<u>A multimodal approach to refine large animal experiments</u>

Introduction

The 3Rs according to Russel and Burch - Reduction, Replacement and Refinement – represents one of the most important and prevailing principle in the area of laboratory animal science for many years.

However, due to the fact that Reduction and Replacement have a greater impact on the entire population, these two were in the spotlight of scientific interest. Looking at their application in science and their promotion in the field, the focus is mainly on substitute and complementary methods or the reduction of animal use. This is in line with an ambitious long-term goal to use as few animals as possible for research purposes: Thus, the first two Rs - Replacement and Reduction - directly affect the total number of animals and the welfare of the entire population. The last of the 3 Rs, Refinement, however, contributes to improving the treatment of laboratory animals in experiments by mainly affecting the welfare of the individual animal. It is therefore always applied when the replacement and reduction of animal use are not possible.

The aim of this study is to showcase how, through strategic balancing, refinement can be implemented during all stages of the experiment in order to improve animal welfare. The consistent application of refinement measures can reduce the stress perception of the animals and at the same time promote and ensure the quality of the scientific results.

Refinement approaches can be planned and should be included in the design and execution of experiments from the beginning, during the experiment and beyond. Refinement should not be a single approach to a specific set of parameters, but a cross-study focus in animal studies. In the following, a concept in the application of refinement strategies will be demonstrated using a clinically relevant large animal model.

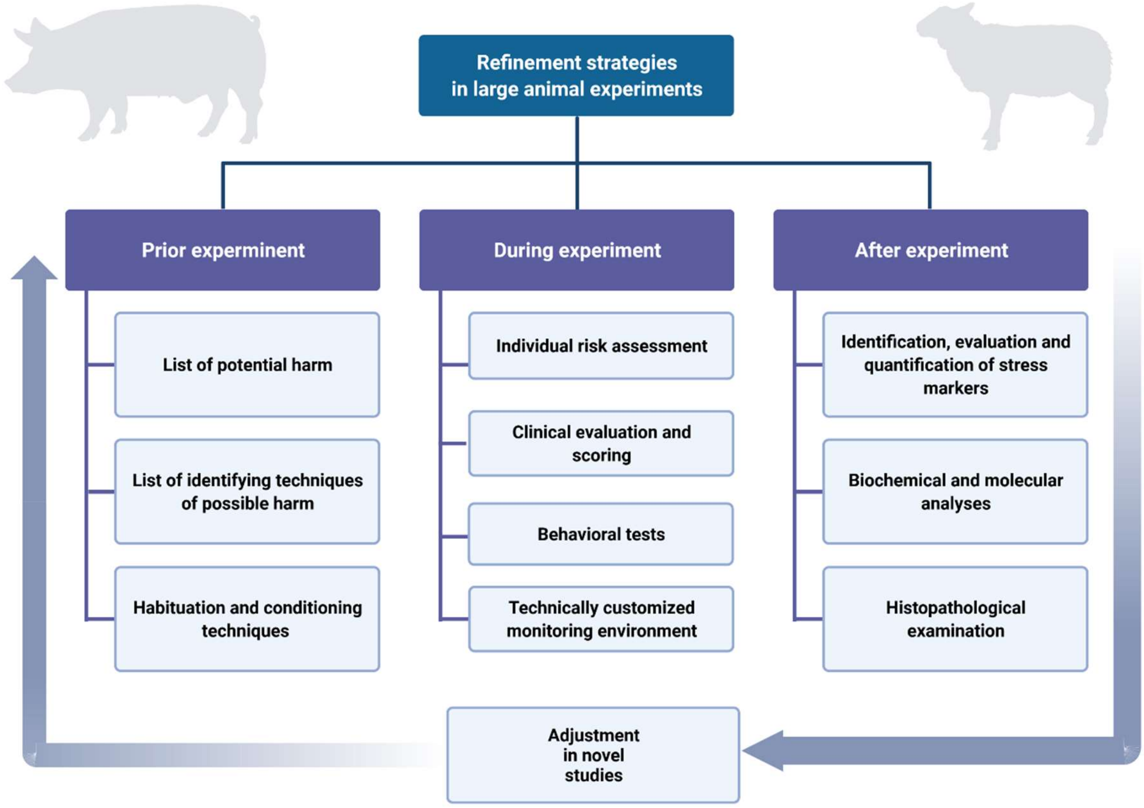

Figure 1 Schematic representation of the strategies for the cyclic implementation of refinement methods in large animal models. Ernst 2021

In order to maintain well-being, both the well-being itself and, above all, any divergences must be objectively detectable at an early stage. In case of divergences from well-being, mainly pain, suffering or other harm have to be specified.

In our working group, the evaluation of different stressors and stress types of laboratory animals in experiments has been a special focus for many years. Here we attempt to develop key parameters for the generation of stress scales that are as close to objective as possible, are non-interventional, and that are independent from the observer. Therefore, we have developed a holistic strategy to be able to gradually intervene in any experimental process and stage. This strategy is shown in Figure 1 and will be demonstrated steb-by-step in a case report using a large animal model of kidney autotransplantation in pigs. Briefly, the kidney is first removed, and then prepared overnight and transplanted to the other side at 24-hour interval the next day, after removal of the contralateral kidney. Afterwards, the animals are observed for 7 days and the function of the kidney is further examined on the basis of clinical and laboratory parameters.

