Home Office

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Non-technical summaries granted during 2013

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Project Titles and key words

- Diagnosis and therapy of toxin-mediated diseases and healthcare-associated infections

  Difficile, immunotherapy, vaccine, HCAI, Gram-negative

- Amygdala hippocampus circuits

  Amygdala, hippocampus, interneuron, GABA receptors, post-traumatic stress disorders

- The neural basis of memory in rodents

  Learning, memory, brain function, plasticity

- Neural basis of memory loss

  Alzheimer's disease, dementia, hippocampus, synapses, memory

- The discrimination of magnitude

  Discrimination learning, spatial learning

- Ecology of non-native fish parasites

  Fish, parasite, non-native, introduction

- Inflammation and epilepsy

  Brain; Inflammation; Seizure; Cytokine; Rodent

- Developing T-cell based therapies for cancer

  Cancer, T lymphocytes, T-cell receptor, immunotherapy, adoptive therapy

- Staphylococcus aureus, pathogenesis to therapy

- Avian Ecology

Diagnosis and therapy of toxin-mediated diseases and healthcare-associated infections
Difficile, immunotherapy, vaccine, HCAI, Gram-negative

- Summarise your project (1-2 sentences)

The programme of work is principally concerned with the study of healthcare-associated infections (HCAIs) with the aims of understanding disease processes and developing vaccines and antibody-based therapeutics. Research is currently focused on infections caused by *Clostridium difficile* and selected Gram-negative bacteria, notably pathogenic *Escherichia coli*.

- Objectives: Explain why you are doing this project. Describe the scientific unknown(s) or clinical or service need you are addressing. Give a brief scientific background or other explanation of why the work is needed.

  C. difficile infection (CDI) is still a major problem as a HCAI with over 20,000 cases reported in 2010-11 in the UK. This bacterium mainly infects elderly patients on broad spectrum antibiotics causing a range of symptoms from mild diarrhoea to severe, recurrent diarrhoea which is often life-threatening. Severe forms of CDI are not particularly well served by current treatment options and there is a need for alternative therapies to be developed. A significant proportion of the work to be undertaken in the current work programme is concerned with the late stage development of an antibody-based therapy targeted at the treatment of severe CDI.

  Gram negative bacterial species represent a significant burden to the NHS as HCAIs and their rapidly growing resistance to frontline antibiotics is severely reducing the options available for effective treatment. Extra intestinal pathogenic *Escherichia coli* are a leading cause of bacteraemia in the UK with 29,777 cases reported in 2011. With few new antibiotics in development, there is now an urgent need to investigate alternative therapeutic options for treatment of Gram negative HCAIs. Current work is focussed on assessing various bacterial protein factors e.g. toxins, bacterial surface components, as potential targets for intervention strategies, such as vaccines.

  The project area also provides a backup emergency response diagnostic capability for assessing the presence of botulinum neurotoxins in samples in the event of suspected outbreaks/incidents involving these toxins.

- Outline the general project plan.

Previous work has shown that a cocktail of antibodies which neutralise the potent toxins produced by *C. difficile* can prevent infection in animals. In the present work programme, the hamster model for CDI will be used to complete the package of data required before first-in-human trials can begin. Studies will include determining the efficacy of product formulations and antibody dose-ranging studies to guide initial clinical studies. Animals (mice) will also be used to validate key assays that will be used to determine product potency.

For work on Gram negative HCAIs, potential vaccines candidate will be identified by genomic and proteomic techniques. Antibodies to these will initially be assessed for protective efficacy in various cellular models. For the most promising candidates, a mouse sepsis model will be used to evaluate their efficacy at attenuating the disease process.

The deployment of the botulinum toxin diagnostic capability will only be used in

emergency response scenarios and it is envisaged that such incidents will be rare.

- Predicted harms: Give a brief description of the procedures to be applied to the animals used in this project and describe the expected adverse effects.

For the CDI hamster model, animals will be given an oral dose of antibiotic followed by a dose of *C. difficile* spores, also given orally. Some animals will be injected with antibodies. Animals develop diarrhoea and become lethargic, sometimes with tender abdomens. For the sepsis model, mice will be injected with bacteria or membrane fractions from bacteria. Animals are predicted to become lethargic and immobile. Botulinum toxins, if present in suspected outbreak samples,may cause severe respiratory distress. For all models a scoring system and regular monitoring will be applied and animals which become distressed or immobile through disease will be humanely killed.

- Predicted benefits: Outline in a few sentences how science will advance, or people or animals will benefit from this project.

The work on antibody-based therapeutics for CDI provides a potentially affordable and effective alternative to antibiotics for treating severe cases which are currently poorly served by available therapies and which have a high mortality rate.

For Gram negative HCAIs, new therapeutic strategies are urgently required to deal with both the causes and symptoms of such infections. The proposed research programme is focussed on early stage research which has the goal of identifying key protein targets which could potentially underpin either vaccine or immunotherapy-based intervention strategies

The botulinum neurotoxins are a diverse family of extremely potent neuroparalytic agents which can cause potentially fatal intoxication which is difficult to manage. While outbreaks of botulism are rare, it is important that sufficient capacity exists to diagnosis these quickly and accurately.

- Estimate the numbers of animals of each species to be used; explain what types of animal will be used and why you chose the particular types of animal. Explain how you will ensure that you use the minimum number of animals.

For the CDI models, it is estimated that up to1000 hamsters and 1000 mice will be used in the disease model and toxin-neutralisation assays, respectively over the 5 year programme. For the sepsis model, up to 1000 mice will be used. Hamsters are considered the ‘gold standard’ for use in the study of CDI and the characteristics of their disease has many parallels with the human infection. Similarly, mice are widely used for the study of sepsis induced by bacterial infection. For the production of antibodies up to 500 mice, 200 hamsters, 200 guinea pigs and 50 rabbits have been allocated over the programme duration. Animal numbers required for diagnosis of botulism cannot be predicted but numbers are likely to be low (<50 mice for the duration of the project). Animal group sizes will be kept to the minimum required to generate statistically significant data.

- Demonstration of compliance with the 3Rs: State why you have to use animals and cannot use non-animal alternatives. Where appropriate, say how you will use non-animal studies in parallel with the project.

CDI is a complex disease, initiated by bacterial spores and it is currently not feasible to assess the efficacy of potential therapeutics without using animal models. It is proposed

to use non-animal, cell-based assays to estimate the capacity of the antibody product to neutralise the *C. difficile* toxins. The results of these assays, however, must be compared initially with toxin-neutralisation assays performed in mice to ensure that they are measuring an activity of the product that is relevant to protection *in vivo*.

