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Review

Zebrafish (*Danio rerio*) meets bioethics: the 10Rs ethical principles in research

O peixe-zebra (Danio rerio) encontra a bioética: os princípios éticos dos 10Rs na pesquisa

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Abstract
Zebrafish (*Danio rerio*) is a tropical fish species widely used in research, worldwide. The development of genetically modified animals and the increasing number of zebrafish breeding facilities due to their emerging use in several research fields, opened room for new ethical challenges for research carried out with this species. It is necessary to raise the scientific community's awareness of the ethical standards and laws in force, on animal research. Thus, the aim of the current study is to describe 10 Rs ethical principles by using zebrafish as model system in research. The classical 3 Rs concerning animal welfare, namely replacement, reduction and refinement; and the added 7 Rs related to scientific (registration, reporting, robustness, reproducibility and relevance) and conduct principles (responsibility, and respect) in zebrafish research are herein presented and critically discussed. The use of these 10 Rs by researchers, institutions and the Animal Ethics Committee is recommended to support regulations, decision-making about and the promotion of zebrafish health and welfare in research.
Keywords: animal health; animal welfare; animal ethics committees; fish; laboratory animals.

Resumo
O peixe-zebra (*Danio rerio*) é um peixe tropical amplamente usado em pesquisas em todo o mundo. Devido ao seu uso emergente em várias áreas de pesquisa, o desenvolvimento de animais geneticamente modificados e o aumento das instalações de peixe-zebra, novos desafios éticos surgem na pesquisa com o peixe-zebra. Além disso, é necessária a conscientização da comunidade científica sobre as normas éticas e leis vigentes na pesquisa com animais. Assim, o presente estudo teve como objetivo descrever os princípios éticos de 10 Rs usando o peixe-zebra como sistema modelo em pesquisa. Os 3 Rs clássicos relativos ao bem-estar animal (substituição, redução e refinamento) e 7 Rs adicionais relacionados aos princípios científicos (registro, relatório, robustez, reprodutibilidade e relevância) e de conduta (responsabilidade e respeito) na pesquisa do peixe-zebra são apresentados e discutido criticamente. Recomendamos o uso desses 10 Rs pelos pesquisadores, instituições e Comitê de Ética Animal na regulamentação, decisão e promoção da saúde e bem-estar do peixe-zebra em pesquisas.
Palavras-chave: saúde animal; bem-estar animal; comitês de ética animal; peixe; animais de laboratório.

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Introduction

Zebrafish, *Danio rerio* (Hamilton 1822), is a small tropical fish species popular in aquariums. In the late 1960s, zebrafish was introduced as vertebrate model system by George Streisinger. Thenceforth, zebrafish has been used as well-established model system in several research fields, such as developmental biology[1, 2], pharmacology and toxicology[3, 4, 5], ecotoxicology[6, 7], veterinary sciences[8], evolution biology[9], nanotechnology and nanomedicine[10, 11], human disease[12], vaccination[13], food safety[14], among others (Figure 1). Zebrafish is also a preclinical screening model used *in vivo* for personalized medicine[15] and nanomedicine[10]. Interestingly, it is an emerging suitable vertebrate model system in the Global South[16], since it accounts for decentralizing scientific publications.

There are several advantages in using zebrafish as model system, such as transparent embryos, high reproduction and fertilization rate, rapid development, short life cycle, small size, low-cost and easy maintenance under laboratory conditions, sequenced genome, genetic similarity to humans, complex behaviour[17, 18], as well as mutant strains, transgenic reporters and knockdown[19]. Furthermore, zebrafish embryos and larvae are an alternative to using adults in toxicity tests[3]. According to international ethical regulations, zebrafish larvae (up to 120 h post-fertilization (hpf)) are considered models *in vitro* (Directive 2010/63/EU)[20]. The Fish Embryo Acute

Toxicity (FET) test can determine the toxic effects of different pollutants (e.g., nanomaterials, pesticides, pharmaceuticals, industrial chemicals, metals, among others)[21]. Using animals in research involves ethical challenges; in 1959, Russell and Burch published the book titled "The principles of humane experimental technique", which became a reference in animal management based on the "3 Rs" (replacement, reduction and refinement) principle[22]. This principle recommends replacing vertebrates by animals who have lower potential for pain perception, based on techniques *in vitro* or computational methods, to reduce the number of animals per experiment and to use techniques that minimize animals' pain and distress[23]. Guidelines were created to determine the ethical procedures for handling animals in research; they incorporated the "3 Rs" principle. Among them, one finds the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes[24], Directive 2010/63/EU on the protection of animals used for scientific purposes [25], Basel Declaration[26], Guide for the care and use of laboratory animals[27], and Code of Ethics[28]. Accordingly, researchers account for the ethical and scientific validity of their studies, whereas institutions and government agencies make researchers follow the ethics codes, to apply appropriate strategies, techniques and facilities to use animals in research[24, 26, 27, 29].

