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Guidelines

of the Working Group of Berlin Animal Welfare Officers

on severity assessment and classification of genetically altered mice and rat lines

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Contents

Background information of the severity classification 4

I. Assignment to a severity degree 5

II. Severity assessment and classification 8

Examples for the classification of the symptoms of genetically altered mice and rat lines into severity degrees 14

1 Lethal factors 16

2 Behavioural disorders 17

3 Alterations of the skin and the coat 19

4 Diseases of the sensory organs 20

5 Neurological diseases 21

6 Diseases of the immune system 25

7 Cardiovascular and haematological diseases 26

8 Diseases of the respiratory tract 27

9 Diseases of the digestive system 28

10 Metabolic diseases 29

11 Reproductive diseases 30

12 Tumour diseases 30

13 Renal diseases 32

14 Alterations of the locomotor system 33

Glossary 36

Authors 39

Imprint 39

References 40

Appendix A - Recommendation on the required number of animals for evaluating an increase in harmful phenotypes in mouse and rat lines 45

Modification of genetic material is an important tool in biomedical research for studying genetic functions, their implications and also disease models. The phenotypical expression is versatile and, depending on the type of genetic manipulation, may affect the well-being of the animals. Under the new Directive 2010/63/EU[1], the investigation and assessment of pain, suffering or distress caused by genetic modifications has come into focus. As intended by the 3R principles (Replacement, Reduction, Refinement), a harmful phenotype must be characterised and reduced to what is strictly necessary for the purpose of the experiment.

These guidelines reflect the initial experiences of defining the severity degrees of genetically altered lines and should provide assistance in the assignment of severity degrees. Line-specific properties, different manifestations of symptoms and institution-specific housing conditions must be taken into account when the severity degree is selected. It is for this reason that the assessment of certain disease patterns may differ from this recommendation.

Professional discussions on the severity degree categories and the provision of further examples are expressly requested and should be addressed to info@ak-tierschutzbeauftragte.berlin. These guidelines will be continuously reviewed and extended on this basis.

Background information of the severity classification

A harmful phenotype as defined by the German animal welfare legislation includes the pain, suffering or lasting harm inflicted on an animal as a consequence of genetic modification.

The Working Group of Berlin Animal Welfare Officers refers to the appropriate needs of mice and rats bred in a laboratory environment when assessing the severity degree of harmful phenotypes.

Deviations from normal behaviour and morphologic appearance must be judged under specific breeding conditions of experimental animals. The consequences for the performance of typical behaviours are assessed. The assessment is made under aspects of experimental animal breeding and pathocentrism and considers all factors leading to pain or distress. Lasting harm is rated as being harmful when it causes pain or distress. The assessment is based on the latest state of scientific research and the principles of the Five Freedoms[2].

If a genetic modification is likely to result in a potentially harmful phenotype, the strain is classified as a harmful phenotype until the opposite is proved by the basic welfare assessment.

Genetically altered animals are all animals with a known genetic alteration in comparison with the standard background strain. These include those resulting from the creation of endonuclease-mediated strains, lines which have stably integrated a transgenic sequence either via homologous recombination (in embryonic stem cells) or by random integration events , strains created via physico-chemical treatments and strains which are developed by identification and selection of spontaneous mutation.

I. Assignment to a severity degree

The assessment of the severity degree of a genetically altered line is often a challenge because of the lack of objective criteria by which the different phenotypic modifications can be assessed. The available collection should serve as a reference for making a comparable and sound assessment and for assigning similar modifications to an adequate severity degree. The assignment is based on the assessments of scientists, animal welfare experts and the available literature.

Criteria for the selection of a severity degree

Non-harmful Phenotype

Following the Directive 2010/63/EU, 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[1] is considered to be harmful. Phenotypic modifications must therefore pass a threshold of phenotypic changes in order to be relevant with regards to the well-being of the animal and animal welfare legislation. If this threshold is not passed, a modification may be categorised as a non-harmful phenotype.

Mild

Directive 2010/63/EU classifies as “mild” those genetic modifications which cause animals to experience short-term mild pain, suffering or distress with no significant impairment of the well-being or general condition of the animals[1].

Moderate

Directive 2010/63/EU classifies as “moderate” those genetic modifications as a result of which the animals are likely to experience short-term moderate pain, suffering or distress, or long-lasting mild pain, causing moderate impairment of the well-being or general condition of the animals[1].

The Working Group of Berlin Animal Welfare Officers considers animals to be at least moderately stressed if it is possible to clinically observe significant deviation from the animal’s general condition[3,4].

Harmful phenotypes must be categorised as at least moderate if

- the lifespan is reduced in comparison with the genetic background strain,
- normal intake of food and movement are impaired,
- a systematic disease occurs which results in an observable deviation in a parameter such as growth rate, body size, anatomy or behaviour[5].

The Working Group of Berlin Animal Welfare Officers recommends checking on a case-by-case basis to see whether the animals result to be in pain or distress.

Severe

Directive 2010/63/EU classifies as “severe” those genetic modifications as a result of which the animals are likely to experience severe pain, suffering or distress, or long-lasting moderate pain, suffering or distress, causing severe impairment of the well-being or general condition of the animals[1].

The Working Group of Berlin Animal Welfare Officers shares this evaluation.

In principle, the indicators for disease states induced by procedures of animal testing also apply to the **categorisation of pain, suffering or distress caused by genetic modifications**[1,4–10]**.** The following may indicate impairment of general condition[3,4,6,9]:

- External appearance, e.g. coat (piloerection, dull coat, dishevelled), skin discoloration (pale, yellowish, reddened), eyes (opaque, sunken, swollen, lids stuck together, lacrimation)
- Pain, e.g. on the basis of the facial expression[11,12], posture (hunched back), behavioural changes, or an altered reaction to manipulation (increased aggression, vocalisation), automutilation
- Mobility, e.g. reduced mobility, including limbs, shifting of weight, uncoordinated movement, limited righting reflex
- Behavioural changes, e.g. isolation from cagemates, reduced spontaneous behaviour,
- Significant loss of body weight
- Reduced intake of food and water

II. Severity assessment and classification

Practical guidance for the implementation of the severity assessment is available in the forms provided by the Bundesinstitut für Risikobewertung (BfR - The Federal Institute for Risk Assessment)[13,14]:

- Assessment of new-born litter
- Assessment of litter at weaning stage
- Assessment of individual adult animals
- Final assessment of genetically altered lines

Time points of investigation and parameters should be adapted to the prospective severity assessment of a line. The expected and the unexpected harmful phenotype should be characterised by systematic examinations at all age stages. This basic examination provides the foundation on which an animal is assigned to a severity degree. Further information on examination criteria and respective forms are provided in the recommendations of the BfR[14].

Information on the genetic background and housing conditions (particularly the hygiene status) should be documented so that differences in the phenotypic manifestation of genetically altered lines can be judged adequately. The designation of the lines should follow the internationally established rules of nomenclature.

Guidelines for Nomenclature of Mouse and Rat Strains[15]

Nomenclature Tutorial[16]

ILAR Laboratory Codes[17]

Characteristics relevant to the harmful phenotype should be summarised in the “Final assessment of genetically altered lines” and passed on together with information on the genetic modification when animals are transferred.

a. Following the BfR recommendations, what lines need a basic welfare assessment?

