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The three Rs – background, legal bases and their application in animal experimentation planning in Germany

Die drei Rs – Hintergrund, Rechtsgrundlagen und deren Umsetzung in der Tierversuchsplanung in Deutschland

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Summary

In 1959, the scientists William M.S. Russell and Rex L. Burch introduced the three Rs (3Rs) – replacement, reduction and refinement – into the scientific community. They published definitions and descriptions of the general principles for the improvement of animal welfare through the implementation of humane techniques in animal experimentation. The concept of the three 3Rs should replace or reduce distress in laboratory animals. Later, the 3Rs were implemented into the European and German legislation. Every scientist who wants to carry out an experiment involving animals in Germany has to adhere to the German Animal Welfare Act (TierSchG) and the regulations on the welfare of animals used for experiments or for other scientific purposes (Tierschutz-Versuchstierverordnung - TierSchVersV). The law demands and specifies the application of the 3Rs on the development and design of animal experiments: First, the availability of alternatives must be excluded; second, the number of animals used for scientific experiments must be minimised and third, the use of refinement methods shall reduce pain, distress, harm and suffering of animals. The fulfilment of these requirements is compulsory for a project to be authorised. Many institutes, organisations and researchers generate alternative procedures and refinement methods. They provide protocols, training videos or guidance documents in journals, databases or on platforms. Nevertheless, there is a great need for research on the 3Rs. It remains very important to continue to raise consciousness of the implementation of the 3Rs into experimental planning.

Keywords: Animal welfare, German Animal Welfare Act, regulations on the welfare of animals used for experiments or for other scientific purposes, Directive 2010/63/EU

Zusammenfassung

Im Jahr 1959 haben die Wissenschaftler William M.S. Russell und Rex L. Burch die drei Rs (3Rs) – replacement (Vermeidung), reduction (Verminderung) und refinement (Verbesserung) – in die Wissenschaftswelt eingeführt. Sie veröffentlichten Definitionen und Beschreibungen der allgemeinen Grundsätze für die Verbesserung des Tierschutzes durch die Umsetzung von humanen Methoden in Tierversuchen. Das Konzept der 3Rs sollte den Disstress bei den Labortieren vermeiden bzw. reduzieren. Später wurden die 3Rs in die europäische und deutsche Rechtsprechung übernommen. Jeder Wissenschaftler, der in Deutschland Versuche mit Tieren durchführen möchte, muss das deutsche Tierschutzgesetz und die deutsche Tierschutz-Versuchstierverordnung einhalten. Das Recht fordert und spezifiziert die Umsetzung der 3Rs in der Entwicklung und Planung von Tierversuchen: Erstens muss das Vorhandensein von Alternativen ausgeschlossen werden; zweitens muss die Tierzahl der für wissenschaftliche Experimente verwendeten Tiere minimiert werden und drittens soll das Verwenden von Refinement-Methoden die Schmerzen, Leiden und Schäden der Tiere reduzieren. Das Erfüllen dieser Anforderungen ist obligatorisch für die Projektgenehmigung. Viele Einrichtungen, Organisationen und Wissenschaftler entwickeln alternative Verfahren und Refinement-Methoden. Sie bieten zum Beispiel Protokolle, Übungsvideos oder Leitfäden in Zeitschriften, Datenbanken oder auf Plattformen an. Nichtsdestotrotz besteht ein großer Bedarf an der Forschung zu den 3Rs. Es ist sehr wichtig das Bewusstsein für die Umsetzung der 3Rs in die Versuchsplanung beständig zu schärfen.

Schlüsselwörter: Tierschutz, Deutsches Tierschutzgesetz, Tierschutz-Versuchstierverordnung, Richtlinie 2010/63/EU

Introduction

In many areas of research, the use of animals is still unavoidable, due to the lack of alternative methods. This is reflected in the current numbers of animals used in research. In Berlin, annually over 250 000 animals are used in research for animal experimentation or killed for scientific purposes. Although a slight decrease is observable, the numbers clearly show that there is a huge need for the development of alternative methods. The main decrease is seen in the section of regulatory use and routine production. Here, many different tests and experiments are already replaced by alternative methods. On the one hand, alternatives have to be developed and animal numbers reduced. On the other hand, severity levels of experiments must be reduced, as well. In Berlin, 21.9 % and 2.7 % of the experiments conducted in 2016 had a moderate and severe degree of severity, revealing a clear demand for an increased application of refinement methods and further research.