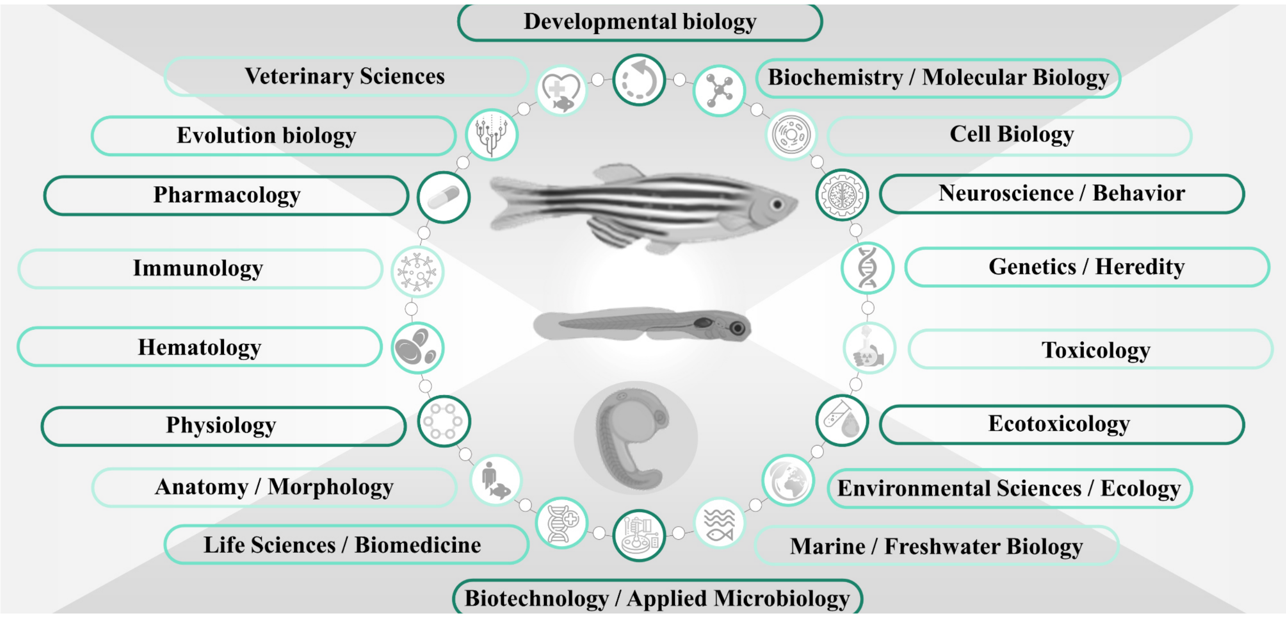

Figure 1. Zebrafish use in several scientific research fields.

Despite the several scientific fields focused on using fish as model system in research, the application of welfare concepts to fish has received less attention than that paid to mammals [30]. However, procedural guidelines, mainly the ones regarding fish, have created Guidelines on the care and use of fish in research, teaching and testing [31], and guidelines for fish use in research [29]. These guidelines aim at ensuring fish well-being in fish breeding facilities and under laboratory conditions; they point out that it is necessary (*i*) maintaining adequate water and food levels, and avoiding malnutrition; (*ii*) animals must be free from disease or injury; (*iii*) behavioural-interactive restriction must not be allowed; (*iv*) animals cannot suffer with stress or physical conditions [29, 31].

A meeting on "Contemporary Topics in Zebrafish Management and Care" was held in the UK in 2014 to bring together researchers, animal technicians and veterinarians who have discussed topics such as (*i*) larvae rearing protocol; (*ii*) 3Rs implementation; (*iii*) management practices, cryopreservation, and environmental enrichment [31]. All these measures helped improving zebrafish's welfare condition in laboratory environment. Furthermore, it is essential standardizing specific lines and protocols to bring benefits to research [32].