- Newly generated lines and new crossbreeds from genetically altered lines,
- Imported genetically altered lines which have not yet been systematically assessed. All information from the last breeder and user should be considered.
- New lines generated by the fixation of spontaneous mutations by positive selection.

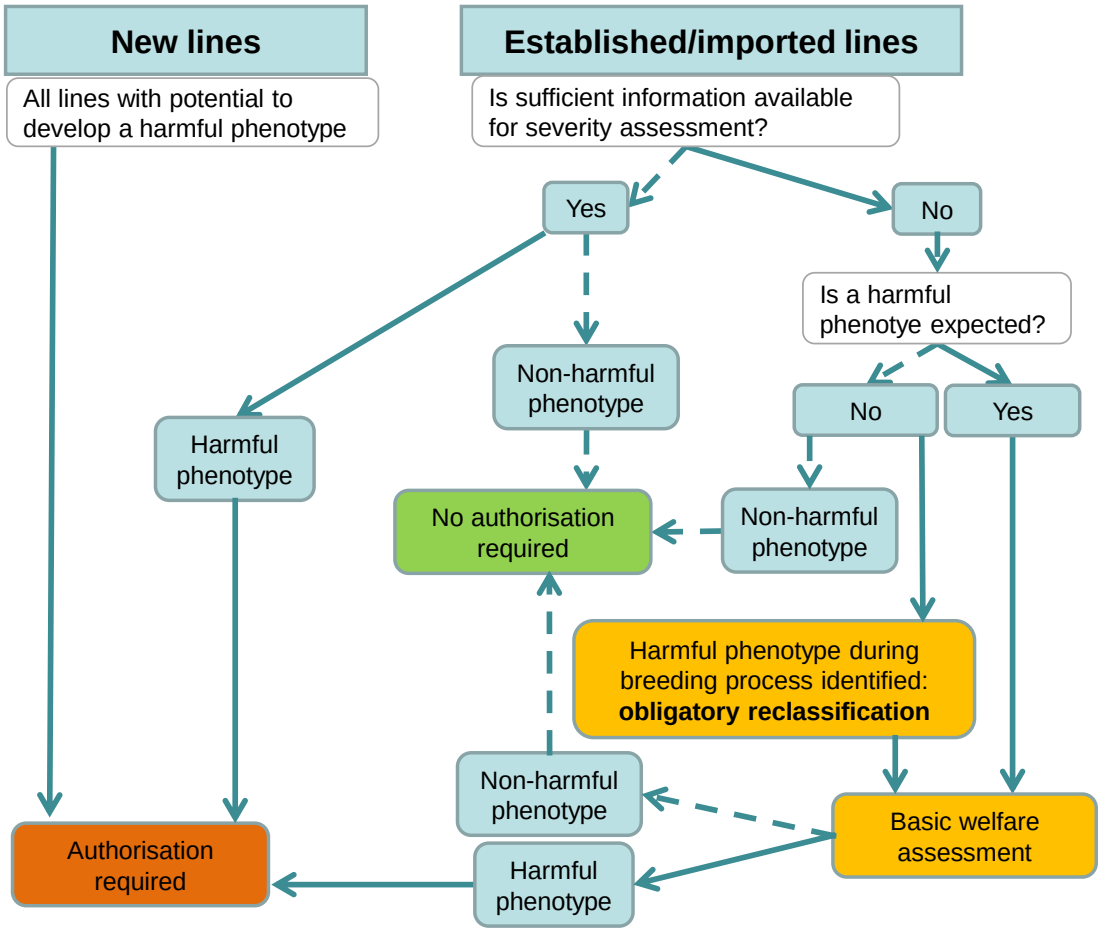
The Working Group of Berlin Animal Welfare Officers recommends a new assessment of the line when the genetic background strain is changed.

b. Following the BfR recommendations, what lines do not need a basic welfare assessment?

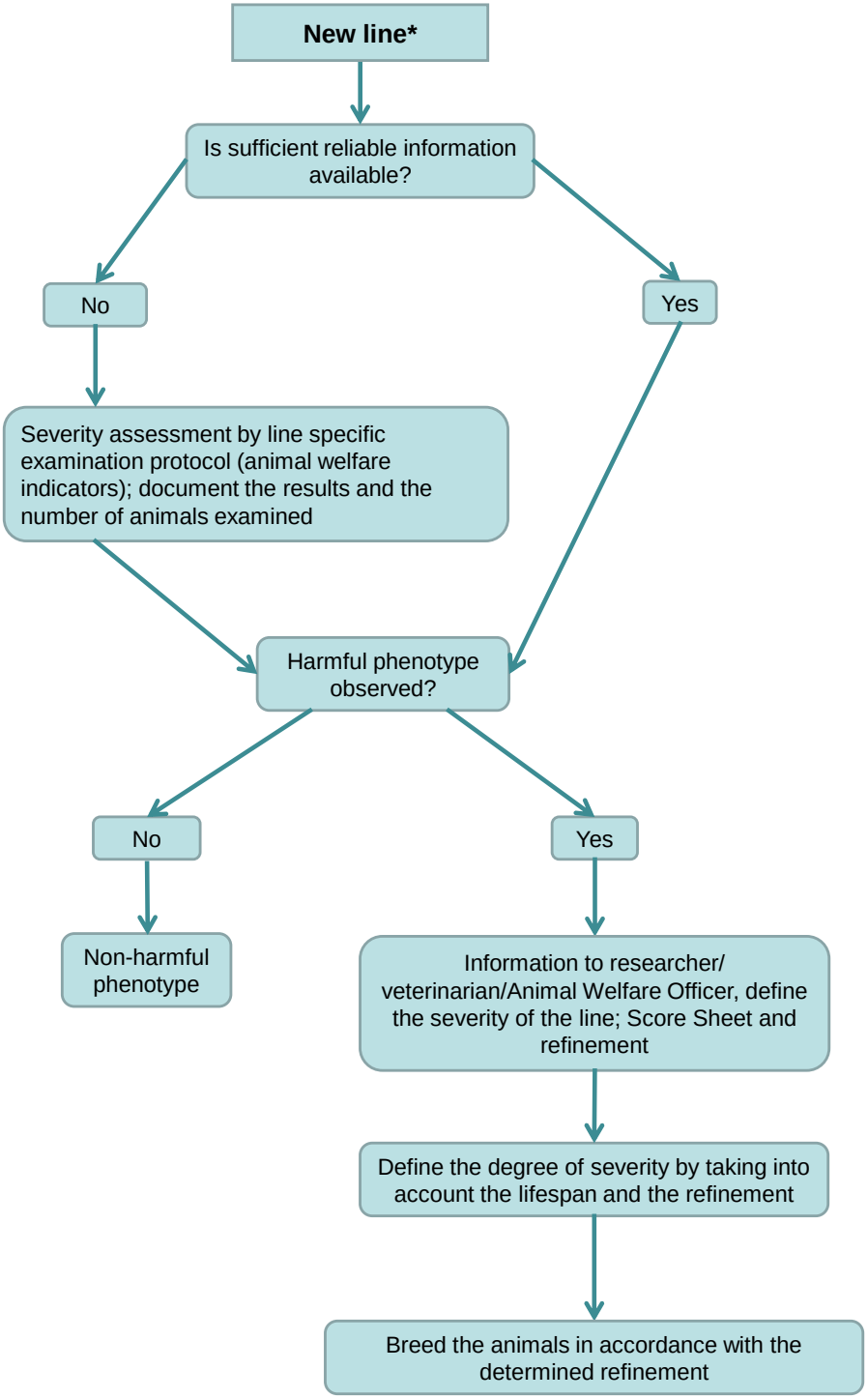
- Lines in which the administration of inductors triggers the altered phenotype (before the induction, e.g. with Tamoxifen).
- Lines in which the type of genetic alteration does not cause any burden (e.g. Cre/loxP system before crossing Cre with loxP (floxed) mouse or reporter lines).
- Wild type lines with or without standardised background or recombinant inbred strains

A final assessment of each genetically altered line should be in place.

