Home Office

NON-TECHNICAL SUMMARY

Enhancing control of poultry diseases

Project duration

5 years 0 months

Project purpose

- (a) Basic research
- (b) Translational or applied research with one of the following aims:
  - (i) Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - (iii) Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes

Key words

Chicken, Parasites, Bacteria, Microbiota, Vaccines

Animal types	Life stages
Domestic fowl (Gallus gallus domesticus)	juvenile, embryo

Retrospective assessment

> The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project is intended to generate new data and knowledge about (1) fundamental biology and genetics of coccidial parasites and intestinal bacteria, (2) host immune responses, and (3) the interactions and co-interactions of parasites and bacteria with the chicken.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Infectious diseases of chickens can compromise meat and egg production, cause severe welfare problems, and be a source of human zoonotic disease. There is a requirement for new approaches to control pathogens that infect chickens for several reasons including pathogen resistance to antimicrobials, legislative restrictions on the use of some in-feed drugs, and consumer concerns about chemical residues in meat. New types of control (i.e. vaccines and alternative compounds such as plant extracts) will be required, supplemented by breeding of poultry with increased resistance to infection, improved immunological responses to vaccines and optimised interactions with gut bacteria. This project is intended to address fundamental issues related to control of parasites with the greatest impact on broiler chickens (*Eimeria*, cause of the disease coccidiosis) and their interactions with bacteria. The project is organized into seven work packages (WP).

WP1: Identification of target antigens for recombinant vaccine development

A panel of proteins has been identified as candidates for use in new anti-*Eimeria* vaccines, but to date none have reached the market. While experimental vaccine performance has been promising, reducing parasite replication and host pathology, improving performance and welfare, these prototype vaccines are not currently competitive with drugs used in routine prophylaxis. Building on the vaccine candidates that have already been identified and will be tested further in WP2, we will continue to identify and test the vaccine potential of new candidates using our established protocols, bioinformatics and cell biology of host-pathogen interactions. This work will include studies of basic parasite biology to identify essential proteins and processes, including genetic manipulation of laboratory parasite lines.

WP2: Development of vectored vaccines for poultry

With a need to vaccinate large numbers of animals against multiple diseases the poultry industry leads the way in take-up of new vaccines, including live replicating and killed recombinant ‘vector’ vaccines. Many vectors have been tested experimentally but commercial usage is limited. There is a need for vaccination strategies that are non-invasive, appropriate for mass administration and have capacity to induce long-lasting protection against a range of different pathogens. We aim to continue development of novel vaccination strategies including use of transgenic parasites and killed yeast lines to deliver

vaccines via the oral route. Parasites produced here will also be used in *in vitro* studies wherever possible to accelerate vaccine development while reducing the use of live chickens.

WP3: Validation of novel anticoccidial feed or water additives

As demands for alternatives to anticoccidial drugs intensify interest in feed or water additives such as botanicals, botanical extracts, enzymes and other molecules is increasing. We currently use an *in vitro* model as a screening tool to identify candidates, but final efficacy will be verified using chickens reared under field conditions.

WP4: Understanding *Eimeria* population biology

The widespread occurrence of anticoccidial drug resistance combined with strong legislative and consumer driven requirements for reduced antimicrobial use in livestock production has increased demand for vaccines to protect against *Eimeria*. Seven *Eimeria* species have long been recognized to infect chickens; however recent detection of three new *Eimeria* species circulating in chickens across much of the southern hemisphere that can escape from current vaccines has revealed unexpected levels of complexity. The consequences of vaccine breakthrough by cryptic parasites can include very high prevalence of sub-clinical infection as well as outbreaks of clinical disease. The occurrence, characteristics and genetic flexibility of *Eimeria* field populations is unclear. We will employ new sequencing technologies and traditional parasitology to improve *Eimeria* genome resources and characterise *Eimeria* field populations, exploring the impact of drug or vaccine selection and mixed infections to improve control of disease.

WP5: The genetic basis of susceptibility/resistance to coccidiosis

Genetic resistance of chickens to infectious disease is well documented, including coccidiosis caused by *Eimeria*. Understanding genetic resistance provides a possible means to breed flocks that are naturally protected against disease. We aim to explore resistance/susceptibility traits during infection including body weight gain, intestinal structure, circulating immune cells and proteins and parasite replication, plus chicken activity and behaviour to identify genetic, biological and visual markers that can be used in selective breeding and routine husbandry of chickens to improve resistance to disease.