The aim of the current study was to describe 10 Rs ethical principles by using zebrafish as model system in research given the growing use of this species as model system and the need of ensuring these animals' welfare and health. Implementing the 7 Rs is an additional initiative to contribute to Animal Ethics Committees at the time to regulate, guide studies, report research carried out with zebrafish, increase the availability of information about this animal model and to minimize unnecessary studies, as well as to contribute to ensure zebrafish welfare and health both in fish breeding facilities and research centres.

The 10 Rs for research carried out with zebrafish

The classical 3 Rs for animal welfare (replacement, reduction, and refinement) were added with 7 Rs related to scientific (registration, reporting, robustness, reproducibility and relevance) and conduct principles (responsibility and respect) in order to help scientists improving zebrafish welfare and health in research carried out with it, as well as experiments' integrity and relevance. These principles encompass different developmental stages of zebrafish used in research, namely, at the embryo, larvae, juveniles and adult stages (Figure 2). The 10 Rs for research carried out with zebrafish have no scale of importance, but they must be recommended for zebrafish use in research.

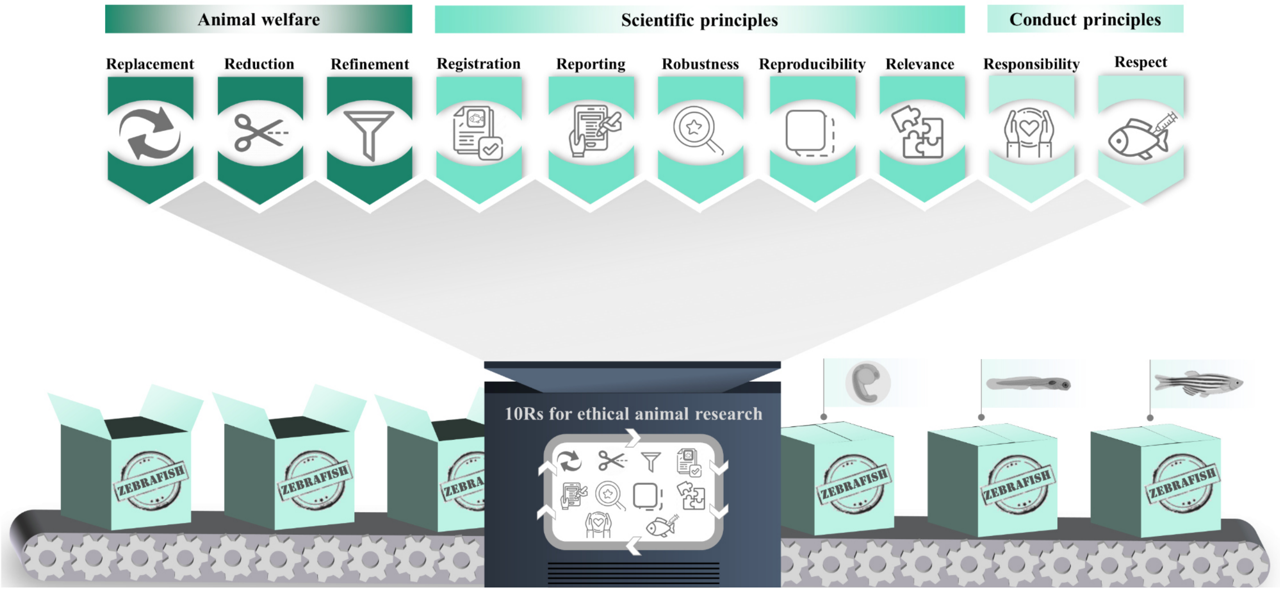

Figure 2. The 10 Rs ethical principles for zebrafish using as model system in research. The principles were classified into three categories, namely: welfare (replacement, reduction and refinement), scientific (registration, reporting, robustness, reproducibility and relevance) and conduct principles (responsibility and respect).

Animal welfare

1R – Replacement

The discussion on ethical principles and welfare in animals' use for educational and scientific purposes lights up the need of replacing model systems in order to eliminate or reduce pain, stress and suffering[25]. Such a replacement can be total or partial; total replacement of animals - without compromising research results - can be accomplished through the adoption of emerging and well-established techniques[33]. Cell lines, tissue and organoid cultures, organ chips, and computational and mathematical modeling are among the commonly applied methodologies[34, 35].