Legal requirements for the severity assessment



Practical approach to the severity assessment



* Also applies to imported lines.
When a new line arrives at the institution, line-specific information should be checked when the animals are taken over. In particular, the source from which the information is coming (e.g. from publications, databases or systematic examinations) and the conditions under which the information was obtained should be verified so that it can be evaluated on the background of local conditions.

c. Which animals should be used for the assessment?

- Animals of the required genotype for the entire breeding and housing period
- No additional breeding or longer housing than planned for the purpose of the experiment

The number of animals to be assessed per line is currently at least 14 with the appropriate genotype (7 ♂, 7 ♀) from different litters[18,14].

This recommended 14 animals to be examined is not based on any statistical analysis. Therefore, the number of animals was recalculated for two probabilities of occurrence, taking into account the expected allele frequencies, the probable penetrance and inheritances and using a Fisher's Exact Conditional Test as a basis. This is necessary for the secure recognition of harmful phenotypes in the modified line (**Appendix A**). This shows that an analysis of 10 animals is sufficient to document a state of higher severity with a power of 80 %. A higher number of animals might be necessary in exceptional cases (e.g. the low penetrance of a specific phenotype).

Animals of corresponding genetic backgrounds or target strains serve as controls. During the establishment of a line, wildtype littermates are particularly suitable if the genetic alteration concerns an undefined genetic background and the generation of a congenic strain is not yet completed.

d. What role do the refinement measures play in the severity assessment?

As soon as a harmful phenotype is detected (also in individuals), measures must be taken to reduce distress. Line and experiment-specific refinement measures should always be developed and implemented in cooperation with the responsible researchers, animal welfare officers and animal care takers.

In case of progressive harmful phenotypes early refinement measures should be implemented. Moreover humane endpoints for housing in the breeding establishment should be defined. If compatible with the objective of the experimental purpose, the animals should be used before a harmful phenotype occurs.

Basically, refinement measures may reduce the degree of severity. Therefore, the categories to which lines are assigned using the applied refinement measures may differ among various institutions. However, a refinement will never lead to exemption from the authorisation requirement.

Refinement measures in breeding and housing

Scoring and humane end-points, e.g.

- Intensive monitoring using score sheets including defined symptoms and appropriate handling instructions.
- Instructions should minimize distress and ensure termination at the earliest point possible

Nutrition, e.g.

- Administering agar packs, moist food, glucose, probiotics or vitamins

Medicinal treatment, e.g. with

- antibiotics or analgesics

Housing environment, e.g.

- additional nesting material for hypothermic animals

Examples for the classification of the symptoms of genetically altered mice and rat lines into severity degrees

1 Lethal factors
2 Behavioural disorders
3 Alterations of the skin and the coat
4 Alterations of the sensory organs
5 Neurological diseases
6 Diseases of the immune system
7 Cardiovascular and haematological diseases
8 Diseases of the respiratory tract
9 Diseases of the digestive system
10 Metabolic diseases
11 Reproductive diseases
12 Tumour diseases
13 Renal diseases
14 Alterations of the locomotor system

More examples are constantly added to the collection by the Working Group of Berlin Animal Welfare Officers. Examples and suggestions regarding the categorisations can be sent to info@ak-tierschutzbeauftragte.berlin.

How to use the table:

The table below is designed for the assessment of mice and rats.

We provide a recommendation for the classification of symptoms into severity degrees of individual animals. The highest degree of severity is the determining factor for categorising a line. The harmful phenotype of an individual animal does not necessarily have to correspond to the categorisation of the entire line. Different genotypes and age groups may exhibit varying severity degrees.

The respective phenotypes should be evaluated according to aspects of duration and magnitude.

If several symptoms from different categories occur, the cumulative effect should be considered, which may lead to a higher severity category.

The category “non-harmful phenotype” comprises phenotypes which do not cause any impairment of well-being under housing conditions which correspond to the current standards for laboratory animals.

Grey fields indicate that no corresponding example is known to us yet.

The following categorisation of symptoms and diseases relates to harmful phenotypes without refinement measures. Distress can and should, wherever possible, be reduced using appropriate refinement measures.

No.	Symptom/disease	Non-harmful phenotype	Mild severity	Moderate severity	Severe severity	Monitoring, Refinement, special housing requirements
1	**Lethal factors[19]**					
1.1	General		Peracute death or death until 5 days post-partum (P5) (due to decreased perception of pain and distress[20–22])	Lethal until 2 weeks post-partum (e.g., underdeveloped, leukopenia, anaemia, microencephaly)	Animals found dead with *unknown*[l] cause of death from 2 weeks post-partum	
1.2	Lethal syndromes				e.g. Morbus Gaucher with fully developed clinical characteristics: growth retardation, neglected coat with dry skin (tail), late opening of eyes on P7, from P14 restricted motor functions, emaciation, paralysis, hyperextension of	

[l] Unless an informed decision can be made that it is unlikely the death was preceded by severe suffering.

No.	Symptom/disease	Non-harmful phenotype	Mild severity	Moderate severity	Severe severity	Monitoring, Refinement, special housing requirements
					the neck, seizures when touched, death at 3 weeks	

2 Behavioural disorders

2.1	Alterations of the activity pattern					
2.1.1	Increased activity, e.g. circling, wire-gnawing, backflipping		Mildly compromised general condition, loss of body weight < 10%	Moderately compromised general condition, loss of body weight < 20 %	Severely compromised general condition, loss of body weight > 20 %	
2.1.2	Reduced activity, e.g. autism					
2.2	Alterations of social behaviour					
2.2.1	Compromised maternal behaviour[23]					
	For the offspring		Reduced nest-building behaviour of dam; prolonged absence from offspring with normal development of young animals; no distinct lactating	No nest-building behaviour, but offspring together with dam; stress because of vocalisation of offspring (cold stress); reduced fluid and nutrient	Separated offspring; infanticide behaviour of the dam; offspring dies because of hypothermia due to absence of maternal care	Group together with an experienced dam or rearing of offspring on nurse, feeding additional mother's milk or milk substitute

No.	Symptom/disease	Non-harmful phenotype	Mild severity	Moderate severity	Severe severity	Monitoring, Refinement, special housing requirements
			posture over offspring (crouching); limited time within the nest, but grooming by the dam after suckling	supply due to reduced maternal care		
2.2.2	Increased susceptibility to stress, leading, e.g., to anxiety disorder, aggressiveness[II]			In social groups: differences in body weight from 15%[III] due to competition for food (dominant behaviour)	Physical lasting harm, e.g., auto-mutilation or injury to cage mates	
2.2.3	Barbering[24]		Lack of tactile hairs without compromised general condition, no abnormal behaviour	Lack of tactile hairs with compromised general condition and abnormal behaviour, classification depends on degree of expression		Separation of affected animals

[II] Alterations of behaviour may not be clearly demarcated.
[III] Comparison between animals of the same genotype.