Step. 1: refinement prior the experiment

Before refinement steps can be taken, potential stressors, pain, suffering or injury in the model must be identified and detected. The first step is to draw up a list of the occurring harms as well as a list of possible identification options. This shows the first refinement possibilities as well as approaches, in which examination procedures have to be considered in particular. In the kidney transplant model, an abdominal surgical procedure is performed, which needs to be studied clinically as well as systemically. An obviously effective analgesia is mandatory. However, possible stressors and harms include pain from the surgical incisions, adaptation to the experimental setting, handling techniques for drug administration and repeated blood sampling, as well as possible kidney dysfunction and associated metabolic problems, to highlight some of the main factors. Refinement approaches at that stage are, above all, the adaptation of the animals to the experiment through habituation and desensitization. With simple conditioning steps, the animals were trained in the basic handling procedures as well as manipulations e.g. injection or ultrasound (Fig. 2).

Days	3	4	5	6	7	8	9	10	11	12	13	14	POD1	POD3	POD3	POD4	POD5	POD6	POD7
Presence of person in box			100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Feed intake from bowl with presence			80	92	94	93	100	99	98	84	100	100	96	96	100	100	100	100	100
Feeding from the hand			62	74	84	82	93	87	89	74	90	91	89	89	92	92	92	92	92
Touching the head			85	97	97	97	100	98	100	84	100	100	100	100	100	100	100	100	100
Touching on shoulder and trunk			37	88	94	92	100	100	100	82	91	97	100	100	100	100	100	100	100
command or food with pausing = stand			4	0	0	0	0	0	0	0	0	0	4	4	4	0	0	0	0
Stand, tolerate touches			48	0	14	5	11	24	32	24	38	41	52	63	69	50	62	50	52
Stand, touch belly			72	81	88	89	89	97	99	82	96	100	100	100	100	92	96	96	96
Stand, touch front legs			72	93	94	84	88	99	100	84	97	97	100	100	100	96	100	100	100
Stand, grab front legs			65	81	91	89	93	97	98	84	97	97	100	100	100	100	100	100	100
Stand, lift one front leg, then the other leg (lift 4 of 4)			43	81	86	85	91	95	94	79	94	94	100	96	96	96	96	96	96
Auscultate (with stetoscope)			35	52	68	72	77	78	81	68	82	92	81	77	82	88	88	90	90
Spray with desinfection, manipulation of the skin on the neck			32	25	18	24	29	30	36	26	56	59	81	70	81	62	73	77	72
Touching under pigjacket at urine catheter hole			18	53	45	55	63	55	67	39	65	78	78	70	85	92	92	85	84
Crawl to lay down, side does not matter, or sit			12	6	20	0	3	0	3	30	0	0	96	89	91	94	88	92	92
Crawl to lay down, wetten the belly			6	18	32	37	29	26	32	34	49	51	67	63	62	76	69	64	72

<10%
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Intended procedure/treatment	Training assignment steps
i.m. Injection (for analgesic administration/initial anesthesia, medication)	Tolerance, short-term pain stimulus; no/low defensive activity, standing still
Blood draw/medication administration (i.v. administration, veneous withdrawal)	Steady position, tolerance of manipulation in and out of the visual field of the animal
Change urine bag	Steady position; opening/closing the pig jacket (noise), grasping under the pig jacket, manipulation of the pig jacket
Change/correct fit of the pig jacket	Grasping and lifting the legs, enclosing the chest/neck, opening/closing the pig jacket
Abdominal ultrasound	Standing still, pressure on the abdomen, wetness/coldness on the skin surface

Figure 2 Stepwise conditioning of the animals over 14 days before surgery and during the 7-day observation phase (POD1-7). Results of the successful conditioning of the steps and breakdown of the individual adaptation phases. As a following result exemplary also shown on the ultrasound diagnostics of the awake pig. (Ernst et. al, 2021 unpublished data)

Step 2: refinement during the experiment

The knowledge gained during the experiment is most valuable for applying refinement strategies to every single animal. Here, direct actions can be taken and evaluated so that the greatest possible adjustments can be made. The interaction between different techniques is of particular importance, assuring that all functional aspects of the animal can be covered. For the assessment of large animal experiments, different aspects of clinical examination, severity scores, behavioral testing, modern monitoring methods and biochemical analysis are combined and exemplarily illustrated in Fig. 3.

