameters can be assessed. For example, zebrafish can predict arrhythmias such as QT prolongation with a high probability. This well-known drug side effect went unnoticed in the past until very late stages of drug development or even after introduction to the market.

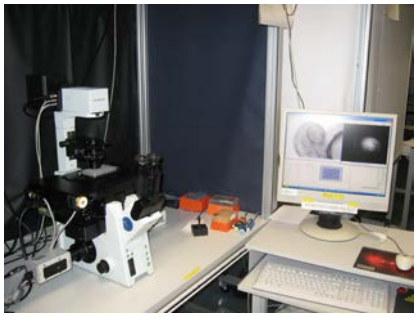

Fig. 28: Automated microscope at EZRC for large screening of zebrafish embryos.

Over the years many mutations have been isolated in the zebrafish that mimic disease states such as cancer, cardiovascular diseases, neurodegeneration and many other human diseases. Zebrafish have become a well-established model for the human genetics community to verify the identification of congenital disease - causing mutations in the human genome. From past genetic screens and the recent large scale tilling effort[188], we have access to mutations in more than 50% of protein coding genes in the zebrafish genome. With the advent of new and highly efficient knock-out technologies based on site-directed nucleases, new mutations can now be generated in any gene of interest. Given that more than 70% of genes are conserved between fish and man[189], many of these mutations will provide models for human diseases. This plethora of disease models opens entirely new avenues for drug

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screening since pathologically disturbed regulatory networks can be probed for drugs suppressing the disease in the context of a living, intact organism. Again pioneering work in the US showed for example the chemical suppression of pathology in a fish model of aortic coarctation[190]. The compounds discovered in this screen are currently being tested for their potential to induce new blood vessels in ischemia models[191].

Fig. 29: Robotic video system at EZRC to monitor feeding behaviour of zebrafish

Such screens typically entail the exposure of zebrafish embryos to thousands of structurally different chemical compounds either supplied by academia or available through commercial sources. By combining these screens with automated procedures ranging from embryo sorting to microscopy and data analysis (**Fig. 28**), the throughput can be increased to tens of thousands of compounds. Besides allowing a high resolution, detailed analysis of overall morphology the sensitivity of these fish embryo assays can be further increased by employing transgenes that label specific cell types or biochemical processes. Increasingly, screens are also carried out to assess the effect of chemicals on the behaviour of zebrafish embryos (Fig. **29**). In addition to providing new candidates for drug development, these data will be valuable sources of information for understanding the interaction of compounds with the developing embryo. Furthermore, results from such screens also represent a major contribution to characterizing the biological effects of many man-made substances that we are exposed to during our daily lives.

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