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EXECUTIVE SUMMARY

Background	The SmartCow project started in 2018 with the goal of integrating major cattle research infrastructures across European countries to support sustainable cattle production. Alongside this broad goal, an important component of the SmartCow work was attention to ethical issues, both within the project work itself and more broadly in cattle research.
Objectives	This report collates examples of the progress made by partner institutions involved in SmartCow in terms of implementation of the 3Rs in research using cattle.
Results & implications	A number of examples of 3Rs improvements within cattle research studies were identified. These were most notably in the areas of refinement of housing conditions and procedures, as well as progress towards reducing the use of fistulated cows, and refining welfare assessment approaches in research and commercial cattle production. These examples from the SmartCow project demonstrate that it is possible to achieve success in attainment of scientific goals, whilst also making progress on implementation of the 3Rs. This demonstrates that livestock researchers across Europe and beyond can conduct high quality science that informs upon major global issues, whilst also improving animal welfare in their study animals.

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Implementation of the 3Rs in SmartCow

1 Introduction

The SmartCow project started in 2018 with the goal of integrating major cattle research infrastructures across European countries to support sustainable cattle production. Alongside this broad goal an important component of the SmartCow work was attention to ethical issues, both within the project work itself and more broadly in cattle research. To support this effort, an ethical board was formed with members from several Smartcow partner organisations. The ethical board served various functions over the lifetime of the Smartcow project: providing ethical appraisals of the TransNational Access (TNA) proposals, providing training, conducting workshops on ethical issues at SmartCow annual meetings, and provided two chapters on ethical issues for the Smartcow book of methods (Veissier et al 2021, Langbein et al. 2022).

As the SmartCow project comes to an end a final goal of the ethical board is to review the success of efforts within the project to advance the ethical acceptability of cattle research. One central principle of ethical appraisal relating to animal experimentation is that the legitimacy (and indeed legality) of any given study should be judged on the basis of the balance of harms imposed to animals involved in the work, versus the benefits of the work itself. An important part of this is on-going efforts to minimise the welfare harms imposed whilst still achieving necessary scientific goals. Ways of achieving this were first outlined by Russell and Burch (1959) in their seminal work on the '3Rs' of humane animal research. This work identified three fundamental principles, which have guided animal experimentation in the subsequent 60+ years. These principles were that wherever possible use of animals should be **Replaced** with non-animal alternatives, and failing that, that animal numbers should be **Reduced** as far as possible, and at all times in their use in research the experience of animals should be as **Refined** as possible. Although the 3Rs should be familiar to all those connected with animal research, it is worth reflecting on how these were originally framed by Russell and Burch. They (1959) defined the 3Rs as follows:

Replacement: *“the substitution for conscious living higher animals of insentient material”*

Reduction: *“reduction in the numbers of animals used to obtain information of a given amount and precision”.*

Refinement: *“any decrease in the incidence or severity of inhumane procedures applied to those animals which still have to be used”*

The use of “inhumane” was intended to refer to the experience of the animal (anything that invoked distress) rather than being an ethical judgment on those imposing the treatment. By outlining these principles Russell and Burch intended to improve the experiences of animals in research (by minimising

the frequency and severity of distress), whilst also maintaining or improving the quality of science conducted (Tannenbaum & Bennett 2015). More generally, refinement is often now taken to mean any improvement in animal experiences that facilitates better welfare, i.e., it includes improvements not just to experimental procedures but also to, for example, housing conditions or animal handling, even when those are unrelated to study goals.

As part of the wider research work a number of SmartCow activities targeted the implementation of the 3Rs in cattle research, and the original proposal stated the goal that this work should “have a positive impact on the ethical acceptability of animal experiments and husbandry by society”. This goal recognised the fact that, whilst improving the scientific understanding of the role that cattle play in key global sustainability and environmental objectives is important, concern about the use of animals in research is also an ongoing societal issue, which engages many members of the public.