Scientists, who want to perform animal experimentation in Germany are bound to follow the German Animal Welfare Act (TierschutzGesetz – TierSchG) (2006a) and regulations on the welfare of animals used for experiments or for other scientific purposes (Tierschutz-Versuchstierverordnung – TierSchVersV) (2013a). All legal bases comprise the claim for the evaluation of alternative methods, the reduction of animal numbers and the minimisation of harm, pain, distress und suffering of animals. Therefore, permission for animal experimentation projects is only granted when these three aspects, the so-called "three Rs" – replacement, reduction and refinement, are implemented in the project design and its application (TierSchG 2006b). The following sections cover the origin, legal bases and application of the 3Rs in animal experimentation.

What is an „animal experiment"?

According to section 7(2) of the German Animal Welfare Act (TierSchG2006a) animal testing is any procedure or treatment for experimental purposes, when it is connected to

1. pain, suffering, distress or harm for animals,
2. birth or hatching of animals suffering pain, distress or harm or
3. pain, suffering, distress or harm for genetically modified animals or their carrier animals.

According to section 3 number 1 of the Directive 2010/63/EU this includes any procedure, "which may cause the animal a level of pain, suffering, distress or lasting harm equivalent to, or higher than, that caused by the introduction of a needle in accordance with good veterinary practice." (European Directive 2010/63/EU 2010a)

Furthermore, animal testing can also be a treatment or action without experimental purposes, if one of the three above mentioned requirements is met and it is carried out for

1. production, isolation, storage or reproduction of substances, products or organisms,
2. total or partial harvesting of organs or tissue for transplantation, cell culture or examination of isolated organs, tissue or cells or
3. performance of experiments for the purpose of training or further education.

The killing of an animal is not considered as an animal experiment, when it is only performed to use its organs or tissue for scientific purposes.

In general, animal experimentation is only allowed for the reasons listed in section 7a (1) numbers 1-8 of the TierSchG (2006a).

The three Rs by Russell and Burch (1959)

In 1959 Russell and Burch published their book "The principles of humane experimental technique" and thereby the definition of the three Rs – replacement, reduction and refinement. Through the establishment of these three concepts, they wanted to improve the treatment of laboratory animals resp. to replace or reduce distress in these animals without decreasing the quality of animal experimentation based research. They coined the concept of a humane treatment of animals used in research. This shall be achieved through the reduction or even better elimination of inhumane, i. e. immoral, cruel, treatment of animals (Russell and Burch 1959).

While applying the three Rs, the degree of inhumanity resp. inhumane procedures of each experiment has to be evaluated. Russell and Burch differentiate between direct and contingent inhumanity. Direct inhumanity is defined as "the infliction of distress as an unavoidable consequence of the procedure employed (…) even if it is conducted with perfect efficiency and completely freed of operations irrelevant to the object in view". Contingent inhumanity is described as "the infliction of distress as an incidental and inadvertent by-product (…) of the procedure, which is not necessary for its success" (Russell and Burch 1959). In the case of direct, unavoidable, inhumanity of a certain technique or experiment, it has to be examined, if the experiment can be replaced or refined by using a different or less directly inhumane technique to get the desired information. Contingent inhumanity has to be avoided at all times, usually by applying appropriate refinement. Inhumane treatment causes distress in animals, which, in all probability, will have negative effects on biological processes and thus the desired results (depicted in Figure 1).

Russel and Burch (1959) define replacement as "any scientific method employing non-sentient material which may (…) replace methods which use conscious living vertebrates". They distinguish between absolute and partial replacement of animal experiments. The latter could be the obtainment of cells and their use in tissue culture by killing only one animal. The ideal state is the absolute replacement of all animal experiments.

