Home Office

NON-TECHNICAL SUMMARY

Genetics of sex differentiation

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

Key words

sex determination and differentiation, reproduction, cell fate determination and maintenance, disorders of sexual development, sex hormones

Animal types	Life stages
Chicken	embryo, neonate, juvenile, adult, pregnant, aged

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?

To understand how cells choose and maintain specific forms and functions (cell states or fates) during development and in the adult animal in specific biological systems, notably the gonads, pituitary and sexually differentiated tissues and how this leads to disease when the processes go wrong.

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?

During the development of an animal, cells go through a sequence of cell divisions and fate decisions, in which they transition from one cell type to another. These changes are driven by complex gene expression programs, that respond to their changing environment (external signals). These decisions of cell fate have to be coordinated in time and space to generate functional tissues, organs and the animal. Although some aspects of certain cell fate decisions can be studied i*n vitro*, and we both use and develop such approaches, it is generally essential to study them *in vivo*, using animal models.

The main purpose of the work to be conducted under this Project Licence is to provide fundamental knowledge on gene networks and cell fate decisions in chicken sex determination/differentiation.

Sex determination refers to the process by which a sexually reproducing organism differentiate as a male or as a female. Central to this process is the differentiation of the gonads into either testes or ovaries, which then operate as the niche for the maturation of the germ cells in either sperm or, respectively, eggs, as well as endocrine organs. The hormones produced, the sex steroids, control the fate of other sexually differentiated structures, such as internal and external genitalia and other body features and are also important for fertility.

In mammals sex is dictated by the inheritance of the sex chromosomes (genetic sex determination system, or GSD) to give XX females and XY males. A single gene on the Y chromosome acts as a dominant inducer of testis differentiation. In its absence the ovarian pathway prevails. From its discovery, many other genes have been identified which have allowed to start building the gene networks that regulate the process.

Most of our knowledge derive from the study of mouse models and from the genetic analysis of human patients with disorders of sexual development (DSD): a wide range of conditions present from birth where the development of internal or/and external sex features are, discordant, different from expected, based on the genetic sex (XY or XX); Despite the advances in the field, the molecular cause of the majority of these cases remain unknown.

A critical feature that has emerged and seems to be common to other vertebrates, is that male and female signal networks are mutually antagonistic. One pathway has to be established while the other has to be continuously suppressed.

This makes the system very plastic. For example, in the adult mouse gonad, altering the expression of key male or female promoting genes, such as *Dmrt1* or, respectively *Foxl2*, causes a switch in the gonad sex identity (gonadal sex reversal). Some examples of naturally occurring plasticity have been

observed in mammals. For example *Foxl2* has a key role in early embryonic ovary determination in goat, but not in mouse, where it assumes a key role in the ovary only after birth. Seasonal plasticity have also been observed in other mammals. For example in the Spanish mole, the female show sex seasonal variation, as, outside breeding season, part of the ovary produce male hormones, which result in some masculinisation of body features, including a more aggressive behaviour.

Many of the molecular players identified in mammals are conserved in other vertebrates, like birds, turtle, fish, reptiles, however they may be expressed in a different order. This fluidity is quite evident at the top of the sex determination cascade where transitions between different trigger mechanisms have been commonly observed even between closely related species. (e.g. in fish and reptiles it is common to observe transition between GSD and environment-dependent sex determination (ESD)). This means that mechanisms of gonad sex determination are rapidly evolving and therefore are quite variable across vertebrates, in contrast to most other developmental processes. Even in mammals, that have a stable GSD system, a few species do not have a Y chromosome (e.g. spiny rat), indicating that the regulator may be replaced by other genes downstream in the pathway.

We are far from understanding the sex determination pathways in vertebrates and we need to study different models to understand the core elements of the process.

The chicken has been chosen as, like mammals, it has a stable GSD system (the female inherits the heteromorphic chromosomes (ZZ/ZW system)) and there is a high degree of homology between the chicken and the human genome. It is a versatile model, that is easily accessible for embryo manipulations and now can also be genetically modified. Due to its sensitivity for endocrine disrupting chemicals (EDCs), it has also been proposed as a bioassay for impact assessment of EDCs on reproductive tissues. We aim to understand the molecular mechanism that control chicken gonad sex determination, how the downstream genetic pathways specify male and female different gonadal cell types and how these instructions are coordinate at organ level.