This report aims to collate examples of benefits to animal welfare, which support this goal. In particular it will focus on improvements in the 3Rs within cattle research that have occurred as a consequence of the SmartCow research programme. Whilst examples of this come from across the SmartCow project as a whole and all partner organisations, particular focus will be given to the work conducted within Joint Research Activities (JRAs) – on the refinement of measurement techniques (Work Package (WP) 5), the development of proxies and non-invasive methods (WP6) and the use of sensors (WP7). Primarily, the beneficial outcomes of the SmartCow work have related directly to the use of animals in research – i.e., 3Rs relating to cows as research animals. However, consideration will also be given to aspects of the work that will benefit cattle welfare outwith the research context.

2 SmartCow and the 3Rs

Research at the various SmartCow infrastructure sites has contributed to current or future improvements in the 3Rs in a number of different areas, including developments in improved housing systems, refinement of procedures, welfare assessment and in relation to alternatives to animal use.

2.1 Improved housing

One central issue pertaining to the use of cows in research studies is the frequent requirement for close containment or restraint during periods of data collection or for specific individual procedures. This issue, of course, applies to some extent in all research with animals, but is particularly prominent in cattle given their size and the additional potential for harm to research staff this creates. Work at the SmartCow infrastructure sites often requires some degree of behavioural restriction – for instance, in facilities for measurement of gas emissions, during collection of urine and faeces, for blood sampling, and whilst conducting procedures with fistulated cows – but improvements have been made in terms of the animal experience during this.

At INRAE a new stall was designed for use in digestibility studies and this was tested under WP5. Restraint in some form of stall is a common requirement in digestibility studies, in order to facilitate sample collection

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(particularly of faeces and urine). The provision for welfare in such stalls can be highly variable and may be poor, given that animals may be maintained in these settings for long periods (a few weeks). Some of the features that may constrain welfare are fundamental to the purpose of the restraint – for instance, behavioural restriction being necessary for the separate collection of urine and faeces – but others are not. The new design supports improved welfare in a number of ways. The stall minimises the restraint requirement for sample collection (and stall size can be altered according to cow size), has improved accessibility via a ramp entrance rather than a step, allows for some degree of social contact between adjacent cows, and provides improved cow lying comfort.

Similarly at Aarhus University a new pen system has been implemented for use with individual fistulated cows. This new pen system replaces tie-stalls (which have normally been used in such studies to allow for sampling, measurement of feed intake and procedures relating to the fistula), allowing the cow more space to move around, some limited social contact (including tactile contact) with other animals and an increased level of cow comfort. Cows can be temporarily placed under full restraint for procedures but are otherwise free to move around the pen. Aarhus University has also established four new respiration chambers during the SmartCow project. Output from the joint workshops in WP5 were considered when the chambers were designed, resulting in larger chambers with enhanced emphasis on air quality, and better accessibility for animals. The intended goal of this new system is the maintenance of normal behaviour in the chamber (for instance, feed troughs are fully transparent so that the cows can maintain visual contact with other animals during feeding).

The University of Reading have also made recent improvement to their cow stalls for research. The new stall has a number of design features that support better welfare and safety for researchers, including easily movable side panels that allow more freedom of movement until restriction is needed for milking and sampling. Most notably the stalls make use of waterbeds to improve cow comfort, based on the recognition that lying surface – particularly for large animals such as cows – is an important environmental feature dictating welfare. The new stall has been notable in not only improving cow comfort but also in improving the resulting quality of collected data: most obviously evidenced by improved stability of feed intake and milk yield during the periods of restriction. These observations tally with research studies at INRAE and Wageningen that suggest these metrics can be an important indicator of cow adaptation to restricted housing. In addition, improvements were made to the equipment used for obtaining total collection and samples of faeces and urine, which minimized cross-contamination and losses during collection and sampling.