No.	Symptom/disease	Non-harmful phenotype	Mild severity	Moderate severity	Severe severity	Monitoring, Refinement, special housing requirements
3	**Alterations of the skin and the coat**					
3.1	Alterations of the coat[25,26]	Lack of coat under thermoneutral housing conditions (temperature, group-housing, environmental enrichment)	Lack of coat (e.g. nude mice) and housing under sub-thermoneutral conditions, classification depends on inside temperature of cage and duration[27–29]			Housing under higher ambient temperatures, provide more bedding and nesting material[30], high-energy food
3.2	Pruritus		Repetitive, short-term scratching, e.g. with scaly skin		No wound healing, permanent scratching	
3.3	**Inflammatory skin diseases**					
3.3.1	Lupus erythematodes (see also 6.1 and **Error! Reference source not found.**)		Inflammatory alteration of the skin, mainly on the upper back, neck and ears, e.g. alopecia, erythema and deep lesions of the skin[31], classification depends on degree of expression			
3.3.2	Comèl-Netherton Syndrom				Erythroderma, severe pruritus, skin detachment, growth retardation[32]	

No.	Symptom/disease	Non-harmful phenotype	Mild severity	Moderate severity	Severe severity	Monitoring, Refinement, special housing requirements
3.4	Dystrophic epidermolysis bullosa				Severe, extensive alterations of the skin (blisters), even limbs may be lost, changes of mucous membranes with compromised food uptake, hyperalgesia[33]	

4 Diseases of the sensory organs[IV]

4.1	Eyes					
4.1.1	Increased sensitivity to light[34], e.g., under albinism	Albinotic strains, if light intensity is adapted to the increased light sensitivity[35]	Increased sensitivity to light with watery eyes, classification depends on degree of expression			Housing animals in dimmed areas
4.1.2	Absence of exocrine glands			e.g., lack of meibomian glands[36], classification depends on the follow-up symptoms (Keratoconjunctivitis sicca)		Tear substitute gel
4.1.3	Microphthalmia, anophthalmia	Blindness[V] (e.g.,				House animals in

[IV] The lack of more than one sense is considered to cause an impairment that should be classified as harmful phenotype

[V] If the animals are kept in a constant environment.

No.	Symptom/disease	Non-harmful phenotype	Mild severity	Moderate severity	Severe severity	Monitoring, Refinement, special housing requirements
		small or no eyes) without impairment of normal behaviour				constant environment
4.2	Hearing disorder	Deafness[V] without impairment of normal behaviour				
4.3	Disorder of the sense of smell		Reduced food uptake due to impairment or lack of the sense of smell. Classification depends on the follow-up symptoms			
4.4	Disorder of the tactile sense		Lack of tactile hairs without compromised general condition, no abnormal behaviour	Lack of tactile hairs with compromised general condition and abnormal behaviour, classification depends on degree of expression		

5 Neurological diseases

5.1	Motoric deficits - general	Altered gait without motoric impairment	Mild motoric impairment without loss of body weight	Motoric impairment without paralysis, with body weight loss < 20%	paralysis, that results in reduced food and water uptake	Moist food on the cage floor, increased energy intake, e.g. glucose

No.	Symptom/disease	Non-harmful phenotype	Mild severity	Moderate severity	Severe severity	Monitoring, Refinement, special housing requirements
						substitution
5.2	Altered pain perception	Hyperalgesia			Hyperalgesia leading to rest of grooming behaviour and reduced activity, vocalisation when handled	
5.3	Seizures		Focal periodic seizures[6]	Spontaneous short-term seizures when the symptoms after the seizure are not more than short-term and mild and the animal recovers completely between the episodes, e.g., short generalized seizures induced by handling; epilepsy with lethal outcome with	Lasting tremor with body weight loss, longer lasting periods of generalized seizures with reawakening[5,6]	Gentle handling, no loud sounds

No.	Symptom/disease	Non-harmful phenotype	Mild severity	Moderate severity	Severe severity	Monitoring, Refinement, special housing requirements
				complete loss of conscience[VI,6]		
5.4	Morbus Huntington		Classification depends on severity of symptoms, e.g., body weight loss, loss of coordination, involuntary and uncontrolled movements, even physical inactivity			
5.5	Rett syndrome[38]		Motoric and behavioural deficits and early death (11th week to 12th month of life), classification depends on phenotypic expression			
5.6	Spontaneous autoimmune encephalomyelitis with ascending paralysis[4,39–44]	No clinical symptoms	Slack tail, impaired gait, without loss of body weight	Paresis of the hind limbs without involvement of the forelimbs for more than 24h, body weight loss < 20%	Paralysis of the hind limbs and paresis/paralysis of the forelimbs, righting reflex > 5 sec, impairment of defecation and urination Body weight loss > 20%, food and water uptake is not possible independently	Longer bottle caps, Moist food on cage floor, additional nest-building material, but no shelter (risk of injury), glucose substitution, fluid substitution, monitoring and if applicable, manual emptying of the bladder, increased

[VI] Cannot be awakened by noise, tactile stimuli, no response to pain stimuli (toe interdigit reflex), Definition for loss of conscience, also see AVMA Guidelines for the Euthanasia of Animals, 2013 [37]

No.	Symptom/disease	Non-harmful phenotype	Mild severity	Moderate severity	Severe severity	Monitoring, Refinement, special housing requirements
						frequency of cage change
5.7	Alzheimer Disease	Motoric and cognitive defects only detectable by specific tests, no impairment within the normal cage environment			Paralysis of the limbs with hunched body posture, food and water uptake is not possible independently[45]	
5.8	Amyotrophic lateral sclerosis (ALS) using the example of transgenic mice for human SOD1^{G93A} [46–49]		Mild motoric impairment without body weight loss	Muscle weakness, paresis of one or both hind limbs for more than 24h, impaired grooming behaviour, body weight loss < 20%	Rigid, spastic paralysis or minimal joint mobility, limb not used for movement, righting reflex > 5 sec, body weight loss > 20%, food and water uptake is not possible independently	Moist food on cage floor, additional nest-building material, but no shelter (risk of injury), glucose substitution, fluid substitution, monitoring and if applicable, manual emptying of the bladder, increased frequency of cage change[39,42]

No.	Symptom/disease	Non-harmful phenotype	Mild severity	Moderate severity	Severe severity	Monitoring, Refinement, special housing requirements
5.9	Holoprosencephaly[50]		Malformation of the forebrain and the facial skull (shortened nose, flattened forehead), Microphthalmia or Anophthalmia, no impairment of general condition or normal behaviour			