As far as feasible, non-animal, cell-based assays are being used and will continue to be used to assess therapeutic candidates. Ultimately, the most promising candidates will require assessment in animal models to determine their effectiveness at attenuating disease progression. For these studies, a mouse sepsis model will be used to evaluate the efficacy of vaccines and therapeutic antibodies.

The diversity within the botulinum toxin family and unknown nature of outbreak samples makes currently available *in vitro* assays insufficiently robust for emergency response applications. As far as feasible *in vitro* assays will be used to minimise animal use.

- Explain why the protocols and the way they are carried out should involve the least suffering.

For all animal models a scoring system and regular monitoring are applied. Animals which become immobile through disease will be humanely killed in order to minimise suffering.

Amygdala hippocampus circuits
Amygdala, hippocampus, interneuron, GABA receptors, post-traumatic stress disorders

- Summarise your project (1-2 sentences)

  I am interested in understanding what nerve cells in the brain underlie emotional-dependent behaviours, and their involvement in fear and anxiety disorders. To this purpose, I mainly study the function of nerve cells of two brain areas involved in these functions, called amygdala and hippocampus, in rodents.

- Objectives: Explain why you are doing this project. Describe the scientific unknown(s) or clinical or service need you are addressing. Give a brief scientific background or other explanation of why the work is needed.

  The brain regions of amygdala and hippocampus are of fundamental importance in cognitive functions such as learning, memory, fear and emotional states in health and psychiatric diseases. The amygdala and the hippocampus are composed of several distinct nerve cell types. About 20% of them release and use a substance called GABA to communicate with other nerve cells. GABA normally reduces the activity of nerve cells and plays key roles in the normal and diseased brain. To define the functions of the amygdala and hippocampus under normal conditions and in experimental models of psychiatric diseases, I aim to characterise the types of nerve cells, their structure and molecular properties. My work identifies specific types of GABA cells which have clear roles in behaviour and are related to psychiatric disorders. To study them may lead to the identification of novel principles of how neurons communicate with each other. For example, I study a type of nerve cell in the amygdala which is of paramount importance for fear extinction, a process that extinguishes fear once fear associations have been learned. Fear extinction is the corner-stone of the psychological therapy of anxiety because the inability to extinguish fear responses is a trait of several anxiety disorders. As the amygdala and hippocampus are associated with post-traumatic stress disorders, this project may also advance not only fundamental neuroscience, but also help to elucidate the mechanisms of action of widely used drugs such as anaesthetics, anticonvulsants and anxiolytics.

- Outline the general project plan.

I will identify the types of nerve cells and their connections in the amygdala, hippocampal and other regions involved in the control of fear memory formation, retrieval and inhibition. I will selectively manipulate the activity of particular sets nerve cell to establish causal relationships between fear behaviour and the underlying nerve cell activity. I will gain insights into the mechanisms underlying learning-dependent changes in fear behaviour by studying molecular and structural changes and how identified nerve cell types contribute to the activity of large populations of nerve cells in interconnected brain areas.

- Predicted harms: Give a brief description of the procedures to be applied to the animals used in this project and describe the expected adverse effects.

Procedures will consist of: non-recovery anaesthesia; under recovery anaesthesia: small skull opening (craniotomy) to insert a glass/metal recording or an injection device to

monitor cells' electrical activity or inject nerve cell markers; recording of nerve cells' activity in non-anaesthetised animals; behavioural test to assess anxiety-like behaviour. Possible adverse effects may include discomfort, pain and infection. The level of anaesthesia will be continuously monitored, and analgesics will be provided prior to, during and/or after surgery. Aseptic technique will be used during surgery to avoid post-operative complications with infection. Behavioural tests may produce mild anxiety but this will be rigorously monitored and controlled.

- Predicted benefits: Outline in a few sentences how science will advance, or people or animals will benefit from this project.

Uncovering the principles of operation of novel GABA nerve cells will significantly progress our understanding of the brain areas of amygdala and hippocampus. Moreover, this research may provide a scientific rationale to develop novel anxiolytic drugs and drugs for the treatment of depression. The project will also provide basis for an animal model to study post-traumatic stress disorders which involve the amygdala and hippocampus of humans.

- Estimate the numbers of animals of each species to be used; explain what types of animal will be used and why you chose the particular types of animal. Explain how you will ensure that you use the minimum number of animals.

For the *in vitro* experiments, I plan to use 3000 rats/mice wild type/genetically altered. I also plan to breed and maintain 5000 mutant and genetically modified mice. Virtually no adverse effects are likely to occur to these animals, since they will be humanely killed by terminal anaesthesia. I also foresee to perform in vivo/behavioural experiments involving up to 2000 rats/mice wild type/genetically altered with or without anaesthesia, and to inject with tracers in vivo up to 500 anaesthetised rats/mice wild type/genetically altered prior to slicing. The experimental design will be performed as much as possible to obtain a good estimate of the within-group standard deviation and of any treatment comparisons which are of interest, without wasting non-necessary resources and animals. Statistical analysis will be planned as much as possible before starting the experiments, and efforts will be devoted to analyse each set of experiment before starting the next one. Care will be taken to assess the normality and homogeneity of variances of the data, and to use ANOVA and non-parametric tests when appropriate. The use of in vitro slice culture technique, one of the two in vitro methods employed, effectively reduces the number of animals used compared to other preparations in vitro, since the slice cultures are maintained and studied up to several weeks after the preparation, and also a good number of slices (usually 12-18) are prepared from a single rat pup.

- Demonstration of compliance with the 3Rs: State why you have to use animals and cannot use non-animal alternatives. Where appropriate, say how you will use non-animal studies in parallel with the project.

There is very little information in the literature on the nerve cell types that I am interested in. Therefore, it is not possible to use a computer modelling approach and experiments on animals are needed to study the functional properties of the neurons of interest. However, some computer modelling based on the experimental results with the aid of one expert in this field will be performed. The use of animals is needed because only experimental results will help to define the physiology, anatomy and neuropharmacology of the cell populations of interest in health and disease. Rats and mice are the lowest vertebrate group in which the organisation of the limbic system including the amygdala, the hippocampus and their cortical and subcortical connections is comparable to the human brain.

- Explain why the protocols and the way they are carried out should involve the least suffering.

I am committed to implement best practice for surgery including aseptic technique, post-operative observation of animals, and rigorous application of endpoints. The behavioural tests will be mild. The animals will be used only for the objectives proposed. I have large experience in animal experimentation with the protocols contained in this licence, and I am confident I will readily recognise abnormal events. The procedures indicated are expected to be the least harmful ones allowing a comprehensive characterisation of the functional and anatomical properties in the nerve cells of interest.