In addition to the development of the new stall, SmartCow studies at INRAE (WP5) have also examined the impact of time in the stall both on indicators of cow welfare and on the implications for data quality. Based on the assessment of the stress hormone cortisol it was found that animals (growing bulls in the

study) experience some stress on initial entry to the stall, but that stress levels were stable thereafter. Bulls reacted to being moved to the stall by spending more time standing-inactive and less time feeding, standing-active or lying-ruminating. During the first 6 days standing-inactive progressively decreased and feeding, standing active and lying ruminating progressively increased, suggesting habituation. However, once urine and faeces collection started (after 6 days), opposite trends were observed, suggesting that the sampling disturbed animals, with no signs of habituation (Bellagi et al., 2022). In this context it is worthwhile noting that, depending on study design, animals may have separate phases of being exposed to a trial diet (typically for two to three weeks) and then being sampled once they have adapted to that diet. The initial adaptation phase may or may not involve a degree of behavioural restriction (e.g in a stall). The INRAE work concluded that, after a suitable period of pre-sampling exposure to the relevant diet, 7 days of containment (for the purposes of sampling) are necessary to get repeatable data, but this represents a refinement of this procedure from the usual duration (Bellagi et al 2021). In other WP5 work at Aarhus it was concluded that for digestion studies with external markers as little as two days of sampling might be sufficient for some studies. From a welfare perspective the best approach is to adapt animals to their diet outside of the restraint setting and then apply the minimal duration necessary for adequate sampling (dependent on study objective).

At Reading, using the new tie stalls and improved faecal and urine collection equipment, it was found that for measurements of N digestion and balance shorter collection periods (4 days) could be used as long as strict attention to detail was employed and patterns of feed intake and diet composition remained stable. It has also been found that gradual acclimation of animals to restraint in tie stalls before experimental measurement periods is important and reduces the negative impact of restraint on feed intake and other behaviours. However, this context may still represent a trade-off between refinement and reduction in terms of the 3Rs, as – under some circumstances – longer periods of containment can still yield better data, possibly leading to reduced need for animals in subsequent studies. This will depend in part on the experimental objectives and design (e.g. change-over or randomized block). Ultimately, the attention to detail in the conduct of experimental procedures by researchers is paramount in reducing error variance and thereby increasing experimental power.

2.2 Improved procedures

SmartCow research, within WPs 5 and 6 has contributed to both general and specific refinement of processes and procedures in cattle research.

The goal of WP5 was to identify, and reduce, sources of measurement error in studies involving feed digestion, nitrogen balance and emissions measurements in order to increase the precision and accuracy of experimental outcomes. This effort to decrease error was aimed at ultimately reducing animal numbers required in this type of research as lowered measurement variance means that fewer animals are required to detect a certain effect size. In addition, work was done to consider non-invasive automated techniques for collection and sampling of faeces and urine, with the goal of refining the animal experience during sample collection.

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WP6 focused on the development and validation of proxy indicators of important animal traits such as feed efficiency, to replace current gold standard measures in digestibility or enteric methane emissions studies, with the goal of reducing requirements for animal handling and restraint to a minimum. Identifying scientifically appropriate proxies for traits such as total tract digestibility, nitrogen excretion and enteric methane emissions was one of the main goals of SmartCow. Valid proxy measures not only open up new potential applications but would also represent a clear refinement within research. Traditionally, assessment of these outcomes requires separate faeces and urine collection with cattle being kept in a metabolic cage in close confinement for as long as 14 days. Although cows do habituate to this setting the degree of confinement represents a substantial behavioural restriction, particularly limiting movement, and impairing ease of lying. The metabolic stall or respiration chamber also inevitably limits social interactions and represents an unstimulating environment for the cows. As part of SmartCow work various alternative methods have been proposed to predict digestibility and urinary nitrogen excretion – including assessment from faeces or blood samples (Andueza et al 2019, 2020; Nasrollahi et al 2019, Cantalapiedra-Hijar et al 2022). This work aimed to determine if total tract digestibility could be successfully predicated using visible/near infrared spectroscopy of faecal samples (collected manually during temporary restraint). To date the studies have shown that the spectroscopy approaches can discriminate between diets on the basis of their total tract organic matter digestibility with a precision close to that of the gold standard method. Similarly, urinary nitrogen excretion can be assessed using different biomarkers analysed either in blood or milk. The urea concentration and the natural ^{15}N enrichment of animal proteins over the diet measured in milk or blood were both associated to changes in the N partitioning and urinary N excretion across diets (Nasrollahi et al 2019), but showed lower (though significant) correlations at the individual level (Correa-Luna et al 2022). In the case of biomarkers of urinary N excretion at the individual level, a metabolomic study conducted in WP6 found 7 plasma repeatable metabolites showing significant correlations at the individual level with total urinary nitrogen excretion (Cantalapiedra-Hijar et al 2022). This kind of research is paving the way to the possibility of phenotyping animals as low or high N polluters without the need to use N balance trials. Proxies based on Infrared spectroscopy to estimate methane emissions in dairy (mid infrared spectroscopy on milk) and beef cattle (near infrared spectroscopy on faeces) have also been studied in SmartCow, using data from GreenFeed as the reference method for this assessment (Andueza et al 2021; Coppa et al Submitted; Coppa et al 2022)