For many years, research was carried out with mammals, because of their common ancestry with human beings; therefore, it allowed extrapolating the results and applying them to human health conditions[33]. However, several regulatory authorities recommended reducing, or eliminating the use of mammal models in research[25, 36]. The United States Environmental Protection Agency (USEPA) will stop conducting, or funding, studies involving mammal models by 2035[36].

Many guidelines to assess chemicals' toxicity have been published; they provide on the use of different animal groups, other than mammals, such as diptera, crustaceans, earthworm species, molluscs and fish[37, 38, 39, 40, 41, 42]. Thus, zebrafish emerged as experimental animal model back in the 1980s, and remained so onwards[43] - one can observe the significant increase in the number of publications based on this model since the 1990s[44]. This small teleost fish presents a whole variety of features that turns it into a gold standard in scientific research, mainly to replace mammals[45]. Zebrafish genome, among other features, was fully sequenced and it shares 70% genetic similarity with humans[46]; moreover, its homology is very similar to that of mouse models (approximately 75%)[47]. In addition, there are thousands of mutant and transgenic zebrafish strains[48].

Several studies have shown that zebrafish embryos and larvae have similar responses to those of adult individuals, and it optimizes model system replacement from the welfare viewpoint, since it saves resources and minimizes waste generation[3, 49, 50]. Furthermore, tests with zebrafish embryos and larvae do not suffer with regulation, since there is no extra-chorionic feeding at these developmental stages[25, 51]. Zebrafish embryos stand out for animal replacement - even at early developmental stages – since numerous biochemical reactions, organ differentiation and morphological formations take place at this stage, and it opens room for evaluating pollutants and drugs in a whole living organism[18], at real-time and at researcher's naked eye, due to their transparency.

2R – Reduction

Reduction is an important principle to be taken into consideration when animals' replacement during scientific research is not possible[33]. Reduction means decreasing the number of animals used in protocols and experimental designs[33]; therefore, the minimum number of zebrafish must be used to achieve scientific or educational aims when no alternative methods such as mathematical models, computer simulation, biological systems *in vitro*, or other non-animal (adjuvant) approaches are available to replace the use of living animals[52].

Researchers must initially choose an adequate model system for the analyses carried out in their experiments in order to avoid the unnecessary use of animals. Furthermore, the number of animals used in experiments must be consistent and well-justified. This principle should be applied in cases where reducing the number of nimals does not impair the scientific rigor of the analysis[30]. Some resources can be used to estimate the fish-sample size needed to start an experiment, among them there are software and power analysis, which aim at determining the smallest sample size necessary to detect a given effect at a specific significance level[30].

In addition, Directive 2010/63/EU suggests that researchers must share organs and tissue of euthanized animals to easily establish new methods *in vitro*[33] or to use the same individuals in different analyses, whenever possible[53]. These are respectful ways to minimize the use of animals in research, rather than to just discard unused biological structures[33].

Zebrafish models reduce the time and resource demands, as well as space demands and costs, in comparison to more popular animal models, such as rodents. Zebrafish also provides greater informational and predictive potential that results from experiments *in vitro*. Thus, this model can reduce the use of mammals in research and mitigate issues related to these animals' well-being[13].

3R – Refinement

Although using non-human models remains necessary in scientific research, excellence standards on the care provided to animals involved in it should fully comply with research conduct[54, 55, 56]. Refining the way experiments are carried out means ensuring that animals suffer as little as possible with them, and it includes better housing, adequate water parameters, regular feeding, adequate anaesthesia and analgesia procedures[57, 58, 59, 60, 61].

Just as all other vertebrates, fish are driven to environmental challenges due to a whole series of adaptive neuroendocrine adjustments known as stress response; moreover, the prolonged activation of this response is detrimental and leads to immunosuppression, reduced growth and reproductive dysfunction. Indicators associated with response to chronic stress (physiological outcomes, disease status and behaviour) are a source of information about fish's welfare state[62, 30]. Developing alternatives to increase welfare not only favours fish health and welfare, but also improves scientific data reliability and repeatability, a fact that has the potential to reduce the number of animals used in research[63].