The neural basis of memory in rodents
Learning, memory, brain function, plasticity

- Summarise your project (1-2 sentences)

  The aim of the project is to investigate how different brain regions, and the cells within these regions interact during the formation of recognition and event memory.

- Objectives: Explain why you are doing this project. Describe the scientific unknown(s) or clinical or service need you are addressing. Give a brief scientific background or other explanation of why the work is needed.

  Discovering the neural bases of memory is one of the foremost challenges of science. We know that memory is not a single function, but that there are different types of memory, for example recognition memory i.e. the memory that allows us to judge whether we have seen something before, and event memory, i.e. the memory that enables us to remember specific events in our lives. However what we now wish to find out is what type of information is being processed during recognition or event memory, secondly where in the brain this information is being processed and stored, and finally how this information is being stored within brain cells.

- Outline the general project plan.

  The general plan of the project is to undertake sequences of studies to discover how specific brain regions are interconnected within the brain and how the cellular processes within these brain regions control learning and the storage of memory information. To achieve this aim we will use rodents (rats or mice) and will disrupt specific brain regions or specific cellular processes within the brain cells by physical or biochemical means in order to understand the effect on memory functions. To examine the effects on memory the rodents will be tested in specific memory tasks that examine either the animals ability to recognise and object that it has experienced before (recognition memory) or that examine the animals ability to remember specific events. To examine the cellular and biochemical processes which underlie changes in neuronal function (plasticity) in specific neural pathways, brain tissue will be used.

- Predicted harms: Give a brief description of the procedures to be applied to the animals used in this project and describe the expected adverse effects.

To examine the role of different brain regions in memory function we will either selectively lesion small areas of brain tissue, or we will implant small cannulae whilst the animals are under general anaesthesia. Once the animals have fully recovered we will test their memory function. To measure recognition memory in rodents they will be placed in an arena and allowed to explore different objects, and their spontaneous behaviour will be measured as it is known that rodents prefer to explore objects that they have not encountered before. To measure memory for places the rodents will be placed in different mazes. There is a small risk of infection through the surgical procedures, however the surgery will be carried out under aseptic conditions and analgesia will be provided at all times. The behavioural tasks have no adverse effects.

- Predicted benefits: Outline in a few sentences how science will advance, or people or animals will benefit from this project.

The results of this project will enhance our understanding of how learning and memory occurs in the healthy adult brain; this knowledge will help us to understand how learning and memory can fail in aging and in neurological disorders such as dementia. The development of therapeutic strategies to treat such conditions depends on a clear understanding of how memory information is stored in different brain networks.

- Estimate the numbers of animals of each species to be used; explain what types of animal will be used and why you chose the particular types of animal. Explain how you will ensure that you use the minimum number of animals.

Rodents will be used as they are the species with the lowest degree of neurophysiological sensitivity that are able to perform the memory tasks upon which the work depends. It is estimated that approximately 400 animals will be used per year of this project licence. To ensure that the minimum number of animals are used we will, where possible, use a within-subject experimental design whereby each animal is its own control.

- Demonstration of compliance with the 3Rs: State why you have to use animals and cannot use non-animal alternatives. Where appropriate, say how you will use non-animal studies in parallel with the project.

An understanding of the cellular processes that underpin learning and memory can only be achieved by studying the intact brain. Hence, the key experiments cannot be done with cultured neurons or by computer simulations.

- Explain why the protocols and the way they are carried out should involve the least suffering.

  For the behavioural studies all the surgeries will be conducted in aseptic conditions, with the animals under general anaesthetic. Analgesia will also be provided post operatively. Throughout the protocols the health of all animals will be monitored daily. To examine the cellular processes which control memory formation we will use brain tissue and thus conduct the experiments in vitro.

Neural basis of memory loss
Alzheimer's disease, dementia, hippocampus, synapses, memory.

- Summarise your project (1-2 sentences)

  We want to understand how Alzheimer's disease starts in the brain using mice as a disease model. We will determine how the earliest signs of memory loss are related to the malfunction of brain cells (neurons) and their connections (synapses), and we will investigate therapeutic interventions.

- Objectives: Explain why you are doing this project. Describe the scientific unknown(s) or clinical or service need you are addressing. Give a brief scientific background or other explanation of why the work is needed.

  Although our knowledge of Alzheimer's disease has greatly advanced, we still don't fully understand how this and other dementias start in the brain. The lack of treatment has devastating consequences for affected individuals and for and their carers. If we understand how a healthy brain becomes affected by dementia we may be able to interfere at an early stage with a therapeutic agent. From studies in post-mortem human brain samples, we know that the number of synapses in the brain is greatly reduced in individuals with Alzheimer's disease. Our work aims to understand how these synapses are lost to prevent that from happening.

- Outline the general project plan.
  We want to establish the earliest signs of memory loss in mice or rats with symptoms similar to those of Alzheimer's disease generated through manipulations such as genetic modifications. We will perform behavioural tests in these mice to understand the progression of memory loss. We will, at the same time, perform analyses of neuronal connections, and relate how the changes in normal function of synapses explain the memory loss. Furthermore, based on our previous work and hypotheses, we will test different therapeutic agents to prevent the loss of normal synaptic function and the capacity to store or recall memories.

- Predicted harms: Give a brief description of the procedures to be applied to the animals used in this project and describe the expected adverse effects.
  The main procedure to be applied to mice or rats in this work is the induction of terminal anesthesia to then kill the animal and isolate its brain tissue. The levels of anesthesia will be carefully monitored to ensure the animals feel no pain at all. We also need to carry out behavioural tests that use the natural behaviours of rodents, such as spontaneous or food-rewarded exploration of a maze. To make sure that animals are fit and healthy for the performance of these tasks, we will constantly monitor their weight and appearance. In a smaller set of experiments we need to carry out surgery to either inject molecules in the brain, or surgery to implant small devices that will release therapeutic agents to investigate a cure to the disease. To prevent the main adverse effects of pain, distress and infection, these surgeries will be performed using strict aseptic technique, excellent surgical practice, peri-operative pain management, and constant monitoring during recovery.

- Predicted benefits: Outline in a few sentences how science will advance, or people or animals will benefit from this project.

Diagnostic methods for dementia, in particular Alzheimer's Disease, are rapidly advancing. It is predicted that in the next couple of decades, routine clinical analyses using spinal taps or even blood tests will identify high risk patients up to a decade before any symptoms appear. It is therefore crucial to establish the mechanisms that operate in early stages of Alzheimer's Disease to be able to stop or slow down memory loss. Whilst this particular work would primary advance our basic understanding of the mechanisms of dementia in neurons, the insights gained may reveal or strengthen the case for pharmacological targets alone or in combination, which can be used to design appropriate drugs for patients.