The validation of spot sampling from easy to access body matrices (milk, faeces, urine, blood) was intended to refine animal experience by reducing requirement for close confinement for lengthy periods during sampling, and by moving to less intrusive sampling methods. The new measurement approaches developed here not only allow for this improvement to the animal experience, but the use of spot sampling also allows for experimental studies that better elucidate sources of variability in N excretion, i.e., the new methods also broaden the potential usage and benefits of this measure in cattle research.

Guidelines for the use of proxies have also been produced, which aim to support and increase the use of these methods in research, in order to refine the experiences of cows in research, but also to allow application in commercial animals (outwith a research setting) which decreases the number of animals in experimental setting.

2.3 Welfare assessment

One important facet of any animal study is the on-going monitoring of animal welfare, both as general best practice and in terms of ensuring compliance with planned experimental severity limits. Methods for practical welfare assessment are also obviously important in commercial practice. In both spheres the use of different forms of technology is increasingly playing an important role in augmenting human monitoring and recording of welfare.

Given this, WP7 had a central focus on developing and validating new automated sensor technologies for recording cattle behaviour, with a view to using these techniques to monitor or predict health and welfare problems. These approaches could contribute to improvements in animal experiments either by reducing animal numbers, or by refining individual animal experiences, or by avoiding more invasive monitoring approaches. But they also have potential for commercial implementation, through novel technologies, or by extracting additional data from existing sensors and thereby providing new services to farmers and could therefore benefit cattle welfare more broadly. Most notably SmartCow work has contributed novel analyses of the circadian rhythm of cow behaviour (Wagner et al 2021). Utilising more complex behavioural traits, such as circadian rhythms has been shown to enhance early detection of health/welfare issues. This work involved automatic detection via sensors of alterations to circadian pattern of activity prior to negative health events. Such application of sensors has substantial potential to improve welfare both in commercial practice and in research.

2.4 Reducing the use of fistulated cows

As well as refining required animal procedures SmartCow researchers have also made progress in developing alternatives to whole animal use that act to reduce animal numbers in cattle research. One particular point of attention is the use of fistulated/cannulated cows in research. This procedure involves an initial surgery to create a fistula between the animal's rumen (or other gut site) and the external environment. Fistulation is used in research to allow regular access to the gut and its contents for sampling or removal of rumen fluid, for the targeted addition of substances (such as feed additives), or for assessment of studies of ruminal degradation / digestibility using an *in sacco* approach. In this approach a small nylon bag of the test substance is placed into the rumen via the fistula – this bag can be retrieved after a set period in order to study the degree of degradation occurring as part of natural digestion in the rumen.