Zebrafish is often kept in water recirculation systems, in commercial polycarbonate tanks with bluish coloration or glass aquarium, at density of up to 5 animals per litter of water. This practical measure enables the formation of social groups typical of zebrafish's behaviour[64, 65, 66]. In addition to using aquarium coloration, it is also recommended to mimic its natural environment, namely: controlled photoperiod and availability of living food in order to stimulate the natural prey capture behaviour and to improve nutritional quality[67, 68, 32]. Zebrafish are kept in tanks at temperature ranging from 26 °C to 28°C, water pH ranging from 7 to 8, alkalinity ranging from 50 to 150 mg of $CaCO_3$/L, water hardness at levels higher than 75 mg/L, salinity close to 0.5-2 g/L and dissolved oxygen levels of at least 4 mg/L[69]. Strains used in laboratory come from strains stabilized with genetic background, since it leads to the loss of natural adaptive capacity and reduces the tolerance to environmental changes, mainly to those related to water physiochemical conditions[70]. Thus, it is necessary to always keep the physicochemical parameters within the reference values recommended in the species' breeding guides and manuals in order to avoid stressful situations[69].

Experimental manipulations that can cause pain or anguish must be carried out under sedation, analgesia or anesthesia, since fish have robust neuroendocrine and physiological responses to stress caused by harmful stimuli[71, 72]. It is necessary using anaesthetic, sedative or analgesic drugs when fish have to be immobilized in order to help handling them, collecting them beforehand, or subjecting them to euthanasia. These are fundamental procedures to achieve fish welfare. It is important highlighting that these vertebrates can show signs of stress and/or pain during handling, transport, marking, sampling and invasive procedures[72].

Using inadequate anaesthetic protocol can compromise not only fish welfare, but also research results' reliability[73]. Studies have been carried out to investigate the most suitable drugs for zebrafish by taking into account the welfare principles as way to refine techniques applied to this species' analgesia and anaesthesia procedures[73, 74, 75, 76]. Tricaine methanesulfonate (60-300 mg/L), clove oil (25-100 mg/L) and 2-phenoxyethanol (2-PE) (0.1-0.5 mg/L) are the most used anaesthetic agents in cyprinid fish. In addition, several factors can interfere with anaesthesia, such as developmental stages and environmental factors[77].

Studies focused on obtaining DNA usually have to cut the fin of adult fish under non-terminal anaesthesia[78]. The fin clipping effect can have negative influence on fish and lead to infection and change its survival, growth and behaviour, as well as decrease feeding and induce cortisol release[65, 79, 80], a fact that affects fish welfare. Efforts to improve and refine DNA collection techniques have increased, since such changes can drastically interfere with the quality of experimental data[81, 82]. Alternative DNA collection methods, such as scaling, mucus and sperm sampling, have been successfully used and they are gaining popularity. Some of these techniques are lesser invasive than fin clipping, in addition to have the potential to improve animal welfare[83]. A new collection technique was developed from skin swab smearing; it is lesser invasive and stressful for fish than fin clipping - such a finding confirms that 'swab' is a refined method for fish DNA collection[84].

Many refinement techniques can be applied during scientific experiments, as well as throughout the animals' lives and they can improve fish living conditions in breeding facilities[85]. Refinement not only favours animals, but it can also improve the quality of research results. Evidence suggests that pain and suffering can alter animals' behaviour and physiology during experimental periods. These changes can lead to variations in experimental results, and it can affect both studies' reliability and repeatability[86, 87]. The development of lesser invasive and more refined methods must be taken into consideration whenever possible.

Scientific principles

4R – Registration

Scientific principles add value to science and society through relevant studies using zebrafish as model system; therefore, we must consider the 4th R: Registration[87]. Adherence to methods focused on registering information available in studies conducted with animals has been inadequate in some cases, as well as has been leading to biased, delayed studies or unreported results[88]. The Declaration of Helsinki includes Registration as mandatory principle. Platforms for these Registries, such as www.preclinicaltrials.eu and www.animalstudyregistry.org, were created by academic initiatives and governmental organizations, respectively. Protocol registrations can be quickly accomplished in these platforms, and it can increase research value. Thus, the use of zebrafish in research must be followed by the prior approval by local or national ethics committees on animals' use in research. Information that is required for experiments' Registration, as described in the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments), are related to study design, sample size, inclusion and exclusion criteria, randomization, outcome measurements, statistical methods, experimental animals, experimental procedures, results, abstract, background, objectives, ethical statements, housing and husbandry, animal care and monitoring, interpretation/scientific implications, generalisability/translation, protocol registration, data access and declaration of interests[88].