We will use the chick (this PPL) and the mouse (separate PPL) to understand conserved mechanisms and help to construct the networks of gene activity required for ovary versus testis development and reveal how certain genes take critical roles within these network in a species specific manner. By comparing aberrant gonadal fate decision in chicken and mouse models due to variation in genes, developmental programming, or hormones throughout different life stages we will gain understanding on causes of DSDs, on the role of the gonadal sex in controlling the development of sexual dimorphism and the potential consequences of environmental endocrine pollutants on reproduction.

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

-Improved understanding of the mechanism of chicken sex determination and of the genetic pathways that drive testis or ovary differentiation. We have recently established that the trigger of chicken sex determination depends on the dosage of the Z gene *Dmrt1* and that the gene *Foxl2* is an essential key gene for ovarian determination in the embryo. We now aim to characterize the interactions between *Dmrt1* and *Foxl2* and to identify the networks regulated by these genes in establishing the testis or ovarian pathway. This work may also lead to the identification and characterization of new genes of the network.

- Improved understanding of the plasticity of the chicken gonadal sex; by perturbing the expression/regulation of potential key ovary or testis genes in the adult gonad (e.g. *Foxl2*) will lead to new insights into cell fate reprogramming and organogenesis.

- Improved understanding of female reproductive function, fertility and premature ovarian failure. We have shown that eliminating one copy of *Foxl2* in chicken results in a phenotype characterized by an eyelid phenotype and, in females, shorter fertility lifespan. These phenotypes resemble the human Blepharophimosis, ptosis, and epicanthus inversus (BPES) syndrome, This model therefore may be used to shed light into human BPES.

-Improved understanding of the hypothalamic-pituitary-gonadal axis. *Foxl2* is a key gene for the adult ovary function, but it is expressed both in the ovary as well as in the pituitary endocrine cells producing the hormones regulating the ovarian cycle. The generation of new chick models where *Foxl2* expression is perturbed in each of these organs separately, will provide new insights into *Foxl2* role in each of these organs and in the sex hormones local and systemic effects on fertility and may inform new strategies to manage fertility.

- Improved understanding of the evolution of sex determination and of the pathways that are conserved.

-Improved understanding of the role of the gonadal hormones and of the sex chromosomes on sexual dimorphism. The chicken model we have generated, carrying a mutation in *Dmrt1*, has shown that ZZ chickens with only one copy of functional *Dmrt1* have gonadal sex reversal (male to female) and sex reversal of the internal and external reproductive organs, but they maintain male body features (e.g. male greater musculature mass, larger combs and wattles, obvious leg spur). This shows that many sexual features of the body in chicken are more influenced by the sex chromosomes of the cells forming the tissues, than by the gonadal hormones. We aim now to analyse the effect of gonadal sex reversal on sexual dimorphism in the opposite direction (female to male, in *Foxl2* knock-out models).

The use of animal models which provide improved understanding of the mechanisms underlying sex bias in human diseases will be of clinical benefit in terms of improved diagnosis and perhaps options for treatments.

We will publish all of our findings in open access journals, with data in a reusable form. Moreover, germ cells from any genetically altered chicken line generated as part of this PPL, will be made freely available to other researchers.

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

There are likely to be multiple beneficiaries from the outputs above.

-In the short term our studies will shed light into unresolved questions in the field of sex determination and its evolution, opening new ways forward to other scientists working in chicken and other systems. Moreover they will be beneficial also to researchers working on the more applied field of reproductive biology in chicken, but also other birds and mammals

- As the chicken is a farm animal, the improved understanding of chicken sex determination will be of relevance to the chicken industry, as it will allow the industry to make more informed decision on issues

related to fertility and to sexual development. Similarly our study may be of benefit to conservation programs.