6 Diseases of the immune system

6.1	Lupus erythematodes[51]		Classification depends on expression of skin alteration (3.3.1) and glomerulonephritis (see **Error! Reference source not found.**)			Regular monitoring for renal insufficiency with urine test strips
6.2	Rheumatoid arthritis see 14.3.1					
6.3	Immunodeficiency[VII]	Without	Classification depends on severity of symptoms, e.g.,			Special hygiene

[VII] Immunodeficient mice which cannot control pathogens, e.g., knock-outs of various cytokines and animals with immuno-cell deficiencies, dysfunctions or -restrictions

No.	Symptom/disease	Non-harmful phenotype	Mild severity	Moderate severity	Severe severity	Monitoring, Refinement, special housing requirements
		infectious disease[VIII]	diarrhoea (see 9.4), rectal prolapse (see 9.1), pneumonia (see 8.2)			management (e.g., SPF barrier housing), killing in case of rectal prolapse and focus on the anal region during routine controls, antibiosis
6.4	Enlarged/reduced lymphatic organs	Normal general condition, no increased or premature morbidity or mortality				

7 Cardiovascular and haematological diseases

7.1	Cardiac arrhythmia, e.g. asymptomatic cardiac channelopathies with structurally normal heart[52]		Short-term arrhythmia with sudden cardiac death			
7.2	Blood coagulation	Coagulation disorder depending on expression and follow-up symptoms				

[VIII] Can only be obtained by a suitable hygiene management

No.	Symptom/disease	Non-harmful phenotype	Mild severity	Moderate severity	Severe severity	Monitoring, Refinement, special housing requirements
7.3	Hypertension using the example of Spontaneous Hypertensive Rats (SHR)[53,54]	Slight hypertension up to 150 mmHg systolic blood pressure	Hypertension up to 160 mmHg[IX] without impairment of the general condition and without strokes	Short-term hypertension > 180 mmHg systolic blood pressure with impairment of the general condition and with occurrence of spontaneous strokes	Progressive deterioration of the general condition with death due to end-organ damage	Define values of blood pressure that reduce the well-being of a line
7.4	Dilated or hypertrophic cardiomyopathy		Transient and short-term intensified breathing after normal activity in the home cage; no permanent impairment of general condition	Global heart failure with permanent respiratory distress and impairment of the general condition, classification depends on expression of symptoms		

8 Diseases of the respiratory tract

8.1	Asthma[55]		classification depends on expression of respiratory distress and follow-up symptoms, e.g., reduced activity			

[IX] Incipient end-organ damage

No.	Symptom/disease	Non-harmful phenotype	Mild severity	Moderate severity	Severe severity	Monitoring, Refinement, special housing requirements
8.2	Pneumonia because of immunodeficiency			growth retardation, no respiratory distress, body weight loss < 20%	Permanent respiratory distress with death, body weight loss > 20%	Antibiotics

9 Diseases of the digestive system

9.1	Rectal prolapse		< 5 mm, moist, no necrosis, not bloody		> 5 mm, permanent	
9.2	Intestinal hyperplasia (of diameter and location)		Enlarged abdomen without impairment of organ functions	Enlarged with impairment of the organ functions and adjacent organs, classification depends on symptoms		
9.3	Diseases of the pancreas			Pancreatitis[56]: classification depends on symptoms		Monitoring of blood glucose serum levels to detect onset of pancreatitis
9.4	Inflammatory intestinal diseases; Colitis[57]		Soft faeces without impairment of general condition, body weight loss < 10%, clean coat	Pasty faeces, body weight loss of 10-20%, reduced activity, temporary hunched back	watery faeces with traces at the anus, contains blood, body weight loss > 20%, permanent signs of abdominal pain (walk on	Regular monitoring on signs of dehydration, e.g., loss of skin turgor, increased change of cages,

No.	Symptom/disease	Non-harmful phenotype	Mild severity	Moderate severity	Severe severity	Monitoring, Refinement, special housing requirements
					tiptoes, hunched back)	probiotics

10 Metabolic diseases

10.1	Hyperglycaemia		Polydipsia, polyuria without impairment of the general condition	Polydipsia, moderate polyuria, loss of body weight < 20%	Insatiable polydipsia, severe polyuria, loss of body weight > 20 %	More frequent change of cages, if applicable 2 water bottles when housed in groups
10.2	Hypoglycaemia (e.g., excessive insulin production due to beta-cell hyperplasia)				Reduced activity as a result of hypoglycaemia glucose	10% glucose in the drinking water, regular blood glucose control
10.3	Obesity[58]	Bred for obesity without impairment of normal behaviour or general condition	Evidence of components of the metabolic syndrome (obesity, lipid metabolism disorder, elevated levels of blood glucose, hypertension), Classification depends on the level of impairment of the general condition			Dietetic food on cage floor, soft bedding when movement is impaired, monitoring the genital health, rat: normal-weight “grooming mate”, more frequent

No.	Symptom/disease	Non-harmful phenotype	Mild severity	Moderate severity	Severe severity	Monitoring, Refinement, special housing requirements
						change of cages

11 Reproductive diseases

11.1	Fertility disorder	Sterility				

12 Tumour diseases

12.1	General		Without impairment of general condition	Tumour diseases if left beyond first detection but animals are killed within conventional limits[5,59]	Tumour diseases if left beyond conventional limits; criteria include e.g., body condition score, tumour diameter, the occurrence of anaemia or ascites, impairments due to tumour growth, necrosis or tumour ulceration[59], Models with spontaneous tumours which are expected to cause progressive fatal disease with long-	Use of body condition score[60,61]

No.	Symptom/disease	Non-harmful phenotype	Mild severity	Moderate severity	Severe severity	Monitoring, Refinement, special housing requirements
					term moderately pain, or distress. Examples include tumours causing cachexia, invasive bone tumours, metastasizing tumours and tumour left to the stage of ulceration[5]	
12.2	Externally visible or palpable tumours (benign, malign): Degree of severity depends on growth, size and location of the tumour		Palpable tumours without significant body weight loss (< 10%), without impairment of general condition and without functional impairments[59,62]		Ulcerated tumours	
12.3	Tumours of the inner organs		Classification depends on location, tumour size or impairment of organ functions and general condition			e.g., monitoring by imaging methods, control of defecation and urination
12.4	Malign lymphoma and leukaemia			Manifest clinical symptoms of		Palpation of the lymph nodes and

No.	Symptom/disease	Non-harmful phenotype	Mild severity	Moderate severity	Severe severity	Monitoring, Refinement, special housing requirements
				tumour disease with impairment of general condition and animals left beyond first detection but killed within conventional limits[59]		the spleen, monitor abdominal girth, regular blood examinations[63]

13 Renal diseases

13.1	Renal insufficiency (e.g., due to glomerulonephritis[64], hydronephrosis, renal fibrosis)		Polydipsia, mild polyuria without impairment of general condition polydipsia, mild polyuria	Polydipsia, moderate polyuria with impairment of general condition	Oedemas, proteinuria and/or > 20% body weight loss, polydipsia, severe polyuria, ascites, with impairment of general condition	regular urine sample for analysis, adapted cage change frequency

No.	Symptom/disease	Non-harmful phenotype	Mild severity	Moderate severity	Severe severity	Monitoring, Refinement, special housing requirements