- Estimate the numbers of animals of each species to be used; explain what types of animal will be used and why you chose the particular types of animal. Explain how you will ensure that you use the minimum number of animals.

  The results of studies in mice and rats are highly relevant to the human condition, as the brain areas involved have conserved anatomy and functions. Nowadays, the scientific tools available to study mice and rats allow unprecedented access to modification of genes and neuronal circuits, which can help us to answer these questions. Per year, we do not expect to reach a maximum of 5000 mice, and 1000 rats. Most likely, as an example, we will use approximately 20-100 mice to produce a peer-review scientific publication that will advance our knowledge in the understanding of how dementia affects neuronal circuits. And in the five-year course of this project license we expect to produce 5-15 of these publications.

- Demonstration of compliance with the 3Rs: State why you have to use animals and cannot use non-animal alternatives. Where appropriate, say how you will use non-animal studies in parallel with the project.

  To advance our knowledge on how dementia starts in the brain, the best available systems are mouse and rat models of neurodegenerative disease. This is because they allow the incorporation of information from many levels of analysis: from how molecules work, to function of brain cells and their connections, to their impact in behaviour. We will interpret our results in the context of human disease to ensure their relevance. Furthermore we will complement this work with collaborations and investigations in invertebrate systems, *in vitro* and computational models. However, investigations in these alternative systems alone, would not answer the proposed questions. Only a live mammalian preparation would permit the levels of analysis required for this work.

  The number of animals required for this work will be kept at the minimum possible by ensuring adequate experimental design consistent with collection of statistically robust and reproducible data. We will use the latest technology to obtain and analyse high levels of information that can be obtained from brain tissue from a single mouse.

- Explain why the protocols and the way they are carried out should involve the least suffering.
  Our protocols strictly follow published Home office guidelines. Pain and distress will be kept at a minimum by using anesthesia and analgesia. High welfare standards will be maintained with good husbandry and environmental enrichment. We aim to identify subtle changes in behaviour that indicate the start of memory problems in mice. Although it may be necessary to allow some progression of the behavioural impairment

to relate it back to the disease, the vast majority of experiments will involve young mice with minor behavioural abnormalities (for example poor memory). *Ex vivo* preparations will be a major component of this work. We will use the brain slice preparation which has great advantages by allowing the analysis of well characterised memory-relevant networks accessible to pharmacological manipulation, and the results from which can be related directly back to the animal behaviour work. The results from this integrative work will advance our understanding of Alzheimer's disease and contribute towards the generation of a cure.

<table>
<tr><td>Project Title (max. 50 characters)</td><td>The discrimination of magnitude</td></tr>
<tr><td>Key Words (max. 5 words)</td><td>Discrimination learning, spatial learning</td></tr>
<tr><td>Expected duration of the project (yrs)</td><td>Four years</td></tr>
<tr><td>Purpose of the project (as in Article 5)[1]</td><td>
<table>
<tr><td>Basic research</td><td>Yes</td><td></td></tr>
<tr><td>Translational and applied research</td><td></td><td>No</td></tr>
<tr><td>Regulatory use and routine production</td><td></td><td>No</td></tr>
<tr><td>Protection of the natural environment in the interests of the health or welfare of humans or animals</td><td></td><td>No</td></tr>
<tr><td>Preservation of species</td><td></td><td>No</td></tr>
<tr><td>Higher education or training</td><td></td><td>No</td></tr>
<tr><td>Forensic enquiries</td><td></td><td>No</td></tr>
<tr><td>Maintenance of colonies of genetically altered animals[2]</td><td></td><td>No</td></tr>
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</td></tr>
<tr><td>Describe the objectives of the project (e.g. the scientific unknowns or scientific/clinical needs being addressed)</td><td>The survival of most, if not all, animals is enhanced considerably by their ability to profit from experience through learning. Thus animals can learn that reward, or danger, will be present in some situations but not others. A long-standing theoretical endeavour has been to understand how animals solve such discriminations, by developing hypothetical models of the changes that occur in the nervous system when animals encounter a cue that signals the presence or absence of reward or punishment. These theories are very good at predicting how animals will learn when they must differentiate between two situations that differ in the kind of stimulation they provide. The theories are not so good, however, when they must account for discriminations where reward, say, is signalled by physically similar stimulation that differs only in magnitude. This shortcoming is serious because it means that we have a poor understanding of how animals learn about such abstract properties of their environment as space, number, time and intensity. With this shortcoming in mind, the proposed project has three goals.

1. To provide much needed evidence about the manner in which animals solve discriminations based on stimulus magnitude.

2. To identify the information that is used to discriminate between two stimuli that differ in magnitude. Is it concrete and tied to the stimuli used for training, or is it more abstract and thus permits a magnitude discrimination to transfer to a</td></tr>
</table>

[1] Delete Yes or No as appropriate.
[2] At least one additional purpose must be selected with this option.

	new set of stimuli? 3. To identify the theoretical mechanisms that are responsible for how magnitude discriminations are solved. The scant evidence that is available suggests they are not solved in the same way as more conventional discriminations. If this is correct, then it will be necessary to incorporate new constructs into existing theories of discrimination learning.
What are the potential benefits likely to derive from this project (how science could be advanced or humans or animals could benefit from the project)?	The immediate benefit of the proposed research will be the development of a full understanding of how animals learn. This knowledge, in turn, will enable us to acquire a complete and accurate appreciation of the nature and mechanisms of animal intelligence. Such an appreciation will have two profound benefits. On the one hand, it will provide an essential step on our journey to understanding how the animal brain works - and ultimately how the human brain works. That is, before we can specify how the animal brain is responsible for intelligence, we must first identify the nature of that intelligence. On the other hand, a better understanding of animal intelligence will allow us to make more informed decisions about how best to care for them when they are held in captivity, and when they are under threat in the wild.
What species and approximate numbers of animals do you expect to use over what period of time?	1600 rats and 1000 pigeons over four years
In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?	All the procedures involving animals will be mild. Indeed, training will consist of creating in the laboratory an analogue of foraging behaviour in the natural environment. In order to encourage animals to respond for food reward, they will be fed a restricted diet, but at a level that has been shown over many studies to have no long-lasting adverse effects. Additional experiments will investigate how rats learn to find important goals in their environment. These experiments will take advantage of their natural ability to navigate in an aquatic environment by requiring them to find the equivalent of dry land in a small swimming pool. Rats are robust swimmers and, once again, no adverse consequences are expected.
Application of the 3Rs	
1. Replacement State why you need to use animals and why you cannot	Because the principal aim of the project is to understand how animals learn, it would be impossible to conduct the proposed experiments