WP6: Understanding the consequences of pathogen interaction and impact on gut bacterial populations

Eimeria infection of chickens can alter, and be influenced by, the composition of the intestinal microflora, in some examples modifying colonisation of specific pathogens such as *Campylobacter jejuni*. We aim to use next-generation sequencing techniques to further define these interactions to (i) improve poultry health in terms of altered colonisation and/or pathogenesis of bacterial pathogens, (ii) reduce the risk of zoonotic transmission to humans through the food chain, (iii) explore the host genetic contribution to variation, and (iv) evaluate interactions in the presence of known anticoccidials or alternative additives.

WP7: Explore the use of embryonated chicken eggs as replacements for hatched chicks in studies with *Eimeria*

A small number of lines of *Eimeria* have previously been adapted to replicate in embryonated chicken eggs. We aim to resurrect this technique and explore its use to replace some studies with hatched chicks.

What outputs do you think you will see at the end of this project?

The work will generate new data and knowledge about (1) fundamental biology and genetics of coccidial parasites and intestinal bacteria, (2) protective host immune responses, and (3) the interactions of parasites and bacteria with each other and the chicken. These outputs are worthwhile in their own right because they contribute to understanding of the pathology and impact of serious diseases. They are also essential for more targeted specific objectives:

Identifying *Eimeria* antigens that induce immune protection or play substantial roles in the biology of the parasite is critical for downstream development of new vaccines that remove the need to produce live parasites for use in current vaccine formulations, and to reduce routine drug use.

Validating the beneficial effects of candidate botanicals, botanical extracts, enzymes and other molecules identified using *in vitro* tests as candidates to improve control of *Eimeria* can improve the welfare of farmed chickens and reduce routine drug use.

Progress towards **new vaccine vectors** that can be used to immunise against multiple pathogens, including species of *Eimeria* in addition to viruses and/or bacteria, and can be administered safely (orally) from day of hatch.

Improving understanding of *Eimeria* parasite population structure will be important to optimise future vaccination efficacy and safeguard vaccine longevity, while also facilitating reduced use of drugs in poultry production.

Breeding for disease resistance is an additive approach that requires understanding of host genetics linked to resistance and improved responses to vaccination. Mapping sequences within the chicken genome linked to resistance/vaccine responsiveness will provide genetic and phenotypic biomarkers that can facilitate downstream development of tools to estimate disease susceptibility and inform future breeding strategies.

Understanding the contribution of *Eimeria* to colonisation of chickens by bacterial pathogens such as *Campylobacter* species can improve control of zoonotic disease. **Understanding interactions with gut bacterial populations** can improve poultry health, welfare and productivity.

Using embryonic chicks to work with *Eimeria* offers opportunities to replace use of hatched chicks and access some parasite lifecycle stages with improved efficiency.

Who or what will benefit from these outputs, and how?

New vaccine vectors offer several benefits to:

- poultry, including fewer vaccinations/injections; better disease protection; less handling; wider uptake of vaccines

- farmers, reducing costs of pathogen control, improving ease of vaccine or feed additive administration

- animal health companies, maintaining a competitive position in markets for control of coccidiosis

- consumers, reducing the cost and improving availability of poultry products

- the environment, reducing use of antimicrobial and anticoccidial drugs.

Commercial development of new anticoccidial vaccines based on vectored proteins would have many benefits including use of none or fewer birds for production of the parasites used in existing live vaccines, cheaper vaccines, and wider uptake. The UK leads the world in manufacture of live attenuated coccidiosis vaccines but there are major issues associated with the need to incorporate many ‘lines’ of parasites in order to induce immune protection. Figures from industry indicate that more than 2 billion doses of Paracox are sold each year requiring sacrifice of >250K birds for parasite production. A prototype multi-valent vaccine will ensure that the UK animal health industry has a solid foundation from which to retain a leading position on coccidiosis control, contributing to overall wealth creation. Inclusion of vaccine antigens protective against other pathogens such as *Campylobacter jejuni* can improve health and welfare further, while reducing the number of vaccinations required. Consideration of new and emerging parasite types may be essential in the absence of drug-based prophylaxis.

Identification of markers that can be used in selective breeding of chickens that are more naturally resistant to *Eimeria*, or respond better to vaccination, offer benefits to poultry, poultry consumers and producers, and the environment, as outlined above. Reducing the occurrence of ill health in chickens will lower the overall cost of poultry products, benefiting consumers as well as production and distribution networks.