14 Alterations of the locomotor system

14.1	**Muscle diseases**					
14.1.1	Paresis		Max. one part of the body up to 24h	More than one part of the body > 24h	More than one part of the body > 24h food and water uptake is not possible independently	Moist food on cage floor, agar pads; additional nesting material; remove shelter (risk of injury); glucose substitution, if applicable
14.1.2	Paralysis				Paralysis of the hind limbs and/or forelimbs regardless of length of occurrence	
14.1.3	Increased muscle mass	Breeding for increased muscle mass without impairment of mobility				
14.1.4	Duchenne muscular dystrophy[65–67]			Reduced mobility from 3-4 month of life, followed by obesity from 12		

No.	Symptom/disease	Non-harmful phenotype	Mild severity	Moderate severity	Severe severity	Monitoring, Refinement, special housing requirements
				month		
14.2	**Bone diseases**					
14.2.1	Shortness of limbs	Short limbs without impairment of mobility			Severely reduced mobility, with impairment of food and water uptake	Food on cage floor, longer bottle caps CAVE follow-up diseases
14.2.2	Polydactyly	Without impairment of mobility, e.g., climbing				
14.2.2	**Deformation of bones**					
14.2.2.	Brachycephalus	Brachycephalus without impairment of general condition or normal behaviour	Classification depends on impairment of food uptake or breathing			
14.2.3	Hydrocephalus			Retardation of growth	Lack of orientation, impairment of food and water uptake	

No.	Symptom/disease	Non-harmful phenotype	Mild severity	Moderate severity	Severe severity	Monitoring, Refinement, special housing requirements
14.2.4	Malposition of teeth	When food uptake is possible without restriction		Dental malposition resulting in impairment of the normal food uptake, classification depends on degree of body weight loss		Shorten teeth, moist food
14.2.5	Dental development disorders (missing teeth)				No uptake of food pellets is possible any more	Moist food
14.2.6	Osteoporosis, osteopetrosis	Mild expression that can be diagnosed by imaging, but animals show no clinical symptoms			In case of fractures	
14.3	Joint diseases					
14.3.1	Rheumatoid arthritis[68,69]	No signs of swelling and erythema, no impairment of mobility			Spontaneous polyarthritis of all four limbs, swelling, erythema	Soft bedding, additional nesting material[70]

Glossary

abdominal	Concerning the abdomen
Anaemia	Loss of red blood cells
Automutilation	Self-harming behaviour
Backflipping	Stereotypy of the animal repeatedly jumping backwards
Barbering	Stereotypy of systematically pulling out coat and/or vibrissae. Concerns individuals or cagemates.
Brachycephaly	A short skull resulting from disrupted longitudinal growth, often leading to disturbed functioning of upper airways.
Circling	Stereotypy of the animal walking in circles.
Dehydration	Water deficit
DIC	Disseminated intravascular coagulation
Dysferlinopathy	Dysferlin-deficient muscular dystrophy
Dystocia	Obstructed labour
Epidermolysis bullosa dystrophica	Hereditary skin disease
Erythema	Localised skin redness

Erythroderma	Skin redness affecting the entire body
Five Freedoms	Comprises aspects of animal welfare and the ability to express natural behaviour – Freedom from hunger and thirst – Freedom from housing-related discomfort – Freedom from pain, injury or disease – Freedom from fear and distress – Freedom to express normal behaviour
Focal	Localised
Glomerulonephritis	Inflammation affecting both kidneys, first affecting the renal corpuscle (glomerulus).
Holoprosencephaly	Malformation of the forebrain and facial skull
Hydrocephalus	Accumulation of fluid in the brain
Infanticide	Killing offspring of the same species
Catarrh	Inflammation of the mucous membranes
Leukopenia	Decreased number of leukocytes
Microencephaly	Small skull accompanied by small brain
Osteopetrosis	Bone resorption disorder resulting in mechanical instability of the bone tissue.
Osteoporosis	Increased bone resorption
Pancreatitis	Inflammation of the pancreas

Paresis	Partial loss of mobility
Paralysis	Complete loss of mobility
Pathocentric	It is assumed that animals are capable of suffering. The well-being of animals, our fellow creatures, must be protected. This rules out causing them pain, suffering or harm without good reason (Section 1 Tierschutzgesetz [German Animal Welfare Act])
Peracute	Occurring suddenly
Piloerection	Hair sticking up
Pneumonia	Inflammation of the lungs
Polydipsia	Excess drinking as a result of disease
Polyuria	Increased urination as a result of polydipsia Not incontinence!
Righting reflex	The animal is placed on its side or back and the amount of time it needs to return to its original position is recorded. This is a simple test to determine motor deficits, for example in the case of muscle weakness or poor general condition.
Thromboembolism	Formation of a blood clot
Growth retardation	Delayed growth
Wire-gnawing	Stereotypy of animals gnawing the cage bars.

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Appendix A - Recommendation on the required number of animals for evaluating an increase in harmful phenotypes in mouse and rat lines

Initial situation

When new mouse and rat lines are developed (genetically or via selective breeding for spontaneous mutations), these lines must be checked for any potential pain, suffering or distress caused by genetic modifications in comparison with the original line. This examination is carried out in two steps, with a check first made to see whether there are any animals with a harmful phenotype in the new line. The potential number of animals with a harmful phenotype must then be tested against the frequency of animals with a harmful phenotype in the original population so that it can be determined whether or not there is really an increased degree of severity (compared with the original population).

First step: Defining the probability that there are animals with a harmful phenotype in the line

It is first necessary to test a representative sample for signs of a harmful phenotype in order to make it possible to prove that there are animals with a harmful phenotype in a line. The size of this sample depends on various aspects which will be considered more closely in the following.

Line to be examined: Case 1 (Defined genotype)

If a mouse or rat line which has been deliberately genetically altered is to be assessed, the genotype of interest which could lead to a harmful phenotype is present and there is molecular-genetic proof of this. This makes it possible to ensure that all of the animals included in the tests really do exhibit the altered genotype which is to be examined.

The most important parameter for the number of animals to be examined is therefore the penetrance with which the genotype which is present also manifests in the

phenotype (as a harmful phenotype in this case). There is almost 100% penetrance in the case of most genetic diseases (polydactyly 90%[1)], piebaldism 90%[2)], Huntington's disease from a certain causal repeat number almost 100%[3)], neurofibromatosis, phenylketonuria). Similar penetrance is to be assumed in genetically altered lines as it is predominantly single gene alterations (or alterations of very few genes) which are aspired to here. However, as crossing often forms the basis for experiments such as these, and there is even a certain variability in the measurement data[4)] for inbred lines, it may be that the actual harmful phenotype is not expressed as a result of genetic modifier of an individual genetic background. In order to accommodate this potential background effect, a high safety margin is used in the calculation and a relatively low characteristic penetrance of 80% is assumed. In the case of lower penetrance multifactorial inheritance or strong environmental influence must be assumed. However, it is then no longer possible to attribute both causes to the genetic alteration alone.