Reduction is defined as "the reduction in the numbers of animals used to obtain information of a given amount and precision" (Russell and Burch 1959). The challenge is, to minimize the numbers of animals, while at the same time keeping the validity and significance of the experiment and including a possible variance in the calculated numbers of animals needed. This variance is based on the individual variance of the used animals. Moreover, "great reduction may occur (…) by the right choice of *strategies* in the planning and performance (…) of research" (Russell and Burch 1959). This implicates,

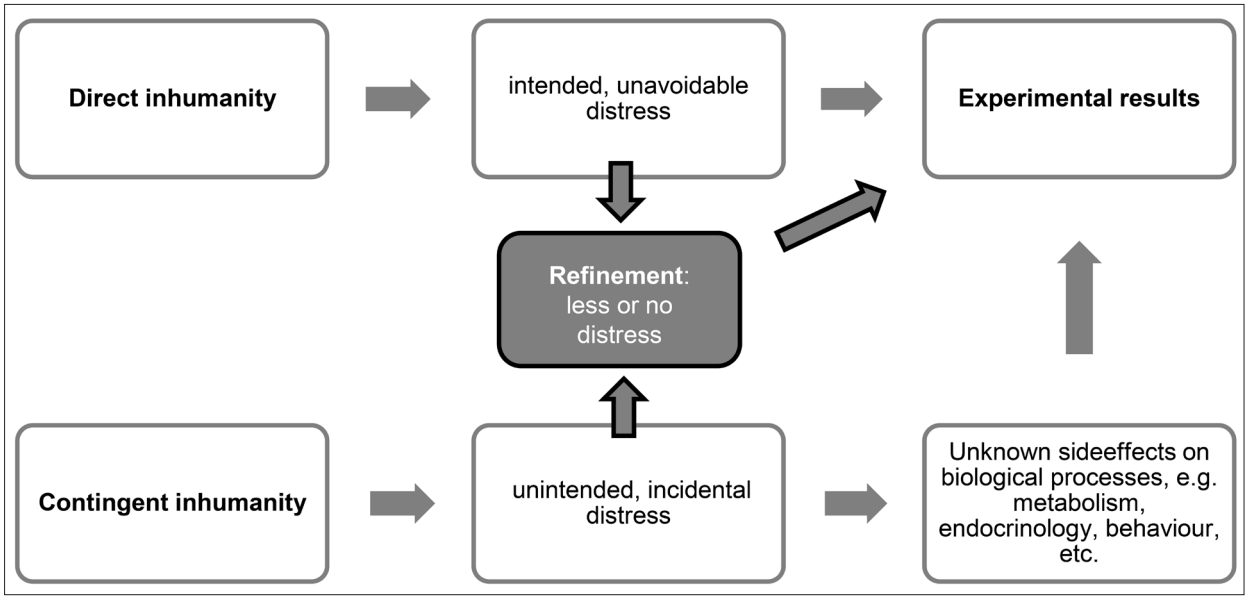

FIGURE 1: *Depiction of the influence of refinement on experimental results. The application of adequate refinement methods on inhumane procedures can reduce or eliminate distress in animals and therefore evade side effects on experimental results.*

that every person conducting research, especially when using laboratory animals, has to question their applied methods constantly.

The third R, refinement, "means any decrease in the incidence or severity of inhumane procedures applied to those animals which still have to be used" (Russell and Burch 1959).

This definition clarifies, that a person, wanting to perform research by using animal experimentation, has to check first, if the experiment can be replaced by using non-sentient material or no organism at all and second, if the numbers of laboratory animals used in the experiments can be reduced. Only if these two modes (replacement and reduction) are verified, researchers are allowed to use animals for their experiments. For these essential animals proven refinement methods have to be applied.

During the last 60 years a variety of interpretations evolved for the three Rs (Tannenbaum and Bennett 2015). Nevertheless, the three Rs originally introduced by Russell and Burch (1959) are still valid and were therefore implemented into the European legislation.