Registration of studies with zebrafish should be free for public access because the registered protocols allow observations on studies presenting the best results[87]. Many controversies and embarrassments can be mitigated by this fast and practical registration of studies with zebrafish[89]. Zebrafish has several strains that may have different responses; thus, it is necessary having the Registry of these features[90]. It is likely thinking about increased administrative tasks or even about stealing ideas through the Register of Studies[89]; however, Registry's aim, and its major outcomes, focus on making research with zebrafish even more efficient.

5R – Reporting

In short, a scientific report is a document to describe research processes and/or results, as well as the status of a problem faced by a technical research. It may also contain recommendations, cautions and conclusions for the research[91]. The aforementioned data can be published in technical notes, film communications and scientific articles; thus, methods and techniques must be disclosed in order to make techniques' standardization easier, and to make sure of results' integrity and reliability [91]. Despite their importance, the 3Rs may be inadequate for experiments run with animals, given their limited focus on the fundamental ethical principle, due to the complexity of the "animal welfare" principle. This situation can lead to lack of practical guiding principles aimed at promoting the second fundamental ethical principle, namely: "scientific value".

Scientific reports play fundamental role in developing methods, standards and parameters that can i) reinforce the 3 Rs, and ii) formulate new thoughts that can lead to discoveries to support new bioethical pillars. Documenting and publishing methods and reports based on models are actions directly influencing ethical and bioethical aspects of animal using. In case there is no detailed record of new methods, techniques, or even studies that have already been tested, there is no possibility of optimizing animals' use and testing; therefore, there is not even the guarantee of compliance with the already established 3Rs. Furthermore, there is intrinsic and dependent relationship among research ethics, reports and records generated from the use of each animal model employed in science.

Accordingly, a meaningful report must have reliable methods and analyses based on statistical reliability to make sure of the quality and reliability of a given experiment or report. However, many experimental scientific papers are not published (50%), or do not leave the pre-print status[87]; these uncertainties only contribute to the well-known 'reproducibility crisis' affecting those seeking new methods and techniques for their experiments[92].

One way to improve reporting lies on considering the ethical and moral responsibilities of researchers, publishers, editors and funders, as well as on dealing with them in a responsible manner: *i*) researchers are in charge of clear and transparent reports; *ii*) Journal publishers and editors help putting improvements in place through their criticism and suggestions[93].

Given the challenges described above, *D. rerio* may be an excellent alternative to develop methods that contribute to reduce unambiguous reports, because its well-assessed biological model provides extensive support through publications and journals. Reports on *D. rerio* are significant due to a model that carries unique combinations of developmental and maintenance traits; it has a well-fused collection of methods that provide a system that has the advantages of running tests *in vitro* and *in vivo*[94]. This process will improve testing and the ability to replicate methods and experiments that can favour researchers in their technical responsibility to publish accurate and faithful reports[94]. This extensive collection of publications contributes to strengthen research and technological development, as well as gives strength to the other Rs, as they precisely depend on dissemination and scientific results to develop and interact with each other.

6R – Robustness

Environmental factors can change zebrafish welfare, health and response. On the other hand, robustness ensures that similar results can derive from previous experiments, even when there are small variations in reagents or experimental designs. Results are conditional and not robust when they depend on specific experimental conditions[95].

Robustness of research carried out with zebrafish can increase when results are confirmed by different methods and collaborative research. The following aspects can improve the robustness of such research: best scientific practices; using guidelines; zebrafish quality and welfare; experimental design; addition of negative, solvent and control groups; sample size estimates; effects of sex differences; suitable statistical analysis; adequate result analysis and interpretation; inclusion of negative and validation data; training and support.

7R – Reproducibility

Study reproducibility is a fundamental science principle. Researchers should be able to recreate experiments and to get to the same (or similar) results and conclusions[96]. However, oftentimes, research cannot be reproduced and they lead to resource, time and scientific credibility losses[97, 98]. Research irreproducibility can highlight experimental design issues, methodology implementation, data quality or integrity, statistical analysis error or study result interpretation[96], as well as animal quality and welfare.