Line to be examined: Case 2 (Selection for spontaneous mutation)

The lines trace back to an unknown, earlier spontaneous mutation and were not created using gene transfer or other methods to induce genetic modifications. If a characteristic which arose spontaneously in this way and has a clearly defined inheritance forms the basis for the deliberate establishment of a special line, a severity assessment must be carried out. In this case, however, there is no marker for only selecting animals with the altered genotype for the examinations of severity degree. However, successful selection for the establishment of a line such as this fundamentally requires a clearly recognisable phenotype which is definitely genetically determined (probably monogenic) and the desired genotype accumulates very quickly as a result of the type of selective breeding/line establishment described. For the selection of animals for the examination of the severity degree, this creates genetic conditions regarding the mutation of interest which are comparable to the pre-selection for the defined genotype. The penetrance of a spontaneous mutation such as this, the diversity of the genetic background and the probability of discovering animals with harmful phenotypes are therefore comparable with Case 1, described above, and there are no differences in the number of animals to be

selected, which takes place just as randomly here. The residual risk of randomly selecting a homozygous recessive animal in a dominant inheritance (from a random, undetected heterozygous breeding pair) is low and covered by the overall low-set penetrance of 80% (it is very likely that this figure is considerably higher in the case of selection for a spontaneous mutation). A line which is built upon a mutation with a recessive inheritance is already homozygous after one generation.

Line to be examined: Case 3 (Syndrome)

Syndromes are always caused by the combined effects of several genes in connection with considerable environmental influence. They can only be examined with respect to harmful phenotypes if there is a main gene which determines the majority of the variance. Generally, effects only become visible during animal experiments under distinct conditions and must then be approved along with the experimental protocol. In such cases disease in animals does not originate from a genetic modification per se.

A line with a genetic modification that is involved in the development of a syndrome must first be examined in the same way as a line with clear gene effects. This is necessary because there is usually no prior information on the extent of the harm inflicted by the phenotype. Under the conditions mentioned, the line under consideration will often only show a small number of compromised animals, which leads to a classification as an unharmed line. However, if there is a stronger link between the genetic modification and a harmful phenotype, this will become obvious during the further establishment and keeping of the line by an above-average occurrence of compromised animals. A first indication of a possible syndrome is if the significance limit only barely missed when evaluating a line for the occurrence of a harmful phenotype. Such a line must be retrospectively assessed. According to the frequency of compromised animals now detected, the required number of animals to test for the causative nature of the genetic modification for the occurrence of the syndrome can be correctly calculated. With this higher number of animals, the comparison to the background line is carried out once again and the line is classified as bearing a harmful phenotype where appropriate. In principle, the smaller the

contribution of the modified gene to the overall variance, the less significant is its genetic modification for the whole organism and the fewer animals will show anomalies due to the genetic modification.

Original line to be examined

In order to determine a harmful phenotype which is potentially increased in comparison with the reference line, it is necessary to also assess the original line with respect to its harmful phenotype. Generally, based on the lack of mutation/genetic alteration, one would assume that this line has no harmful phenotype. As spontaneous mutations always remain with a low allele frequency, including in the original population, or can only be present in genes which have just been genetically altered, a certain “harmful phenotype background” of 5% animals with a harmful phenotype should be assumed. This value is higher than the occurrence of significant genetic diseases in animal breeding which, with a maximum defective allele frequency of 8%[5)] in the recessive inheritance, leads to a frequency of <1% animals with a visibly harmful phenotype. It should be considered that inbred rodent strains may possibly exhibit a harmful phenotype by themselves. It is recommended to generally assume a proportion of 5% animals with a harmful phenotype for a background line as long as no specific data is available.

Second step: Calculation of animal numbers to be examined

A significance test can be used to check whether the new line differs considerably from the original line with respect to the frequency of animals with a harmful phenotype occurring, which is why it is necessary for the same number of animals to be examined once in the original population for the comparison. The original population signifies the genetic target background or the backcross population here. In the case of F1 or F2 populations, a population with the same genetic construction without the tested genetic alteration should be used for comparison.

Note: During screening, all abnormalities and forms of burden should generally be considered and not only those which are to be expected as a result of the genetic

alteration, as there may be unexpected consequences in combination with the whole genome.

The number of animals to be examined was determined using sample size planning for a comparison of two probabilities (program: proc power).

The following conditions were set:

- Test: Fisher's Exact Conditional Test for two probabilities
- Distribution: exact conditional
- One-sided test
- Alpha: 0.05
- Power: 0.8
- Probability of a harmful phenotype in the original population: 5% (see above)
- Probability of a harmful phenotype in the altered population: 80% (see above)

With these conditions, a difference between the two populations in the probability that animals with a harmful phenotype will occur must be assumed to be 0.75 (80% minus 5%). This means that 7 animals would be sufficient for an analysis to significantly ensure this difference with a power of 0.8 (Fig. 1). However, as animals can only be included in the calculations in whole numbers, an animal with a harmful phenotype in the original population among 7 examined animals already represents a probability of occurrence of 14.3% (in comparison with the calculated 5%) or, with 5 animals with a harmful phenotype in the altered line, of 71.4% (in comparison with the calculated 80%). For 7 animals this results in a realistic difference in the probability of occurrence of only 57.1%. This shows that the number of animals calculated was too low and the risk of false-negative results is too great. 8 examined animals also achieve a difference which is too low (62.5% to 70% limit of detection) and 9 animals, at 66.7%, only just achieve the limit of detection (65%). Therefore, 10 animals should be examined per population, for which the expected difference in probability of 70% can be detected with a safety margin of 10%. Furthermore, a greater burden can still be detected with 10 animals with an unexpectedly high burden in the original line (up to 20%) under corresponding circumstances (difference of 65%).