Implementation of the three Rs in the Directive 2010/63/EU

In 2010 the EU adopted Directive 2010/63/EU, on the use and protection of animals for scientific purposes, which replaced the Directive 86/609/EEC from 1986. It took full effect in January 2013. The aim of the Directive 2010/63/EU is to strengthen the legislation in the different European countries in order to improve animal welfare for laboratory animals, which still have to be used in research. Therefore, the Directive applies to all animals used for scientific or educational purposes. Furthermore, it firmly fixes the principles of the three Rs in EU legislation:

"The care and use of live animals for scientific purposes is governed by internationally established principles of replacement, reduction and refinement. (…) the principles (…) should be considered systematically (…). When choosing methods, the principles of replacement, reduction and refinement should be implemented through a strict hierarchy of the requirement to use alternative methods." (European Directive 2010/63/EU 2010b).

Among other duties, according to the sections 4 and 13 of the Directive 2010/63/EU, every Member State shall ensure

1. wherever possible, to use a scientifically satisfactory method or testing strategy without using live animals,
2. the reduction of the number of animals to a minimum without compromising the objectives of the project,
3. experiments involve animals with the lowest capacity to experience pain, suffering, distress and lasting harm,
4. refinement of breeding, accommodation and care, and of methods used in procedures, eliminating or reducing to the minimum any possible pain, suffering, distress or lasting harm to the animals and
5. that death as the end-point of a procedure shall be avoided as far as possible and replaced by early and humane end-points. Otherwise the procedure shall result in the painless deaths of as few animals as possible with the minimum possible suffering beforehand.

Further details on the requirements for the care and accommodation of animals can be found in Annex III. Among other things, this part points out further aspects of refinement, e. g., demanding a species-specific housing and enrichment to minimise pain, suffering, distress and harm in animals.

Importantly, "the elimination of pain, suffering, distress or lasting harm" by the application of the three Rs "shall not exclude the use of an animal in procedures from the scope of this Directive" (European Directive 2010/63/EU 2010c).

Implementation of the three Rs in the German Animal Welfare Act

After the adoption of the Directive 2010/63/EU, it was implemented in the German Animal Welfare Act in 2013. The sections 7 to 11b and 15 deal with animal experimentation. Every person wanting to conduct animal experimentation in Germany is bound to uphold this Act. Consequently the three Rs must be applied.

Replacement: In line with section 7a(2) no. 1 and 2, it must be verified if the objective pursued can be achieved by using other methods or procedures, always based on the current scientific knowledge.

Reduction: The number of animals used for animal experimentation has to be limited to the essential degree (TierSchG 2006c). Duplicate or repeated experimentation can only be authorised, when the necessity for the verification of existing results is scientifically justified (TierSchG 2006d).

Refinement: In pursuance of section 7(1) sentence 2 no. 1a and c and section 7a(2) no. 4, the inflicted pain, suffering, distress and harm on animals and the species-specific capacity to suffer under the experimental exposure has to be limited to the essential degree. Animals whose species-specific capacity to suffer the impact of experimentation is more strongly developed may only be used if animals whose capacity is less strongly developed are not sufficient for the pursued purpose (TierSchG 2006e). The animals which are intended for use in animal experimentation or whose tissue or organs are intended to be used for scientific purposes shall be kept, bred and cared for so that they are only stressed by the extent essential for the use in scientific purposes (TierSchG 2006f). Moreover, species-specific requirements for housing, feeding, behaviour and movement have to be provided to prevent unnecessary pain, suffering, distress and harm in the animals (TierSchG 2006g). In order to restrict the inflicted pain, suffering, distress and harm to the absolute necessary degree and to reduce animal numbers through an optimal experiment realisation, persons holding or in charge of animals and conducting animal experimentation must possess the required knowledge and abilities (TierSchG 2006h).

Implementation of the three Rs in the regulations on the welfare of animals used for experiments or for other scientific purposes (TierSchVersV)

The TierSchVersV (2013a) was implemented in August 2013. The Ordinance serves to define and implement particulars from the German Animal Welfare Act and the Directive 2010/63/EU for laboratory animals and animal experimentation.