Reproducibility is particularly important for emerging model systems that have available a limited number of protocols and information[99]. The Organization for Economic Cooperation and Development (OECD) currently provides guidelines for Acute Toxicity Testing in Fish[100], Fish Embryo Acute Toxicity (FET)[21], Fish, Juvenile Growth test[101], 21-day Fish Assay[102] and Fish sexual development test[103]. However, many research fields lack specific protocols for fish, such as genotoxicity[4], mutagenicity[5], and neuroscience and behavioural studies[99]. Gerlai[99], for example, describes methodological issues and solutions to ensure the reproducibility of behavioural neuroscience studies with zebrafish. Furthermore, although the 3Rs have been incorporated to the ARRIVE guidelines, additional efforts are needed to comply with laws in force, in different countries, to make sure of research integrity[104].

Thus, measures such as (*i*) ensuring access to protocols used in the studies; (*ii*) conducting well-designed reproducible research and minimizing experimental error and the number of animals used in experiments; (*iii*) being transparent and clear about results found in studies; (*iv*) correcting analysis of complex data; (*v*) avoiding cognitive bias; (*vi*) identifying gaps in knowledge about studies carried out with zebrafish; (*vii*) standardizing protocols to studies with zebrafish[98, 32, 105] must be applied to ensure the reproducibility of studies with this species.

8R – Relevance

Animal species must be able to meet all study aims when animals are used. Accordingly, planning must be carried out before any experiment is implemented in order to avoid the unnecessary use of animals. Therefore, researchers must know the features of the model system they intend to use, such as its anatomy, physiology, developmental stage, specific behaviours, among others[22]. Even if the aim of the study is to investigate human diseases, to perform toxicity tests or environmental monitoring, the relevance of the herein assessed principle must concern the transferability of an animal test. Experiments should only be carried out with a given species after taking into consideration its relevance to biological knowledge advancement[106].

Zebrafish has many relevant features for research on animal development[107], genetics[108], behaviour[109], drug discovery[110], diseases[111], vaccination[13], food safety[14], among others. Unlike other vertebrate models, its embryos have a body plan, whose beating heart, blood vessels and rudimentary intestine 48 hpf can be observed[17]. Despite the early formation of the cardiac and metabolic systems in zebrafish, experimental manipulations are possible as long as embryos get oxygen from water through passive diffusion and nutrients from the yolk sac[112]. Furthermore, fertilization and zebrafish embryo development are external, and embryos are translucent – a feature that turns them into an ideal model system for imaging during development[113, 114]. Another relevant feature lies on the fact that zebrafish's innate immune system develops in the first two days of embryogenesis[115]. The development and maturation of their adaptive immune system occur between 4 and 6 weeks after fertilization[116] - such feature makes them an ideal model system for immunology studies.

The zebrafish genome was sequenced in 2013 by Cambridge's Welcome Trust Sanger Institute. Zebrafish has 26,206 protein-coding genes, and approximately 70% of them have at least one ortholog in humans[45]. It should be noticed that approximately 84% of genes known to be associated with human diseases have zebrafish orthologs[45]. Currently, due to the development of tools - such as CRISPR-Cas9, TALENs, ZFNs - for gene editing, several mutant zebrafish strains, transgenic reporters and knockdown have been developed for studies on diseases, developmental biology, toxicology, among other fields[19]. Finally, information on zebrafish's anatomy, physiology, development and genetics was deposited in a database to make scientific communication about this species easier (www.zfin.org). All these features turn zebrafish into a relevant model for scientific research.

Principles of conduct

9R – Responsibility

Everyone involved in research with animals account for the care provided to, and use of, them. Institutions, animal ethics committees, investigators and animal caretakers must understand and accept their responsibility[67, 117, 118]. Institutions account for supporting animal ethics committees, investigators and animal caretakers. Ensuring access to veterinary advice, education and training, policies and procedures, in the process to comply with the principles of research with animals are examples of such support[67, 117]. Animal ethics committees account for validating, approving and monitoring the care provided to, and use of, animals for scientific and teaching purposes[117, 118]. Investigators and animal caretakers, including associated veterinaries, are responsible for applying high care and management standards to animal use in research[117, 118, 119, 120, 121].