Understanding interactions between *Eimeria* and bacterial zoonoses can improve poultry product quality, reducing risks to consumers and increasing confidence in food supply. Understanding interactions with the 'healthy' gut bacteria found in chicken intestines can improve chicken health, welfare and productivity. Improved broiler chicken gut health is expected to lower demand for antimicrobial intervention, reducing drug use in livestock production. Lower antimicrobial consumption will reduce selection for antimicrobial resistance in enteric and environmental microbial populations, and reduce antimicrobial flow into environments around chicken production systems.

Indirect benefits include staff and students working on the project who will receive training in laboratory and simulated farm level settings, including a range of protocols that can only be applied with live animals and can also be used to answer a variety of experimental questions beyond the remit of this work. The national and international scientific community will benefit from improved understanding of *Eimeria*, their interactions with bacteria, and provision of improved vaccine vectors for poultry.

How will you look to maximise the outputs of this work?

All data produced from these studies will be published in Gold Open Access peer reviewed journals, as mandated and supported by the funding bodies. In addition to data, protocols and standards developed or applied will be described, providing resources and benchmarks for comparative studies. Data such as DNA or RNA sequences (e.g. defining bacterial populations or genes that are active) will be

submitted to open repositories, specifically the European Nucleotide Archive (ENA), linked to the DNA Data Bank of Japan (DDJB) and GenBank. Published studies will include results of null or unassociated measures to share awareness. Results will be shared with peer audiences through national and international conferences (e.g. British and World Veterinary Poultry Association meetings, British Society for Parasitology).

Results and progress will also be reported in industry journals and magazines, as well as live events such as the Pig and Poultry Show, to ensure dissemination to relevant target audiences.

A series of collaborations with partners in industry and academia will enhance outputs for the work, including links to researchers in Asia, Africa and Central/South America, as well as Europe and North America.

Species and numbers of animals expected to be used

- Domestic fowl (Gallus gallus domesticus): 5500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Chickens will be used throughout these studies, recognising that they are the target animal and not just a model. A range of chicken types will be used, including (i) specific pathogen free (SPF) chickens to permit optimal parasite production and accurate assessment of measures with minimal background variation, (ii) commercial broiler and layer chickens, representing target populations and providing real-life examples that can be reared under simulated industry conditions, and (iii) genetic knockout (KO) chicken lines lacking specific immune functions to assess the impact of the host immune response on parasite replication, pathogenicity and vaccine response. Chicks will typically be received at day of hatch for vaccination and equivalent characterisation studies, or 2-3 weeks of age for parasite production and selection studies. Studies with embryonated chicken eggs will conclude prior to hatching.

Chicks will typically be used up to six weeks of age (maximum of ten weeks old), recognising that coccidiosis is primarily a problem during the early phase of chicken growth.

Typically, what will be done to an animal used in your project?

Experiments will usually fall into one of four types, with some variation around specific procedures.

1. Parasite amplification, selection or characterisation

SPF chickens will typically be used for these studies, although a range of inbred or genetically modified chickens may be used (e.g. the range available at the National Avian Research Facility [NARF]).

Chicks will usually arrive between two and three weeks of age and undergo a minimum seven day settling in period. Once settled, chicks will receive a parasite dose (usually by oral inoculation, mimicking the natural route of infection). Doses are carefully managed based on previous experience to prevent the occurrence of clinical disease. Chicks will usually be kept in groups in cages with wire gridded floors and a range of environmental enrichments. Primary outputs include recovery of fresh parasites from faecal material collected below the wire gridded floors or from tissues collected post-mortem. Supplementary steps may include the addition of selection during parasite replication (e.g. dietary drugs, previous controlled infection), and measures of performance (e.g. body weight gain), parasitology (e.g. faecal excretion of parasite 'eggs' – known as oocysts), immunology (e.g. blood collection from the wing vein), gut integrity (e.g. faecal or blood markers), and pathology (e.g. lesion scoring) during or after infection. Measures that are being assessed for potential to replace invasive procedures or improve endpoint precision include use of accelerometers to measure chicken movement. Chickens in this type of experiment will usually be kept for seven to ten days after parasite inoculation, undergoing a single procedure (oral dosing) or two if blood is collected.