-Our studies on the genetic pathways driving sex determination/differentiation will be of relevance to DSD in humans and have an immediate impact. DSDs represent a major paediatric concern and a significant healthcare burden due to the difficult clinical management of these conditions and, in some, the association with gonadal cancer and infertility. It is estimated that the incidence of new-borns with ambiguous genitalia is 1 in 4,500-5,500 births. More commonly, 1 in 300 new-borns present with some sort of developmental abnormality affecting the somatic sex phenotype. The range of pathologies of DSDs is quite extensive and, as of yet, the molecular cause for the majority of these cases is unknown. Only 50% of the cases receive a definitive clinical diagnosis and only for 20% of those a specific molecular diagnosis. A better knowledge of the genes and genetic pathways involved in the process through the study of animal models is essential to improve the ability of making correct diagnosis of DSDs, and apply the right treatment, which may include surgical intervention and/or lifelong hormonal treatment.

-Improving our knowledge of sex differences may ultimately improve gender based healthcare in humans, as relevant to the pharmaceutical industry for gender-based drug development In the longer term (perhaps in 5 to 15 years). There are sex differences in food consumption, metabolism and obesity, with concomitant differences in the risk and manifestation of obesity-related conditions such as atherosclerosis and diabetes and in cardiovascular disease and hypertension. It is therefore crucial to determine the factors that lead to the sex differences for the development of novel therapeutics.

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

Alongside the publication of primary research papers in open access journals, we will present our work at meetings, ranging from small focused workshops to large international conferences. Critically, we will communicate negative results and approaches as well as those that are positive.

We collaborate, both internally, within the host Institute, and externally with scientists and clinicians based in the UK and abroad.

We share details of approaches and data generated with our collaborators, which allows for improved development of methods and better synthesis of findings.

Where we develop new transformative methods, we will publish the protocols as papers and post them on bioRxiv.

Species and numbers of animals expected to be used

- Domestic fowl: No answer provided

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.

We will use chickens for all the projects covered in this PPL. These studies will be complementary to the studies in mice that are covered by a separate PPL. This is done for multiple reasons. First, these studies will improve our understanding of the evolution of sex determination/differentiation.

There is much to learn about sex determination pathways in vertebrates, including even in mice and humans. Data obtained from the chick will indicate additional conserved mechanisms and help to construct the networks of gene activity required for testis versus ovary development and reveal how certain genes take on critical roles within these networks in a species-specific manner.

Secondly, recent advances in the understanding of sex determination/differentiation have raised doubts as to the universality of the mouse as mammalian model of sex determination. For example, studies in mouse have shown that the gene *Foxl2* has a key role in the adult ovary to maintain the ovarian fate, but it is dispensable in the embryo for ovary differentiation. However in another mammal, the goat, *Foxl2* is necessary for early ovary differentiation. Moreover the differentiation of the early ovary in the mouse is atypical, as it proceeds in the absence of any estrogen and the germ cell niche is only formed quite late, compared to other mammals. It is therefore important to study other model systems to better understand ovary determination and differentiation. The chicken is chosen because, like mammals, it has a genetic sex determination system and it is an amniote. Most genes important for the differentiation of the ovary or testis are conserved, including *Foxl2*.

The chicken has many experimental advantages. The embryo is easily accessible for manipulations, such as tissue ablations and tissue grafts, administration of substances and gene mis-expression. It is particularly suitable to study gonadal differentiation as the gonad is one of the organs that can be easily and specifically targeted in the embryo. Moreover, it is now possible to generate genome modifications that can be transmitted via the germline.

With respect to life stages, our work ranges from early embryo in the egg, all the way to ageing adults. This range reflects, in part, the focus of the lab on certain genes, for example *Foxl2*, that function in cell fate decisions throughout many or all these stages, but also the importance of understanding the effect of changes that take place during organ development in the adult.

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

The main work covered by this PPL involves breeding, including genetically altered (GA) animals and harvesting tissues from embryos or from post-hatching animals after they have been killed (using a schedule 1 procedure) for detailed analysis of phenotypes.

Some embryos from wildtype or GA lines may be subject to cell/tissue grafting aimed to generate gonadal chimera. A few of them may be hatched and killed at different stages of gonad differentiation.