use non-animal alternatives	with non-sentient subjects.
2. Reduction Explain how you will assure the use of minimum numbers of animals	Based on my extensive investigations of learning and memory in rats and pigeons, appropriate statistical techniques can be used to identify the minimum number of animals that will be required if an experiment is to yield meaningful results. This strategy will be helped by using multivariate statistical tests to analyse the results from each experiment. Numbers of animals will also be kept to a minimum by using, where possible, experimental designs in which each subject acts as its own control condition.
3. Refinement Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.	Rats and pigeons will serve as subjects for two principal reasons. First, I have considerable experience of investigating the mechanisms of learning in these species. It is thus possible to design experiments that yield the maximum scientific gain while causing the minimum of harm to an animal. Second, it is of considerable theoretical importance to know to what extent the conclusions derived from one species apply to another. If the experiments should reveal similar findings with species as diverse as rats and pigeons, then it will indicate that their results are of widespread, and fundamental significance.

<table>
<tr><td>Project Title (max. 50 characters)</td><td>Ecology of non-native fish parasites</td></tr>
<tr><td>Key Words (max. 5 words)</td><td>Fish, parasite, non-native, introduction</td></tr>
<tr><td>Expected duration of the project (yrs)</td><td>5</td></tr>
<tr><td>Purpose of the project (as in Article 5)[3]</td><td>
<table>
<tr><td>Basic research</td><td>Yes</td><td></td></tr>
<tr><td>Translational and applied research</td><td>Yes</td><td></td></tr>
<tr><td>Regulatory use and routine production</td><td></td><td>No</td></tr>
<tr><td>Protection of the natural environment in the interests of the health or welfare of humans or animals</td><td>Yes</td><td></td></tr>
<tr><td>Preservation of species</td><td></td><td>No</td></tr>
<tr><td>Higher education or training</td><td></td><td>No</td></tr>
<tr><td>Forensic enquiries</td><td></td><td>No</td></tr>
<tr><td>Maintenance of colonies of genetically altered animals[4]</td><td></td><td>No</td></tr>
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<tr><td>Describe the objectives of the project (e.g. the scientific unknowns or scientific/clinical needs being addressed)</td><td>The objectives are to:

1. Determine the environmental and biological conditions under which UK resident fish become infected with non-native parasites
2. Identify the individual consequences of the infections of non-native parasites for the behaviour, feeding and growth of UK resident fish
3. Identify the population, fish community and ecosystem level consequences of infection by non-native parasites for UK freshwaters.

Each objective addresses a significant scientific unknown.</td></tr>
<tr><td>What are the potential benefits likely to derive from this project (how science could be advanced or humans or animals could benefit from the project)?</td><td>The potential benefits are the derivation of scientific knowledge that is used to develop policy and procedures that better regulate the movement of fish and their parasites across UK freshwaters, and minimise the infection of native fish by these parasites</td></tr>
<tr><td>What species and approximate numbers of animals do you expect to use over what period of time?</td><td>The species are fish encountered throughout UK freshwaters, including some non-native species. These are roach Rutilus rutilus, common bream Abramis brama, rudd Scardinius erythrophthalmus, sunbleak Leucaspius delineatus, topmouth gudgeon Pseudorasbora parva, fathead minnow Pimephales promelas, common carp Cyprinus carpio, crucian carp Carassius carassius,.barbel Barbus barbus,dace Leuciscus leuciscus and chub</td></tr>
</table>

[3] Delete Yes or No as appropriate.
[4] At least one additional purpose must be selected with this option.

	Leuciscus cephalus Over the 5 year project, a maximum of 4250 fish will be used. An individual fish is likely to spend no more than 150 days under a Protocol.
In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?	The expected adverse effects are low grade, chronic infections of parasites. In conjunction with these are the use of anaesthesia, insertion of tags for identification and the effects of environmental manipulations. The expected level of severity is mild and moderate, depending on the Protocol. The severity is measured according to a scoring of the behaviour of the fish during daily observations. This is in relation to their respiration, feeding, swimming behaviour and response to external stimuli. Severity is also measured according to the weight loss of an individual fish (10% loss for protocols with mild severity, 20 % for moderate severity) The animals will be killed at the end.
Application of the 3Rs	
1. Replacement State why you need to use animals and why you cannot use non-animal alternatives	Non-animal alternatives cannot be used in the project as the work is specific to investigating how non-native fish parasites impact UK fish species. Some steps in protocols are not necessary for all parasites however, as previous work has already been completed on these and reported in the scientific literature.
2. Reduction Explain how you will assure the use of minimum numbers of animals	Minimum numbers of animals will be used in the project by ensuring each experiment if designed with the advice of a statistical expert. That exert will advise on the number of treatments, replicates and fish being used, with multi-factorial designs used where possible to minimize numbers. The aim of the statistics to is indicate the minimum number of animals required to provide statistically robust data.
3. Refinement Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.	The parasites being used in the project are those that are non-native to the UK and have been identified as potentially harmful to UK freshwater fish by regulatory authorities. As such, they are already refined for meeting the project objectives. To minimise harm, the fish to be infected with the non-native parasites will be sourced from aquaculture facilities, with the Environment Agency's fish farm facility used wherever possible where the fish are maintained to high husbandry standards that are usually free from any

	confounding infections of other parasites, and are checked regularly for this. Thus, confounding issues relating to multiple parasite infections will be avoided. Animal handling will be minimised to periods of data collection when they will be under general anaesthetic. Where temperature and environmental conditions are being varied within a trial then these will be within the range of those experienced by the animals in natural situations. Appropriate periods of acclimatisation to tank and pond conditions will be used and where tagging is used to monitor fish performance, those used will be the most appropriate and least invasive. The use of a behavioural and weight loss scoring system within daily observations also minimises welfare costs to the animals through clear definition of endpoints.