2. Efficacy assessment of novel anticoccidial controls (experimental vaccines, candidate feed additives)

A wide range of chicken types will be used for these studies. SPF, inbred or genetically modified chickens will be used to assess immune responses and responses to infection or vaccination with limited host genetic variation, permitting increased reproducibility and small group sizes. Commercial broiler and layer chickens will be used as representatives of the target populations, providing data that is directly relevant to field populations. Chicks will usually arrive within one day of hatch and be accommodated in groups in floor pens or cages following industry standard practices. Chickens may be accommodated at commercial stocking densities to mimic field conditions. Most chicks will undergo a minimum seven day settling period unless a commercial vaccination strategy is followed, in which case vaccination/feed additives may be administered at any time after arrival. Vaccines/feed additives will usually be delivered by oral inoculation, but for vaccines alternatives include administration into muscle or under the skin. Chicks will be infected by oral inoculation, mimicking the natural route of infection, and doses will be carefully managed based on previous experience to prevent the occurrence of clinical disease. A range of measures will be used to define the effects of parasite infection and/or vaccination including measures of performance, parasitology, immunology, gut integrity, and pathology during or after infection, as described above. Chickens in this type of experiment will usually be kept for five to seven weeks, including periods of vaccination or additive supplementation, infection and recovery. The number of procedures will vary depending on the protocol (e.g. regularity of repeat vaccination or blood collection), but rarely exceeds five per bird.

3. Parasite interaction with host microbiota

In most studies commercial broiler or layer chickens will be used as representatives of the target populations, providing data that is directly relevant to field populations. SPF, inbred or genetically modified chickens may be used to assess responses with reduced host genetic variation, permitting increased reproducibility and small group sizes. Chicks will usually arrive within one day of hatch and be accommodated in groups in floor pens or cages following industry standard practices. Chickens may be accommodated at commercial stocking densities to mimic field conditions. Most chicks will undergo a minimum seven day settling period unless a commercial vaccination strategy is followed, in which case vaccination may be administered at any time after arrival. Chicks will be infected with

Eimeria by oral inoculation, mimicking the natural route of infection, and doses will be carefully managed based on previous experience to prevent the occurrence of clinical disease. Co-infections with bacteria such as *Campylobacter coli* or *C. jejuni* may be initiated by separate oral inoculation. A range of measures will be used to define the effects of parasite infection and/or vaccination including measures of performance, parasitology, immunology, gut integrity, and pathology during or after infection, as described above. Variation in specific bacterial colonisation and shedding, or in composition of bacterial populations in the gut, will be quantified by microbial culture and molecular techniques from faeces or tissue/intestinal samples collected *post-mortem*. Chickens in this type of experiment will usually be kept for up to four or seven weeks, depending on age at arrival. The number of procedures will vary depending on the protocol (e.g. regularity of repeat inoculation or blood collection), but rarely exceeds five per bird.

4. Parasite inoculation into embryonated chicken eggs

Embryonated chicken eggs will receive up to two injections containing antibiotics and purified parasites to grow egg-adapted parasite lines. Eggs will be incubated at 41^{o}C to support parasite development. Embryos will be harvested for purification of parasites to study parasite cell biology. Each study is expected to last seven to ten days and chicks will not be hatched.

What are the expected impacts and/or adverse effects for the animals during your project?

Oral inoculation is straightforward in chickens and very well tolerated. Parasite doses are carefully managed based on previous experience to prevent the occurrence of clinical disease. Clinical signs are non-specific but include ruffling of feathers, paleness of comb and wattles, consistently closed eyes, wet droppings, diarrhoea and/or bloody faeces, or reluctance to move. Chickens exhibiting two or more of these signs will be removed and euthanasia will not be delayed for any experimental or procedural reason. Chickens will also be removed if a single sign persists for > 24 hours. Bacteria inoculated in co-infection studies are well defined strains known to be non-pathogenic for chickens.

Blood collection from the wing vein can result in a localised haematoma but these are tolerated well. The risk is far lower than sampling from the jugular vein and consequences usually resolve within 24 hours.

Other adverse effects include conditions common during commercial chicken production including lameness and feather plucking. Regular inspections and good husbandry will be used to minimise the occurrence of these effects.

Injection of embryonated eggs is routine in the poultry industry during *in ovo* vaccination and is well tolerated.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The impacts and adverse effects of the procedures described here are expected to be mild for most individuals. However, a moderate severity is indicated in recognition that host susceptibility can vary

considerably, especially in hybrid commercial chickens, possibly reaching ~5%.