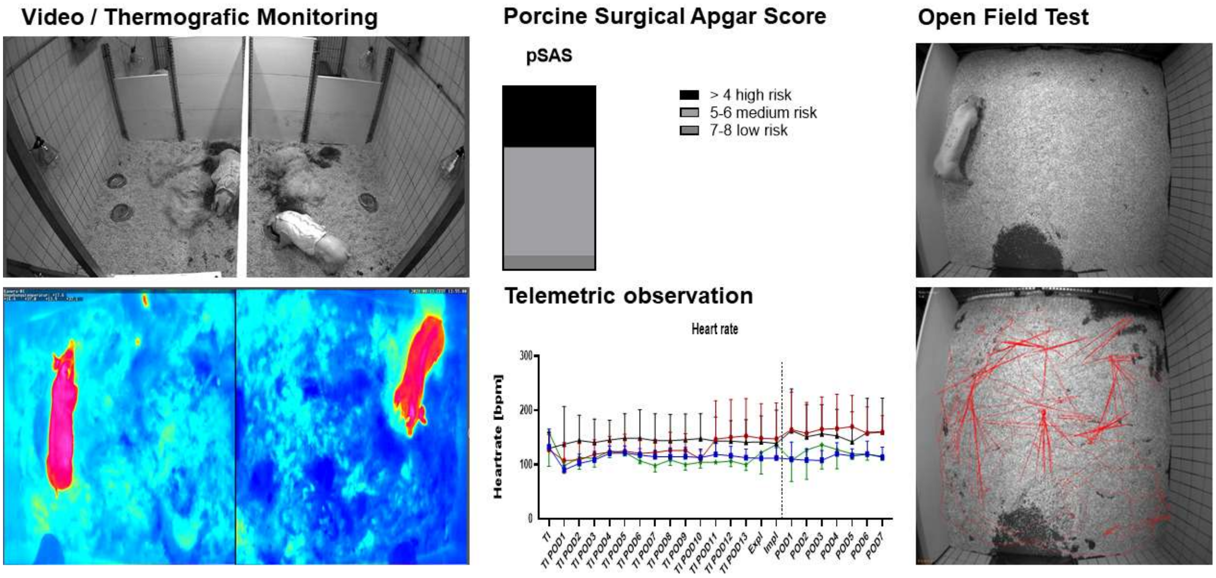

Figure 3 Exemplary presentation of monitoring methods during the experiment by video surveillance and thermography, classification by surgical apgar score, the use of telemetry data and video tracking systems in behavioral testing for example using the open field test.

In the case of surgical interventions, it is also necessary to conduct an assessment of the impact of the intervention on the well-being of the animals. The Surgical Apgar Score (SAS), which is used in human medicine, has appeared to be an objective assessment tool, which our working group recently validated in the course of this project for pigs (Ernst et. al 2021 ESR under review with revisions). Furthermore, clinical evaluations are performed using laboratory parameters (e.g. kidney parameters from serum samples) and clinical examinations, score sheets, as well as special monitoring methods such as the implantation of telemetry transponders or around-the-clock monitoring through thermographic observation. All these examinations provide indications for the early detection of pain, suffering and harm and approaches for the implementation of refinement methods.

Step 3: Refinement after the experiment

As refinement measures after the experiment the retrospective evaluation of different parameters is intended. Biochemical and laboratory chemical parameters, as well as histological examinations, can detect and evaluate inflammation and changes that are not visible as obvious damage or harm in vivo. An example of the use of a novel retrospective stress evaluation is the detection of feacal corticosterone metabolites (FCM) from animal feaces.

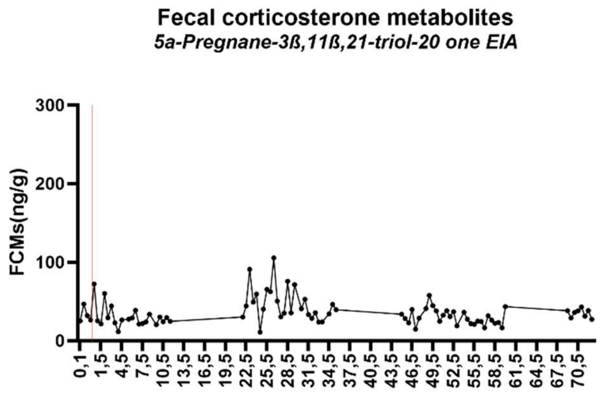

Figure 4 FCM analysis after i.m. administration of a specific stressor = ACTH injection. Increase of fecal corticosterone metabolites after 24 - 28h. Ernst et. al 2021 unpublished data

As shown in Figure 4, the occurrence of stress, especially in the chronic setting, can be shown with the help of this assay. Approximately 24-28 h after exposure to a stressor, the increase of the FCM value in the faeces is visible.

This is a retrospective non-invasive method of stress evaluation in with no direct animal contact required. Through this, conclusions can also be drawn in the husbandry of the animals, independent of the experiment. By using retrospective evaluation methods, refinement methods can be reviewed and adapted for subsequent projects. This is important because the process of refinement involves a continuous cycle in which further findings should be incorporated into upcoming experiments.

Conclusion:

The goal of my research present here, is the future development of pain and stress management tools that detect and display pain and harm objectively, independent of the observer and specific to the animal species. This may also create a basis for an application outside of the area of laboratory animal science, as most of these methods can be applied independently of the experimental setting. For large animals, this may help to build a bridge for implementation in conventional livestock production, and thus to improve the conditions of animal welfare with a higher economic efficiency of animal use.