Experimental animal use, social issues, scientific integrity and animal welfare are related to responsibility in research with animals[122]. Responsibility to the experimental use of animal concerns using them for scientific and teaching purposes, ad it must be justified and conducted based on ethical acceptability[118]. Responsibility to social issues includes sharing with the public the reasons why research with animals is required in order to improve health issues of animals, humans and the environment[123]. The aim is to show the impact of scientific purposes on stances that go beyond developing scientific knowledge, such as on societal consequences, as well as to share the practices and procedures adopted in research with animals[124]. Responsibility to scientific integrity comprises the design and conduct of research with animals, including legal accountabilities. It is recommended to apply the Planning Research and Experimental Procedures on Animals (PREPARE) to ensure the adoption of nowadays best practices concerning the care and management procedures for animals used in research proposals[121]. Finally, responsibility for animals' welfare requires procedures about the proper care and management provided to the selected species throughout its lifetime[118], and it includes number of animals, animals' source, biological status, transport, prevention of and relief from pain, suffering, distress and disease, appropriate housing and husbandry, as well as final disposal[22].

The principles of research with animals remain a challenge; however, well-established guidelines and clear legislations allow "R" responsibility enforcement[22]. Knowledge on zebrafish biology, physiology and behaviour is crucial at the time to apply the best practice for its care and use for scientific purposes, since it ensures zebrafish welfare over its life cycle[125, 31]. Therefore, responsibility in research with animals is associated with the duty to respect animals.

10R – Respect

According to the bioethical imperative defined by Fritz Jahr, "every living being [must be respected] on principle as an end in itself and treated, if possible, as such"[126]. Respect can be demonstrated when everyone who uses animals considers both the quality and dignity of their lives[121]. Scientific and social impact through research with animals should be cautiously evaluated based on the necessity and justification of animals' use for scientific proposals, and on animals' welfare[118]. Accordingly, "R" (Respect) in research with animals also consists in minimizing harms to animals; applying the 10Rs at all animals' care and management stages; applying the best practice for animals' care and management; and understanding and accepting one's responsibilities, as well as following recommendations of national laws and regulatory guidelines[121].

For research using zebrafish, "Respect" can be applied to knowledge on zebrafish life-history traits; to choose the appropriate life cycle stage for the scientific proposal and to adopt recommendations for animals' acquisition; maintenance, such as water quality parameters; feeding; environmental enrichment; as well as all aspects involved in animal welfare[31] in order to use these spaces in research experiments, besides the use of the 10 Rs presented in the current study.

The growing use of these animals for scientific and educational purposes reinforces the need of regulating scientific research based on using fish as model, including zebrafish[127]. Furthermore, some previous studies described zebrafish as "tool" in several research fields[128, 129, 130], as well as educational "tool". However, since ethical concerns should guide animals' use in scientific and educational proposals, the herein presented initiatives focused on the 10 Rs must be considered and implemented in order to ensure zebrafish's use in research, as a well-established as animal model, rather than just as "tool". Thus, it is essential reinforcing the urgent need of adopting "model system" or "animal model" as standard term. It is important emphasizing that respect for animals should underpin research with animals. Therefore, guaranteeing the ethical research conduct in studies with zebrafish, as well as animal welfare and compliance with scientific principles must be the very basis of principles of conduct.

Conclusions

The emerging use of zebrafish as model system in several research fields opens room for new ethical challenges. The current study describes 10 Rs ethical principles for zebrafish using as model system for research. They were classified into three categories, namely: welfare (replacement, reduction and refinement), scientific (registration, reporting, robustness, reproducibility and relevance) and conduct principles (responsibility and respect). Implementing the 10 Rs can help researchers, institutions and Animal Ethics Committees in regulating, making-decisions and promoting zebrafish health and welfare during research protocols. Standards and laws must be followed by researchers in order to ensure research integrity, as well as its relevance for human and animal heath development.

Conflict of interests

The authors declare no conflict of interest

Author Contributions

Conceptualization: T. L. Rocha; *Data curation:* A. Canedo, P. Saiki, A. L. Santos, K. da S. Carneiro, A. M. de S., G. Qualhato, R. da S. Brito, F. Mello-Andrade, T. L. Rocha; *Formal analysis:* A. Canedo, P. Saiki, A. L. Santos, K. da S. Carneiro, A. M. de S., G. Qualhato, R. da S. Brito, F. Mello-Andrade; *Methodology:* A. Canedo, P. Saiki, A. L. Santos, K. da S. Carneiro, A. M. de S., G. Qualhato, R. da S. Brito, F. Mello-Andrade; *Supervision:* T. L. Rocha; *Writing-reviewing and editing*: A. Canedo, P. Saiki, A. L. Santos, K. da S. Carneiro, A. M. de S., G. Qualhato, R. da S. Brito, F. Mello-Andrade, T. L. Rocha.

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