Replacement: Section 31(1) sentence 2 no. 2a refers to the corresponding sections in the TierSchG (2006i). The screening of alternative methods must be depicted in every application for authorisation of animal experimentation.

Reduction: Section 31(1) sentence 2 no. 2a refers to the corresponding sections in the TierSchG (2006j). The number of laboratory animals must be reduced to the essential minimum.

Refinement: In addition to the TierSchG (2006), the ordinance specifies refinement for laboratory animals through

- demanding that laboratory animals, their housing conditions and the functionality of the facilities have to be checked at least once a day, that animals are transported without pain, suffering, distress and harm and that deviating terms are changed immediately (TierSchVersV 2013b),
- the demand for analgesia and anaesthesia before, during and after pain-, stress- and harmful experimentation (TierSchVersV 2013c),
- pointing out the possibility of and defining the requirements for a placement or release outside an institute, death is not naturally the end of an experiment (TierSchVersV 2013d),
- the definition of strict conditions for a re-use use of laboratory animals, i. e. the animal is healthy, a veterinary check-up was performed, the former and the new experimentation is not causing severe harm (TierSchVersV 2013e),
- the definition of the measures after achieving the objective of the experiment, esp. concerning the immediate reduction of pain, suffering, distress and harm to the essential minimum and demanding for reasonable grounds to sacrifice an animal and a death with the least pain and suffering of the animal with the required knowledge by using certain methods of killing (TierSchVersV 2013f),
- the demand for defining humane endpoints (TierSchVersV 2013g).

Advices for the application of the three Rs based on practical values

A person wanting to conduct animal experimentation needs the project approval from the competent authority (TierSchG 2006k). In Germany, these authorities have designed a special application form. It summarises and provides all the required information according to the TierSchG (2006l) and TierSchVersV (2013h) for a project application. The competent authorities of each German federal state supply the application form. Additionally, the European Commission provides guidance documents on different topics, including the preparation of projects. Guidance documents are published online (European Commission 2016). Among other things, projects will be permitted only, when the three Rs are implemented into the development and design of experiments. For each of the three Rs, this has to be reasonable and easily comprehensible. The fulfilment of these requirements is compulsory for a project authorisation. Apart from experienced researchers or university courses on experimental planning, different organisations and institutes offer guidance for the design and planning of projects on their webpages with incorporated consideration of the 3Rs. The National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) has designed a free Experimental Design Assistant (https://eda.nc3rs.org.uk/) that supports researchers in the planning of their experiments (Percie du Sert et al. 2017). Moreover, it provides the ARRIVE guidelines (https://www.nc3rs.org.uk/arrive-guidelines), developed by Kilkenny et al. (2010), that shall help and "improve the design, analysis and report-

TABLE 1: *Selection of databases (db) and reference webpages (wp) for the search of alternatives to animals and animal experimentation*

Scope	Title	Type	Webpage
Regulatory tests	AltTox	db	http://alttox.org/mapp/table-of-validated-and-accepted-alternative-methods/
	CheLIST	db	http://chelist.jrc.ec.europa.eu/
	European Commission	wp	https://ec.europa.eu/jrc/en/research-topic/alternatives-animal-testing-and-safety-assessment-chemicals
	European Pharmacopoeia	wp	https://www.edqm.eu/en/european-pharmacopoeia-background-50.html
	JRC (Q)SAR	db	https://eurl-ecvam.jrc.ec.europa.eu/databases/jrc-qsar-model-database
	OECD	wp	http://www.oecd.org/chemicalsafety/testing/animal-welfare.htm
	TSAR	db	https://tsar.jrc.ec.europa.eu/
generic	ALTBIB	db	https://toxnet.nlm.nih.gov/altbib.html
	Altweb	db	http://altweb.jhsph.edu/
	AnimAlt-Zebet	db	https://apps.bfr.bund.de/animalt-zebet/index.cfm
	AWI	wp	https://awionline.org/content/alternatives
	AWIC	wp, db	https://www.nal.usda.gov/awic
	Bf3R	wp	http://www.bfr.bund.de/en/german_centre_for_the_protection_of_laboratory_animals.html
	DB-ALM	dp	https://ecvam-dbalm.jrc.ec.europa.eu/
	EURL ECVAM	sg	https://eurl-ecvam.jrc.ec.europa.eu/databases/search-guide
	Google Scholar	db	https://scholar.google.de/
	GV-Solas	wp	http://www.gv-solas.de/
	NC3Rs	wp, db	https://nc3rs.org.uk/
	Norecopa	wp, db	https://norecopa.no/alternatives
	PubMed	db	https://www.ncbi.nlm.nih.gov/pubmed/
	RSPCA	wp, db	https://www.rspca.org.uk/adviceandwelfare/laboratory
	Web of Science	db	https://webofknowledge.com/
	3R Guide	db	https://norecopa.no/3r-guide-database
education and training	HSVMA	db	http://alted.hsvma.org/
	InterNICHE	db	http://www.interniche.org/
	LAL	wp	http://www.lal.org.uk/education-and-training
	LAWTE	wp	http://www.lawte.org/
	NORINA	db	https://norecopa.no/norina-database