Some embryos from wildtype or GA lines maybe administered viral particle, marker molecules, or DNA. Most embryos will be killed before the first two thirds of embryogenesis.

We may use substances such as doxycycline, to induce a genetic alteration, e.g. a conditional loss- or gain-of-function of a specific gene, as well as to follow cell fates, or to isolate specific cell types (e.g. by activation of a fluorescent reporter gene).

We may also use signalling pathway modifiers, such as Fadrozole or Estrogen, to interfere with the sex determination process, or challenge the ovarian or testis pathway and/or use labelling agents, to look at cell processes (e.g. EdU or BrdU for cell proliferation) in tissues after harvesting.

These substances may be administered in the egg, or in post-hatch animals in diet or by injection, and may be carried out multiple times over a few days, followed by a variable period prior to the animal being killed and the tissues analysed.

Most of the manipulation work is carried out and terminated in embryos before the first two thirds of embryogenesis.

Some animals may be used as surrogate hosts to transmit exogenous germ cells that may have been genetically modified. Those surrogates may be GA with defective germ line. We may use drugs to kill the endogenous germ cells. These procedures are done at early embryonic stages and the hatched animals are kept as founders for breeding and for generating embryos for experiments.

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

We are investigating the genetic pathways affecting the development of the reproductive organs, secondary sexual characteristics and the fertility of the birds. Therefore we work with lines with specific gene mutations affecting gene expression or regulation which may result in male-to-female or female-to-male gonadal sex reversal and/or sterility. Those chickens should experience no more than mild effects. The birds will be closely observed to monitor any unexpected adverse phenotypes. With mutations affecting some genes, there may be broader phenotypes, which can lead to more severe adverse effects (e.g. Foxl2 knock-out line has also a chronic eyelid phenotype of moderate severity already present in heterozygotes). All those lines with harmful mutations, or leading to sterility, will be normally maintained for breeding as heterozygotes, or whenever possible, will be maintained by cyclic generation of germline chimeras (chicken surrogate hosts carrying modified germ cells of interest). Some animals may be crossed to generate homozygotes or compound mutations, where stronger phenotypes occur, including embryonic or postnatal lethality, or reduced lifespan. It will be necessary to study embryonic stages and to keep some animals with harmful mutations until the phenotypes develop, in order to study how they arise. We will kill animals before end points for the relevant severity band are reached.

The frequency, type and severity of any adverse event depends on the procedures being used, together sometimes with genetic status, including the genetic background of the strain. We endeavour to minimise the chances of these occurring; for example we will generate new GA lines by designing genome editing strategies that perturb gene expression in a defined temporal window and/or specific tissue, when the gene under study may have roles in multiple tissues within the body. However the cause of adverse effect sometimes may be unknown. This is also the case of the occasional death after administering signalling modifiers (e.g. tamoxifen).

In the case of new GA lines the phenotype will be analysed in steps, starting with the egg at different stages of embryogenesis and then in the hatched chicken at different timepoints. GA lines with known adverse effects will be closely monitored. One such a line is the Foxl2 knock-out line which has a chronic eyelid phenotype of moderate severity developing from hatching even in heterozygotes and therefore affecting the chick welfare for all its lifespan.

Any animal will be killed prior to it reaching the relevant end points as defined in the protocols.

Surgical procedures are generally performed in the early embryo and therefore should not cause discomfort. All embryonic manipulations may have variable success rate which is difficult to predict. However their outcome is normally established by monitoring the embryos growth, and embryos that continue with their normal development may be incubated up to hatching.

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 expected severities for the chicken experiments are:

Mild; about 50% of the animals.

Moderate; about 49% of the animals.

Severe; less than 1% of the animals.

The germline chimera chickens (chickens derived from embryos injected with exogenous germ cells) and the gonadal chimera chickens (chickens derived from embryos transplanted with tissue that give rise to the gonad) are falling in the mild category. The GA line mutated in the *Foxl2* gene (*Foxl2* knock-out) falls in the moderate category due to an eyelid defect that compromise the ability to blink. Those animals are under eye treatment regime to avoid infections. The severe category includes animals with unexpected severe effects; those animals would be killed if found.