<table>
<tr><td>Project Title (max. 50 characters)</td><td>Inflammation and epilepsy</td></tr>
<tr><td>Key Words (max. 5 words)</td><td>Brain; Inflammation; Seizure; Cytokine; Rodent</td></tr>
<tr><td>Expected duration of the project (yrs)</td><td>5</td></tr>
<tr><td>Purpose of the project (as in Article 5)[5]</td><td>
<table>
<tr><td>Basic research</td><td>Yes</td><td></td></tr>
<tr><td>Translational and applied research</td><td>Yes</td><td></td></tr>
<tr><td>Regulatory use and routine production</td><td></td><td>No</td></tr>
<tr><td>Protection of the natural environment in the interests of the health or welfare of humans or animals</td><td></td><td>No</td></tr>
<tr><td>Preservation of species</td><td></td><td>No</td></tr>
<tr><td>Higher education or training</td><td></td><td>No</td></tr>
<tr><td>Forensic enquiries</td><td></td><td>No</td></tr>
<tr><td>Maintenance of colonies of genetically altered animals[6]</td><td>Yes</td><td></td></tr>
</table>
</td></tr>
<tr><td>Describe the objectives of the project (e.g. the scientific unknowns or scientific/clinical needs being addressed)</td><td>Epilepsy is one of the most common neurological disorders, affecting nearly 1 in every 100 of the population. There are many different types of epilepsy but all have the common feature that sufferers undergo ‘seizures’. Seizures are caused by a sudden burst of increased electrical activity in the brain. At present there is no cure for epilepsy as current medications only control the seizures. These treatments work in around 70% of patients but the remainder still suffer from seizures. There are also unwanted side-effects with current treatments. To develop better treatments we need a better understanding of mechanisms that cause seizures. Studies in rodents and humans have shown that molecules that are normally produced by immune cells during inflammation may contribute to epilepsy. One molecule, called interleukin-1 (IL-1), is particularly important. Stopping IL-1 actions reduces seizures in experimental studies, but exactly how inflammation, IL-1 and other inflammatory molecules contribute to seizure is not known and what we aim to find out.</td></tr>
<tr><td>What are the potential benefits likely to derive from this project (how science could be advanced or humans or animals could benefit from the project)?</td><td>It is not always easy to gauge the success of basic research in terms of benefit. However our research to date has led directly to the first clinical trial of a cytokine inhibitor (IL-1Ra) in stroke patients. Although IL-1Ra is unlikely to be tested in epilepsy, other means of blocking IL-1 action (i.e. inhibition of the enzyme caspase-1 which is required to produce the biologically active IL-1 protein) has recently completed a Phase II trial in epileptic patients. Overall therefore we believe that there is a very real</td></tr>
</table>

[5] Delete Yes or No as appropriate.
[6] At least one additional purpose must be selected with this option.

	chance that our studies will identify key mechanisms affecting brain excitability and seizure that could be targeted for the development of new treatments.
What species and approximate numbers of animals do you expect to use over what period of time?	The proposed experiments will be conducted on rats and mice. Over the period of the licence (5 years) it is estimated that approximately 1500 mice and 100 rats will be used, well over half for breeding.
In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?	To induce seizures in rodents chemicals (known as convulsants) are injected directly into the brain or body. Behaviour is monitored for signs of seizure activity (e.g. twitching) and brain activity recorded (electroencephalogram, EEG). The EEG allows detection of seizures and is used clinically. At different times after seizure animals are sacrificed by appropriate means to assess gene/protein expression. In some experiments drugs will be administered or genetically modified mice used to determine effects on the severity of seizures. Seizures can result in significant behavioural effects in the animals, as epilepsy does in patients. However, as far as we know the animals are not aware of the seizure or suffering, similarly to humans. Usually seizures are short-lived and animals do not show obvious signs of pain or discomfort post-seizure.
Application of the 3Rs	
1. Replacement State why you need to use animals and why you cannot use non-animal alternatives	Studying the mechanisms involved in seizures is extremely complex and involves understanding the interactions between different cell types within the CNS. There are no *in vitro* models or systems that replicate the complex interactions and architecture of the CNS, and its communication with other potential influencing factors such as the immune and vascular systems. Thus, the aims of this project could not be met in full by studies in cell culture alone and require *in vivo* studies. However, wherever possible the *in vivo* studies will be complimented by *ex vivo* (brain slices) and *in vitro* (primary cell culture) approaches. Studies in lower organisms will also be utilized wherever possible and we are already doing work in flies to inform on possible combination treatments.
2. Reduction Explain how you will assure the use of minimum numbers of animals	Experiments are designed on the basis of our own findings, published data or new hypotheses based on indirectly-related observations. In designing studies we will refer to the ARRIVE guidelines (http://www.nc3rs.org.uk/page.asp?id=1357). Experimental interventions will always be randomised and, whenever possible, blinded. In many cases, and always with interventions with unknown or uncertain outcomes, pilot studies might

	be undertaken on a small number of animals. Prior to any new study, a full evaluation of previous/pilot data and power calculations will be performed, such that the minimum number of animals required to provide valid data are used. If there is any doubt on experimental design or statistical analysis, advice will be sought from a statistical advisor.
3. Refinement Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.	The fundamental mechanisms of neuronal communication, disruption of which leads to epilepsy, are the same (as far as we know) in these species to humans. The cerebral and cerebrovascular anatomy is similar in rats, mice and humans and seizure behaviour is also comparable. Seizures are most typically associated with behavioural changes but we feel we can use a lower degree of severity and still achieve our aims, thus minimising any animal suffering. The proposed studies could not be undertaken in lower species because they do not show such similarities to humans, and *in vitro* experiments do not allow the complete study of complex interactions between different cell types in the CNS or body systems (i.e. immune and nervous). Studies in rodents have proven successful in identifying some of the underlying mechanisms of seizure generation and the hope is that even greater understanding will lead to the development of new treatments for epilepsy. Animal welfare will be continually monitored throughout and seizure exposure controlled to prevent large and persistent convulsions. If these are seen the experiments will be stopped or drugs given to reduce the seizures. In any surgical procedure animals will be given analgesics (pain killers) after surgery. We will always use the lowest number of animals possible to meet our aims.

Developing T-cell based therapies for cancer
Cancer, T lymphocytes, T-cell receptor, immunotherapy, adoptive therapy

- Summarise your project (1-2 sentences)

Some immune cells have the capacity to recognise tumours and destroy them. However, tumours have also developed ways to avoid destruction by these immune cells. Therefore we wish to explore ways of boosting and refining the immune response so that it can effectively and safely target cancer cells for destruction.

- Objectives: Explain why you are doing this project. Describe the scientific unknown(s) or clinical or service need you are addressing. Give a brief scientific background or other explanation of why the work is needed.

Approximately one third of the British population will develop cancer and 1 in 4 of them will die from it. Thus, there exists an urgent need to develop more effective anti-cancer therapies. The immune system has the potential to selectively destroy cancer cells and over the last 10 years studies, initially in animals and subsequently confirmed in humans, have shown that immune cells can be used to treat a few types of cancer. This has encouraged further work to improve these approaches, for example, to expand their use to treat many more cancer types. However, this requires several hurdles to be overcome. These include (i) generating immune cells that are able to recognise and destroy the different cancers, (ii) ensuring, following injection, that they reach the tumour site, (iii) ensuring that the tumour cells are not able to evade the immune cells and finally (iv) ensuring that any new approaches are safe.