ing of research using animals". The Norwegian platform Norecopa offers the recently published PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines for planning animal experiments, reducing waste and increasing reproducibility in animal experimentation (Smith et al. 2017, Norecopa 2017).

According to our experience, as employees of the State Office of Health and Social Affairs in Berlin the three Rs often miss out in animal experimentation planning. Especially the replacement of experiments is often dismissed with a few sentences or even not mentioned at all. Therefore, the common allegation of missing availabilities of alternative methods, techniques or models must be substantiated with reference sources or search results from different databases. Furthermore, it must be recorded that and where the search was conducted and why alternatives cannot be applied. Over the last years many alternatives have been established in research. Webpages and databases inform and help finding these for specific topics (e.g. regulatory tests, research in general or training and education) (Tab. 1). Upon this search, applicants have to examine and discuss whether it is possible to replace their projects in total or at least partially. Among other things, the application should at least provide answers to the following questions:

- Which alternatives will be used?
- Which alternatives were considered and why are they not applicable?
- Which sources were used to find possible alternatives?

Applicants also have to check, if and how the number of animals can be reduced to an essential minimum. Although biometrical calculations are mostly attached to the application form, in our experience, they are often not transparent or faulty. The basis of calculation has to be reasonably explained, e.g., by using former results or similar experimental setups. Computations have to be done for each part of the project, according to the specific efficiency, dependent variable, variance etc. For this, it is highly recommended to work in close cooperation with statistical consulting units of research institutes or universities for the study design and data analysis. While the calculation of the sample size is the fundament for reducing animal numbers to the essential minimum, further aspects, like the breeding scheme, genetic backgrounds, the experimental design or the origin of the animal, organ or tissue must be considered. In the process of project evaluation reoccurring questions and suggestions arise:

- Is it possible to use litter mate controls in order to reduce the number of bred animals?
- How is the breeding scheme? Animals with what kind of genotypes are mated?
- Is it possible to have the control in the same animal (using imaging procedures over a period of time or comparing right and left side, e. g., in transplantation or implantation experiments)?
- Is it possible to perform several experiments with identical control groups at the same time, to reduce the number of control animals?
- Is it possible to structure the experiments sequentially (later parts will only be done, if the preceding parts were successful)?
- Starting point of dose-finding studies: Is it possible to start in the middle and increase or decrease the dose to avoid that all dosages are being tested?
- Is it possible to collaborate with other research groups or institutes in order to reduce the number of used animals (joint projects, joint use)?
- Is the background of the different animal strains identical?
- Is it possible to get more information from the same animals?
- Is it possible to share organs and tissues of killed animals?

Ideally, all of these questions should be addressed by the applicants. Concerning the last question, it is advisable to consult sharing-platforms, like "AniMatch" (https://www.animatch.eu/). Its intention is the exchange and

sharing of organs and tissue of laboratory animals used for scientific purposes in order to reduce the animals used in research. To avoid the repetition of experiments due to unknown and unwanted effects based on genetic differences, animals with identical backgrounds should be used. Kelmenson (2016) and Sacca et al. (2013) explain the importance of the same genetic background by taking the example of the C57BL/6 mouse strain and its substrains.