Industry data indicate that 10-30% of pedigree and commercial broiler chickens will experience enteric dysbiosis by five weeks of age when reared under standard commercial conditions in the absence of antimicrobial prophylaxis. Chickens that experience dysbiosis will be removed from the study, not retained and treated.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Eimeria parasites do not grow productively in cell culture and can only be obtained from infection of live animals. Similarly, studies of anti-parasite control and host-pathogen interactions require use of live animals to assess effects on parasite growth and consequences of infection. Over the past two decades our lab has significantly improved propagation of *Eimeria* in cell culture, and we routinely use immortalised cell lines for the study of early invasion events in the parasite's lifecycle and effects of potential interventions, significantly reducing the use of chickens. We will continue to use cell culture whenever possible, but studies beyond the first step in parasite replication cannot be supported in this manner. We will also continue to test new methods for full propagation of the parasite life cycle in cell culture or tissue explant systems. However, currently the only way to amplify parasites, perform genetic crosses, maintain pure strains, study pathology and host immunity, generate novel transgenic lines of parasite, or evaluate vaccine efficacy is to carry out infections of animals.

Which non-animal alternatives did you consider for use in this project?

Non-animal alternatives for *Eimeria* replication are not currently available. Compartmental models such as fermenters (gut lumen models), organoids and tissue explants represent incomplete systems and are not currently fit for purpose. The topic has been reviewed during preparation of this application (e.g. targeted searches of the published literature via PubMed, Web of Science and Google Scholar). Progress with explants and organoids has been made in recent years (e.g. publications including Nash et al, doi: 10.1038/s42003-021-01901-z), but lack the ability to replicate interactions between host, environment and gut bacteria.

Considerable progress has been made in cell culture of the initial invasion and early development steps for one *Eimeria* species that infects chickens, however, the system is not productive (i.e. does not complete the lifecycle) and is not suitable for the other *Eimeria* species that infect chickens. Further, parasites to be used in these limited cell culture studies can only be produced by prior replication in chickens.

Why were they not suitable?

Non-animal alternatives cannot currently be used to re-create the complex interaction between parasite and host and cannot be used to reproduce *Eimeria*. Similarly, interactions with the environment, host immune response and gut bacteria cannot be replicated.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals requested has been estimated based on our own and other published experience of studies with *Eimeria* species parasites in a range of inbred, broiler and layer commercial chicken systems. Data defining the variation expected within experimental groups and the magnitude of responses to treatments such as prior exposure or vaccine application are used in power calculations to identify optimal group sizes with sufficient statistical power using the minimum number of animals. Input figures vary considerably between chicken types (e.g. inbred SPF chicken lines to outbred commercial layer lines), parasite species (e.g. caecal dwelling versus small intestinal species) and even some parasite strains. Further details are available in the relevant protocols.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will follow ARRIVE guidelines for experimental reporting and study design to ensure the most relevant, reduced and transparent experimental design. We will also apply community consensus guidelines when available for reporting study design to ensure all sampling is fit for purpose (e.g. adapting the human microbiome STORMS checklist to poultry; Mirzayi et al., 2021, doi: 10.1038/s41591-021-01552-x).

For parasite amplification infectious doses are calculated to yield high numbers of progeny without inducing clinical signs based on our experience and taking into account replicative potential and known pathogenicity. For all parasites the doses and numbers of animals are calculated from theoretical and actual data. For each study groups of 5-20 animals are typically used, and to minimise the overall numbers the research group operates a pooled resource so that each batch is utilised with minimal wastage. The number of animals used in studies for parasite production is determined based upon the number of parasites required for downstream applications (e.g. purification of genomic DNA for genome sequencing, protein for mass spectrometry, selection of transgenic sub-populations, use for cell culture studies), not statistical comparison between groups.

For comparative studies such as definition of vaccine or feed additive efficacy, immune responses, cross-protection between *Eimeria* species and impact on gut bacterial populations, animal numbers are the minimum required based on experience supported by statistical power calculations (at 5% significance, 80% power). For example, immunisation/challenge experiments are carried out using the minimum number of animals required for statistically significant results identified by power calculations (usually n=5-10, dependent on the trait and the parasite species). Data such as parasite replication are usually analysed using a parametric method, while measures such as rating of pathology require non-parametric analysis.

For research on alternative additives every candidate will be tested using our *in vitro* model before moving to trials in experimental animals to reduce the number of studies and experimental groups. We have estimated that use of this model as pre-screening for novel additives can help to reduce the use of chickens by at least 30%.