- Outline the general project plan.

This project aims to validate novel immune-based therapies designed in our laboratory to target the tumour cells or the blood supply that serves them. The study will use mouse models of cancer and of new blood vessel formation. These involve injecting tumour cells or implanting small pieces of sponge under the skin (into which new blood vessels, resembling those in tumour tissue, will grow). Immune cells are then injected to see if they can prevent tumour growth and/or recognise the new blood vessels.

- Predicted harms: Give a brief description of the procedures to be applied to the animals used in this project and describe the expected adverse effects.

In addition to normal mice, some genetically altered strains of mice will be used in this project that spontaneously develop tumours (and therefore more closely model human cancer) or that lack their own immune system (and thereby allow us to introduce a human immune system). In this project some mice will be injected with tumour cells or have small sponges implanted into the skin. These procedures will be conducted under general anaesthesia. The mice will then be injected with immune cells and other therapeutic agents as well as agents that enable us to see the growth of the tumour cells. Occasional blood sampling using a needle will also be required. These procedures will induce some stress due to restraint and transient discomfort from needle insertion. Injecting human T-cells into mice may result in the immune cells attacking the host tissues and the growth of cancer cells could eventually prove fatal to the mice. Some mice will also be exposed to limited doses of radiation to partially deplete their own immune cells and thereby allow us to efficiently introduce “tumour-targeted” immune cells. High doses of radiation can cause sickness in mice therefore we will use doses that are well tolerated. However, any animal

showing signs of distress or pain reaching a moderate severity limit will be culled to avoid further suffering.

- Predicted benefits: Outline in a few sentences how science will advance, or people or animals will benefit from this project.

This project will advance our understanding of immune-based therapies for cancer. It will also help to validate much needed new treatments for cancer which can then be trialled in cancer patients.

- Estimate the numbers of animals of each species to be used; explain what types of animal will be used and why you chose the particular types of animal. Explain how you will ensure that you use the minimum number of animals.

This project will only use mice bred for research purposes. Mice are the most suitable animal in which to study immune responses to cancer because we understand the immune response in this species more than any other animal and this response is sufficiently similar to that in man to justify use in this way. Furthermore, specialised strains of mice are available that enable us to perform well controlled experiments that will yield high quality information. Some strains also allow us to use human cells in these models so we can study the response of human T cells to human cancer cells. We estimate that we will use 160 mice per year. Where necessary we will initially use pilot studies with small numbers of mice to help determine the minimal number of mice required for an experiment to reliably answer the question being addressed. Also, where possible experiments will be designed to derive the maximum amount of information from the minimum number of animals.

- Demonstration of compliance with the 3Rs: State why you have to use animals and cannot use non-animal alternatives. Where appropriate, say how you will use non-animal studies in parallel with the project.

All potential immune-based therapies to be tested in mice as part of this project will firstly have been tested in vitro (i.e. without animals) to demonstrate that they look promising. However, these in vitro assays are limited in what they can test since they cannot adequately reproduce the challenges we face in treating cancer in a patient. For example they do not replicate the 3-dimensional architecture of tumours, through which T-cells must spread. They also fail to reproduce conditions for T-cells circulating in the blood stream to stop and move into the tumour tissue. When using T-cells to target blood vessels in tumour tissue, animals are again required to reproduce the defective nature of such vessels, including their unusual blood flow properties. Animal studies can also identify potential side effects of these treatments where the immune response reacts to normal tissues. Finally, the complex interactions that occur between components of the immune response and other tissues cannot be adequately modelled without using animals.

- Explain why the protocols and the way they are carried out should involve the least suffering.

The methods used are designed to involve the least suffering by limiting the number of procedures involved to that required for generating a reliable answer (e.g. the number of times a mouse is imaged will be limited to the minimum required to plot the growth dynamics of the specific tumour line involved). Furthermore, animals will be anaesthetised before injecting tumour cells or implanting sponges into the skin and the size of needles used will be kept to a minimum. Finally, mice will be monitored daily or more frequently if close to maximal tumour size or when using rapidly growing tumours

with risk of ulceration. The advice of the named veterinary surgeon and named animal care and welfare officers will be taken to ensure that any animal suffering is minimised where possible,

Staphylococcus aureus, pathogenesis to therapy

- Summarise your project (1-2 sentences)

  The project intends to develop novel treatments against a major animal and human pathogen, the bacterium *Staphylococcus aureus* (including its well known methicillin resistant variety, MRSA).

- Objectives: Explain why you are doing this project. Describe the scientific unknown(s) or clinical or service need you are addressing. Give a brief scientific background or other explanation of why the work is needed.

  The bacterium *Staphylococcus aureus*, particularly its Methicillin-Resistant variant (MRSA), is a major animal and human pathogen with a tremendous health impact and a remarkable financial burden to society worldwide.

  It is endemic in hospitals, spreading in the community, and present in approximately a third of all individuals; carriage is a risk for infection. Antibiotics are failing, the pipeline of new therapies is poor, and no vaccine is available. An effective prevention or treatment regime is essential and urgent.

  The project intends to develop an innovative vaccine or immunotherapy based on novel antigens and formulations. The ideal formulation should have a heightened potential for success in clinical trials, which will reduce costs, and the time to market and patients. Such optimized selection of a prophylactic or therapeutic candidate will be attained through implementing new evaluation criteria. Physiological/immunological determinations will be added to typical measurements to define the establishment and progression of disease as well as the efficacy of the tested vaccines and immunotherapies.

- Outline the general project plan.

  - Staphylococcus aureus strains (wild type or mutant) will be injected into animals.
  - Parameters to be assessed and quantitated:
    - during the experimental course:
      - behaviour, appearance, body weight and temperature
      - physiological and immunological measurements (from serum)
    - at the endpoint: bacterial loads in organs and immune cell responses
  - Preventive or treatment regimes will be applied before and/or during infection
  - Infection-only experiments will enable to determine the bacterial/host components and physiological/ immunological parameters involved in infection (OBJECTIVE 1)

- Prevention or treatment plus infection will allow evaluation of the protective efficacy of specific formulations, and the physiological/immunological parameters associated with disease protection (OBJECTIVE 2)
- A novel Health Status Factor derived from new parameters measured above will be developed and implemented to evaluate disease and protection, increase robustness of the model, and early intervention
- Studies will be undertaken mainly using the mouse model of septic arthritis (Animal Model 1; usually interpreted as a chronic model)
- The acute Survival Model of mouse infection will be established at the onset of the license (OBJECTIVE 3) and will be used exclusively to corroborate selected significant and reproducible results obtained in the mouse model of septic arthritis.