Another important step is the publication of negative results after project completion, to prevent repeated failure and experimentation. For this, the Public Library of Science (PLOS) started a collection called "Missing Pieces" (http://collections.plos.org/missing-pieces) and publishes negative, null and inconclusive results. Moreover, scientific journals like "Negative Results" (http://www.negative-results.org/) pave the way for the exclusive publication and discussion of negative findings. Likewise in the past, different journals, like the Journal of Negative Results in Biomedicine (https://jnrbm.biomedcentral.com/) or New Negatives in Plant Science (https://www.journals.elsevier.com/new-negatives-in-plant-science), published negative results. Although these journals are now discontinued the published data remain available for the exchange of information.

Despite the European and German legal bases repeatedly demand the implementation of refinement, the influence of refinement on the wellbeing of animals and thereby on the experimental outcome still seems to be underestimated and thus, in our experience, is often neglected in experimental planning. In order to find the best refinement, it is important to know the amount of pain, suffering, distress and harm that an animal experiences during the application of specific techniques, through disease models, housing conditions, breeding, genotypes etc. For this purpose, Annex VIII of the Directive 2010/63/EU defines different severity categories and lists and grades a number of common examples. In addition, the European Commission published a guidance document on prospective severity assessment, its integration in project planning and examples (European Commission 2012). Furthermore, specialists offer support by publishing their experience, results, statements, tutorials etc. online (Tab. 1 and 2). Different studies (Hawkins et al. 2011a, Hawkins et al. 2011b) or even software prototypes, like SONET (http://www.gv-solas.de/index.php?id=61), help planning and designing protocols for the assessment of animal welfare and severity of procedures. In general, recognising and evaluating symptoms of pain and distress in different animal species can be difficult and is still widely under discussion. Hence, a lot of research on this topic is in progress. Published examples and recommendations can help with this task (e.g. general guidance documents by the National Research Council (2008, 2009) or by Jones et al. (1998) and webpages and databases listed in Tab. 1 and 2). Scientists also published recommendations on the welfare assessment of genetically altered mice and rats (Wells et al. 2006, Zintzsch et al. 2017). Using validated animal species-specific grimace or pain scales helps identifying pain and distress during the ongoing experiment (Tab. 3). The identification of pain and distress can and should lead to a fast refinement and thereby reduction of severity.

Using the prospective severity assessment helps finding the best refinement strategies. A lot of refinement recommendations and training modules for the realisation of procedures, the experimental design, handling, the appropriate analgesia and anaesthesia regime, the housing conditions during an experiment, genotyping techniques and the definition of humane endpoints are published and widely available (Tab. 4). Some of them can be found in specific refinement and enrichment databases, in books and on platforms and webpages (Tab. 2, in addition to Tab. 1). The daily check or the husbandry in an especially protected environment (i. e. cage system) are not representing a special refinement method. By law, animals and their facilities have to be

TABLE 2: *Selection of databases (db), reference webpages (wp), platforms (pf) and books on welfare and pain assessment and for the search of refinement and enrichment methods*

	Type	Topic	Webpage/reference
Animal Welfare Institute	db	Environmental Enrichment and Refinement of Husbandry for Nonhuman Primates	https://awionline.org/content/primate-enrichment-database
	db	Refinement of Housing and Handling Conditions and Environmental Enrichment for Animals Kept in Laboratories	https://awionline.org/content/refinement-for-animals-kept-in-labs-database
	pf	Refinement Forum (LAREF)	https://awionline.org/content/refinement-forum-laref
NC3R	wp	Anaesthesia	https://www.nc3rs.org.uk/anaesthesia
		Analgesia, pain assessment	https://www.nc3rs.org.uk/analgesia
		Grimace scales, pain assessment	https://www.nc3rs.org.uk/grimacescales
		Macaque welfare assessment	https://www.nc3rs.org.uk/macaques/welfare-assessment/
		Welfare assessment	https://www.nc3rs.org.uk/welfare-assessment
GV-Solas	wp	Anaesthesia, analgesia	http://www.gv-solas.de/index.php?id=33
AHWLA	wp	Welfare and pain assessment, handling	http://www.ahwla.org.uk/site/Tutorials.html
PennState	wp	Training videos	https://www.research.psu.edu/arp/training/videos.html
	book	Anaesthesia	(Flecknell 2015)
		Anaesthesia and analgesia	(Fish et al. 2008)
			(Hawk et al. 2005)