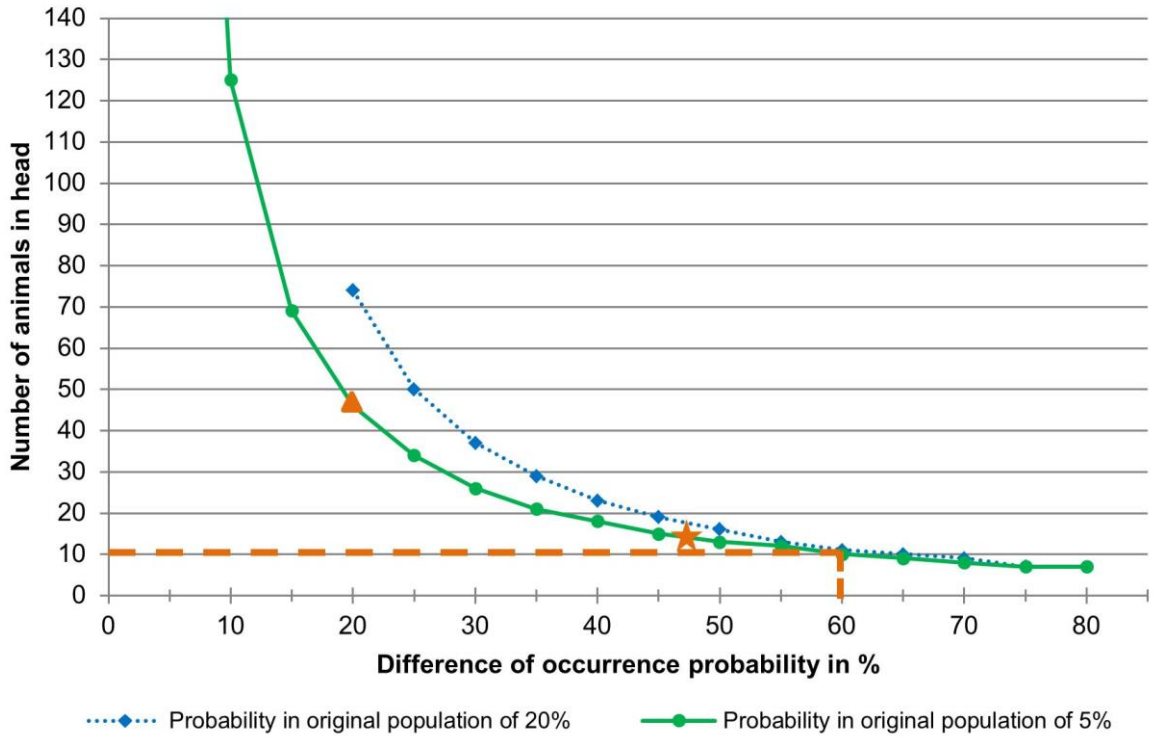

Fig. 1: Number of animals required to detect a certain difference in the probability of occurrence of animals with a harmful phenotype between two populations showing distinct probability of occurrence of compromised animals against a background line with a proportion of 5% (green) and 20% (blue) animals displaying a harmful phenotype. For an animal number n = 10, a difference of 60% can be discriminated (brown lines). The presently used number of 14 animals to be examined makes it possible to significantly discriminate two lines (star), even with a difference as low as 47.5%. The detection at a difference of 20% (e.g., in the case of a syndrome) requires to increase the number of animals to 46 (triangle).

Third step: Significance test against the original population

After both populations have been examined, the proportions of animals with a harmful phenotype are calculated for each population. The difference between the proportion of animals with a harmful phenotype in the altered population and that in the original population is then formed. The value in Fig. 2 shows whether there is a significant difference with a power of 0.8 and an alpha of 0.05. If this is the case, the genetically altered line exhibits a greater severity degree and further breeding will require approval.

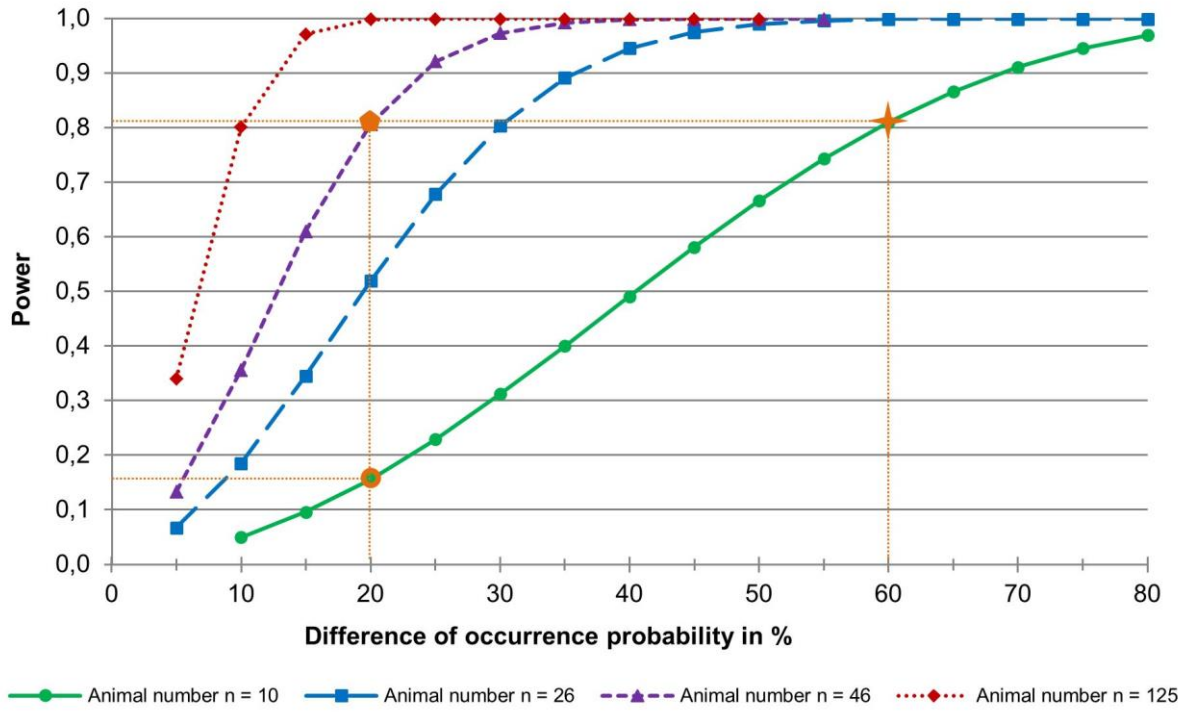

Fig. 2: Power to detect a significant difference between two populations with a difference of occurrence of a harmful phenotype of >60% between the background and the genetically modified population, the power of 0.8 is ensured by the examination of n = 10 animals (star). With smaller differences, more animals have to be examined as illustrated for 20% difference, where n = 46 (pentagon), since there is insufficient power with 10 animals (circle).

Example 1: When 10 animals are examined, 1 animal with a harmful phenotype is found in the original population and 8 animals with a harmful phenotype are found in the altered population. This corresponds to a probability of harmful phenotypes occurring of 10% in the original population and 80% in the altered population. The difference is 70% and exhibit a power of detection of >0.8. This shows that the severity degree of the altered population is significantly increased.

Example 2: Out of 10, no compromised animal is found in the background population while there are 2 in the genetically modified line. The difference of 20% between the two populations represents a power of only 0.16. Hence it has to be concluded that the genetic modification does not result in a harmful phenotype.

Example 3: 1 out of 10 animals with a harmful phenotype is discovered in the original population, while there are 4 in the altered population. The difference of 30% is not sufficient to separate both populations safely with regard to the occurrence of animals with a harmful phenotype, since the power of only 0.31 is too low. However, during further breeding of the line, it becomes obvious that animals with a harmful phenotype occur with a low frequency but on a regular basis. It is for this that the line must be revaluated. Since the information from the first study assumes a probability of occurrence of 30%, 26 animals are to be examined. In the background population, 2 animals with a harmful phenotype are found during the new evaluation, while there are 11 in the genetically modified line. This results in a difference of approximately 35%. Out of the group of the 26 tested animals, the difference between the two groups can now be secured with a power of 0.89. Hence, the lines has to be considered as one that displays a harmful phenotype caused by a genetic modification and therefore further breeding needs authority approval. The presence of a syndromic disorder can be assumed.

Summary

The calculations above show that there is a high degree of certainty (power 0.8) of recognising lines with an increased severity degree when a total of 10 animals is examined. As the gene effect can be modified via the sex and a genetic background that is not completely reproducible, 5 male and 5 female animals, preferably from 5 different litters of different parents, should be selected for the examination. In case inheritance is clearly limited to one sex or if there are epigenetic effects, 10 animals of the affected sex must be chosen in order to achieve reliable results. In conclusion, it can be noted that the currently widely accepted number of 14 animals to be included in the evaluation of a new genetically modified line is sufficient to easily detect an increased severity degree on a reliable basis already at a difference in the probability of occurrence as low as 47,5%. Only in cases with very low penetrance (syndromes) a revaluation may be needed.

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