<table>
<tr><td>Project Title (max. 50 characters)</td><td>Avian Ecology</td></tr>
<tr><td>Key Words (max. 5 words)</td><td></td></tr>
<tr><td>Expected duration of the project (yrs)</td><td>5 years</td></tr>
<tr><td>Purpose of the project (as in Article 5)[7]</td><td>
<table>
<tr><td>Basic research</td><td>Yes</td><td></td></tr>
<tr><td>Translational and applied research</td><td></td><td>No</td></tr>
<tr><td>Regulatory use and routine production</td><td></td><td>No</td></tr>
<tr><td>Protection of the natural environment in the interests of the health or welfare of humans or animals</td><td>Yes</td><td></td></tr>
<tr><td>Preservation of species</td><td></td><td>No</td></tr>
<tr><td>Higher education or training</td><td></td><td>No</td></tr>
<tr><td>Forensic enquiries</td><td></td><td>No</td></tr>
<tr><td>Maintenance of colonies of genetically altered animals[8]</td><td></td><td>No</td></tr>
</table>
</td></tr>
<tr><td>Describe the objectives of the project (e.g. the scientific unknowns or scientific/clinical needs being addressed)</td><td>The overall aim of this project is to examine links between the environment experienced by an organism and its physiology, behaviour and performance. We willspecifically address the following objectives:

(1) Consequences of environmental circumstances on physiology (stress), behaviour (dominance and foraging strategies) and gene expression.

(2) Consequences of conditions experienced during development and adulthood on fitness.

(3) Interaction between endogenous rhythms and external environmental circumstances and their influence on physiology, behaviour and reproductive performance.

(4) Changes of resource utilisation in response to environmental changes
The work will help us to better understand the physiological mechanisms underlying the organism’s response to environmental change.</td></tr>
<tr><td>What are the potential benefits likely to derive from this project (how science could be advanced or humans or animals could benefit from the project)?</td><td>This work will improve our understanding of the interactions between individual variation in behaviour, physiology and genetics with variation in environmental factors. The work will help us to understand the complex links between environment, physiological responses, behaviour and fitness in free-living birds. The results will help us to better understand the mechanisms underlying the organism’s response to environmental variability. It is vital to address possible proximate pathways of adaptation in order to understand the</td></tr>
</table>

[7] Delete Yes or No as appropriate.
[8] At least one additional purpose must be selected with this option.

	ultimate, evolutionary causes of patterns observed in nature. The work is thus of importance for conservation as it will provide a better understanding how organisms will cope with changes in their environment. The work potentially also has welfare implications through the validation of a novel non-intrusive technique to assess response to stress and disturbance in wildlife.
What species and approximate numbers of animals do you expect to use over what period of time?	The work will initially focus on common woodland passerines (e.g. blue and great tits (*Cyanistes caeruleus* and *Parus major*) and seabirds (e.g. herring and lesser black-backed gulls *Larus argentatus* and *L. fuscus*), but additional species may be added as the work progresses. *Over the 5 years a maximum of 5000 individuals across all species will be involved, but not all of those birds will have experienced all measurements.*
In the context of what you propose to do to the animals, what are the expected adverse effects and the likely/expected level of severity? What will happen to the animals at the end?	*We propose that we will measure reproductive performance and survival, behavioural traits (dominance behaviour, response to novel objects and response to increased perceived predation risk), physiological state (physiological and oxidative stress, metabolic rate, body temperature) and health status (immune functions, parasite burdens). Some of these measurements involve observation and may include experimental provision of specific clues and some may include the need to take a small tissue sample (blood, feathers, skin).* *All planned procedures are considered to be mild. Birds may be caught and temporary stress caused by handling will be minimised by good technique and rapid release carried out by experienced licensees holding the appropriate wildlife permits. For blood sampling, no adverse effects are expected other than mild discomfort due to venipuncture. Blood sampling may result in haemorrhage, haematoma and/or thrombus formation. These adverse effects will be minimised by good technique. Only experienced licensees will carry out sampling. Applying gentle pressure on the site of venipuncture before the bird is released will ensure haemostasis. Haematomas will be avoided using different veins for sampling in cases where more than one blood sample is required. Digital pressure will be applied to the site of blood sampling to control haemorrhage. Birds will not be released or returned to the nest until they have stopped bleeding and are looking alert. The sampling volumes and frequencies do not cause anaemia or hypovolaemia.* *All individuals that are deemed to be healthy will be released back into the wild at the end of the*

	procedures. In the unlikely event of animals suffering accidental damage that would compromise their survival (e.g., broken bones), they will be humanely killed.
Application of the 3Rs	
1. Replacement State why you need to use animals and why you cannot use non-animal alternatives	We plan to assess the response of an organism's behaviour, physiology and fitness to changes in its environment and so there is no alternative to use live sentient animals. However, the animals will be studied in their natural environment and manipulation of environmental conditions will be within the range they naturally experience and the protocols are generally non-invasive and where they are invasive they are mild and so the degree of pain and suffering imposed on the animals will be small.
2. Reduction Explain how you will assure the use of minimum numbers of animals	We will use power analyses to determine the sample size required to show statistically significant tests. By following the same individual through two or more breeding seasons, and thus being able to use each individual as its own control, we can increase the statistical power of our test and thus use fewer individuals. Typically, the experiments will not use more than 150 families per year, depending on population size, and we will attempt to leave some proportion of the population unmanipulated and unaffected from invasive procedures to serve as a control. Smaller subsets of families may be used for more specialised measurements like metabolic rate. We will avoid pseudo-replication by using the same individual or individuals from the same family by applying mixed model statistical analyses and specifying 'individual' and 'family' as random effects.
3. Refinement Explain the choice of species and why the animal model(s) you will use are the most refined, having regard to the objectives. Explain the general measures you will take to minimise welfare costs (harms) to the animals.	By using small electronic chips glued to the bird's ring deploying a small antenna invisible to the bird in the nest cavity we can record both identity and visiting rates of individual birds without the need to capture and handle a bird, and so minimising disturbance to the birds. Over the course of the next few years we will record surface temperature of individual birds remotely using infrared thermal imaging with the aim of comparing the data with stress physiological parameters gained from intrusive sampling. If the correlation between these two sets of measurements is good, we hope to be in a position to make recommendations for the general uptake of non-intrusive imaging in favour of intrusive blood sampling in similar studies.