TABLE 3: *Selection of grimace and pain scales for different animal species*

Animal species	Reference
Cat	(Reid et al. 2017)
Dog	(Reid et al. 2007)
Horse	(Dalla Costa et al. 2014)
Lamb	(Guesgen et al. 2016)
Macaque	(https://www.nc3rs.org.uk/macaques/welfare-assessment/health-indicators/)
Mouse	(Langford et al. 2010)
Piglet	(Viscardi et al. 2017)
Rabbit	(Keating et al. 2012)
Rat	(Sotocinal et al. 2011)
Sheep	(McLennan et al. 2016)

TABLE 4: *Selection of journal articles on refinement of different disease models and scientific procedures*

Disease model/ scientific procedure	Reference
Abdominal tumour models	(Paster et al. 2009)
Experimental autoimmune encephalomyelitis	(Saul et al. 2017, Wolfensohn et al. 2013b)
Genotyping techniques	(GVG Genetic Monitoring GmbH (http://www.gvg-gm.de/en/products/snooplex-fastprep/), Mitrecic et al. 2008, Otano-Rivera et al. 2017)
Models involving biotelemetry	(Hawkins 2014)
Models involving seizures, convulsions and epilepsy	(Wolfensohn et al. 2013a)
Rheumatoid arthritis	(Hawkins et al. 2015)
Sepsis and septic shock	(Lilley et al. 2015)
Stroke	(Lourbopoulos et al. 2017)
Toxicity evaluation of insulin analogues	(Jensen et al. 2017)

TABLE 5: *Selection of training programs as refinement and enrichment for laboratory and shelter animals*

Animal species	Reference
Cat	(Kogan et al. 2017)
Dog	(Berns et al. 2012, Meunier 2006)
Mouse	(Leidinger et al. 2017)
Primate	(Perlman et al. 2012, Westlund 2015)

inspected on a daily basis (TierSchG 2006m) and the housing conditions have to ensure, that animals do not suffer pain, distress or harm (TierSchG 2006n) and that they have to conform with Annex III of the Directive 2010/63/EU (TierSchVersV 2013i). Therefore, further refinement could be more nesting material, play and hiding places, larger cages and/or checking the animals more than once daily. Different studies show that this environmental enrichment can improve animal health for different animal species, like fish (Wafer et al. 2016) or rats (Castelhano-Carlos et al. 2017). Various suggestions for enriched housing conditions can be found, for instance, for mice (Slater and Cao 2015), rodents and rabbits (Baumans 2005) or non-humane primates (NC3Rs 2017). Special enrichment of the environment can be used to habituate animals to specific experimental setups, as head fixation, restraining, different tastes of diets and water or different litter or cage bottoms, to reduce distress during the experiment. The preceding training of certain handling or application techniques (Tab. 5) could be another important stress reducing refinement and enrichment step before starting the experiment. Additionally, camera assisted surveillance can reduce handling times, shift experiments to the periods of activity (e.g., the night for mice) and provide video training material for other researchers.

Concerning refinement, the central question is: How are animal pain, distress, harm and suffering minimised in order to achieve the research objectives? This question must be addressed in experimental planning and the project proposal.

The bottom line is that there is still a lot of potential of research on the 3Rs and their application. It remains very important to continue raising consciousness of the implementation of the 3Rs into experimental planning permanently.

Conflict of Interests

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Ethical approval

The ethical approval is neither applicable nor required.

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Authors contribution

A.K. designed the study and wrote the manuscript. J.H. contributed to the study design and proof-reading.

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