Whilst use of fistulated animals has greatly added to knowledge relating to the basic biology of the bovine gastrointestinal tract, and its responses to different feeds and additives, particularly in terms of methanogenesis in recent years (Beauchemin et al 2020), concerns about animal welfare remain. Such



concerns include the acute impact of the initial surgery, and associated pain (Durand et al 2021), as well as possible longer-term issues relating to maintaining a small number of animals in a research setting for many years, in circumstances where housing can remain sub-optimal at some institutions. SmartCow partners (INRAE) have been working on an *in vitro* fermentation method, which allows the rumen conditions to be replicated in an artificial environment (Niderkorn et al 2020) providing a partial (since apparent dry matter or crude protein degradation is not entirely equivalent to *in sacco* degradability) alternative to *in sacco* methods. This method represents a reduction in animal use (only a limited number of donor animals are required to generate the rumen juice used) and means that key parameters such as pH, gas production, and dry matter loss can be measured without the requirement to use cattle for each study. This approach may be used to provide pre-screening of alternative feeds or products *in vitro* – limiting the number of treatments that require to go forward for full *in vivo* validation.

More generally, the use of fistulated cows remains a procedure that may divide public opinion. Fistulated cows continue to be used at a number of SmartCow infrastructure sites – for *in sacco* studies, digestibility work, enteric methane emission studies, and for rumen inoculum harvesting. However, a number of SmartCow partners have discontinued their use or intend to do so in the near future and this has led to programmes of research examining alternatives to the various purposes this procedure is conducted for. This includes: replacing *in situ* measurement of feed values with lab based enzymatic methods; collection of rumen fluid direct via tubing (Muizelaar et al 2020); application of sensors for measuring rumen parameters (e.g., pH which is an established method (albeit with some limits to applicability: Dijkstra et al 2020), but also measuring ammonia or other parameters); alternative techniques to measure digestive flow / passage; investigating marker techniques (for collection in faeces) and procedures for freezing/thawing rumen content collected at slaughter, allowing preservation for further *in vitro* fermentation. However, whilst these approaches have led to a reduction in the requirement for fistulated cows, there remain some scientific constraints on a complete move to alternatives. For instance, some of the work exploring alternatives has identified interactions between method of fluid collection and the treatment applied (van Gastelen et al 2019); that this is an interaction rather than just a measurement bias means that fistulated cows are still needed for the time being, in order to get good samples and biologically relevant measurements for specific experimental objectives.

3 Summary

Since they were first proposed the 3Rs have become a central feature of legislation relating to animal experimentation in Europe. Whilst support for the use of animals in research is variable within society, the basis for such societal and political support as does exist is that animal use will be minimised in terms of both the numbers of animals used and the negative affective states that they experience (Maestri 2021).

This expectation arguably forms the basis of an implicit social contract between the public and researchers, as well as the regulators and legislators that govern animal research.

Research studies using cattle are conducted for a variety of reasons, including animal health and welfare, livestock performance and – increasingly – to address pressing societal concerns, particularly relating to environmental impact. Cattle are a prominent part of global livestock production, with cattle production representing a major component of agricultural economic output in many countries around the world. As such cattle production play a major role in the viability and sustainability of many communities and regions. Balancing competing interests between environmental impact and economic value in cattle production will be a key challenge for agricultural policy makers over the coming decades, and research work using cattle, such as that done within SmartCow, is likely to remain as a critical source of information and solutions in this context. As such, cattle will continue to be used in a research context for the foreseeable future. Given this, it remains important to continue developing scientific approaches that yield the necessary high-quality information whilst giving due respect to the 3Rs and to the experiences of the sentient individuals that are the subjects of the work. Whilst some problems remain to be solved, the SmartCow project has shown that it is possible to achieve success in attainment of scientific goals, whilst also making progress on implementation of the 3Rs. This demonstrates that livestock researchers across Europe and beyond can conduct high quality science that informs upon major global issues, whilst also improving animal welfare in their study animals. In many cases – such as the use of improved housing conditions – these advances allow simultaneous improvement in cow welfare and in data quality (accuracy, precision, and applicability). It is also important to note that, whilst advances at individual institutions are important one notable additional achievement of SmartCow – via the online book of methods – has been to improve communication on methods and standards across relevant research sites conducting cow research in Europe.

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