Our recent development of oral dosing using a non-toxic fluorescent dye as a quantifiable measure of gastrointestinal damage is intended to permit repeat sampling from living birds, potentially replacing terminal measures such as intestinal lesion scoring. Piloted on an existing licence, this strategy will be developed further here and can reduce the number of chickens required in an experiment since it will not be necessary to cull multiple birds to assess different timepoints. The quality of the data will also be improved when repeat measures are made from the same individuals, reducing background variation.

Previous relevant innovations include development of molecular tools to quantify parasite replication, replacing traditional parasite counting by microscopy. Application of the assay has permitted reduction of group sizes by up to 50% in some studies.

Testing *Eimeria* amplification in embryonated chicken eggs may offer opportunities for reduction in future studies if background variation and inefficiencies in harvesting some parasite lifecycle stages from hatched chicks can be reduced.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The studies proposed here have been based on current commercial knowledge, extensive experimental data collected over more than 20 years of work, and consideration of the published literature. Background data defining the level of variation expected in measures of infection, parasite replication, pathology and performance, including the influence of distinct host breeds/lines and parasite species/types, are used in power calculations as outlined in each protocol. Samples collected from these studies will be blinded for laboratory analyses wherever possible. Tissues and data will be shared with other projects within the group and made available to others within the wider community to maximise their use.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare

costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Chickens are the natural hosts of the *Eimeria* species to be used and the data generated is of direct relevance for development of improved control strategies.

Methods for infection of chickens with *Eimeria* have been refined over many years and are carried out with a minimum of stress to the animals. We minimise animal suffering by thorough monitoring, well defined end points and carefully controlling doses of parasites. For the vast majority (~95% of animals used) it is expected that suffering will be within a mild severity band; we require a moderate severity limit because we cannot rule out rare occasions where animals may show clinical signs of coccidiosis. The inclusion of a behavioural measure of chicken health (using accelerometers to measure movement) adds a new quantitative trait that can be assessed without a terminal or invasive procedure and offers opportunities for repeated measures. Further, data produced using accelerometers may be appropriate for use as a new clinical end point, permitting intervention before pathology-based measures become apparent.

Other procedures such as oral inoculation, blood sampling from the wing vein rather than the jugular, and vaccination by injection into muscle or under the skin are well established and selected to minimise the need for (more) invasive procedures. For example, final blood sampling will be undertaken immediately *post-mortem* whenever possible rather than from live birds to minimise the number of procedures per individual.

Chickens will be housed in the RVC Animal Welfare Barn (AWB) facility that was refurbished in 2018. Chicken environments are enriched with perches and materials for activities such as reflective discs, balls or cable ties to peck including caged and commercial conditions studies.

Why can’t you use animals that are less sentient?

The study of *Eimeria* in chickens cannot be accurately replicated in any other less sentient animal. Each *Eimeria* species is specific to an animal host species, therefore, the species of interest to combat chicken coccidiosis can only be studied in chickens, infections cannot take place in any other host species.

The use of embryonated eggs is planned to replace some chicken use for studies with specific parasite lines and lifecycle stages, but is not currently applicable for non-adapted lines (i.e. most current parasite lines, all field isolates).

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Chickens will be habituated to experimental staff from arrival, including routine ‘pen walk throughs’ that will be used to detect chickens experiencing the effects of *Eimeria* infection.

Based on experience with defined doses of the *Eimeria* species that infect chickens it is clear when the consequences of infection are most likely to first occur (e.g. ~112-130 hours for *Eimeria tenella*). We increase monitoring during periods of elevated risk, acting to remove individuals if they approach protocol endpoints.

Research staff involved in any experiment will be trained by researchers experienced in each procedure and assessed by the Named Veterinary Surgeon (NVS), Named Training Competency Officer and/or other competent personnel following established practice.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow expert guidance on husbandry from industry and veterinary practitioners, benefitting from interactions with the British and World Veterinary Poultry Associations to ensure that best practices are always followed.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The applicant and all team members who will work on the project frequently interact with the NC3Rs, checking the website regularly and attending seminars and webinars when they occur. The host organisation works with a NC3R Regional Programme Manager, providing opportunities for periodic training in 3R related topics and one-to-one meetings for advice. The host organisation is also very active in dissemination of 3Rs relevant news, providing training and updates via a newsletter, emails and online notifications.