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

- Used in other projects
- 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?

Most, if not all cell fate decisions in the embryo and adult animal take place within a complex environment, where events inside the cells are influenced by a variety of signals, whether these are from neighbouring cells, involve molecules, such as growth factors, cytokines and hormones (which can act over considerable distances), are commensal with the animal, such as gut microbiota, or are part of the environment, i.e. are external to the organism. Moreover, most tissues develop in a complex way in three dimensions over time in a carefully orchestrated manner. Therefore, although some aspects of certain cell fate decisions can be studied *in vitro*, and we both use and develop such

approaches, it is generally essential to study them *in viv*o (as a minimum to judge the suitability of *in vitro* systems to give meaningful information).

(i). Several distinct cell lineages give rise to the developing gonads and their continued interactions are required for appropriate gene activity leading to the development of either testes or ovaries. It is currently not possible to mimic all of these cell-cell interactions using cells maintained *in vitro*. Moreover even if we can culture the intact early gonad for periods of few days, which does allow us to follow some events in real time (and reduce animal numbers), this still requires breeding to produce the animals.

(ii). To study postnatal gonadal sex reversal, cannot meaningfully be studied in any current *in vitro* system. While it is possible to culture isolated specific type of ovarian cells for a limited time, they tend to lose expression of critical genes and their normal features. The same is true of cells in the testis. Without a robust and reliable culture system that could maintain the properties of different cellular types, it would be impossible to address the consequences of deleting *Foxl2* in the adult ovary in meaningful way. Moreover, it would not be possible to investigate how other testicular cell types differentiate, nor the complex morphological changes occurring from ovary to testicular-like structures.

(iii)The pituitary develops through a complex series of events involving different tissues and there are no actual protocols to reproduce its cellular organisation and proper hormonal function *in vitro*. Progresses have been made in growing pituitary organoids, from stem cells, however also these structures don't have sufficient complexity to model the *in vivo* organ, or to be useful to address the complex interactions of the pituitary with the gonad, to regulate reproduction, ageing and other key physiological processes, which requires whole animal study.

(iv). There is now increasing evidence that many aspects of anatomy, physiology, behaviour, pathologies and responses to treatment, differ between the sexes and even when these appear similar, the underlying mechanisms may be different. These differences are likely to be due to direct effects of sex chromosome-linked genes, to sex hormones made by ovaries or testes, or both. Moreover, these effects can be organisational (i.e. they are established during development, perhaps prior to any obvious difference), or activational (require constant input). Experiments to understand the mechanisms involved, the importance of which have been widely recognised in recent years, cannot be conducted *in vitro*.

The use of the chicken as a model species itself addresses "replacement". The chicken offers advantages over using a mouse model, as the mother does not need to be killed to obtain the embryos and, as germ cells can be introduced in the embryo, there is no need of any surgical manipulation of the hen to generate new GA lines. Moreover the majority of experiments to study sex determination will also be carried out at unregulated developmental stages (before day 14.5 of incubation).

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

We complement our *in vivo* analyses in chicken with tissue culture models and organ culture.

Why were they not suitable?

We can gain a certain amount of information from these culture systems, but there are serious limitations. It is not currently possible to replicate the complexity of tissue structures in culture models. Moreover such cell and organ cultures cannot permit research on aspects of biology such as reproduction or physiology.

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?

This estimate is based on several factors. It is based on our experience of working with chick embryos and the experience over the past 4 years with generating and maintaining chicken lines (e.g. *Foxl2* and D*mrt1* knock-out GA lines).

We also continually re-evaluate the numbers of animals required for each experiment. This will allow us to determine the number of animals required per experiment to give statistically valid results.

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

(i) When designing specific experiments within the overall project, we estimate the minimum number of animals required to give robust answers. Most often this can be based on our prior experience, or on published data. Often it is not necessary to use statistics (e.g. three transgenic lines giving the same result shows that this is correct), however, we perform statistical analysis whenever necessary. Where experiments result in consequences, for which we have little or no prior information, usually around 5 or 6 animals per treatment group (which will include sex as a variable when relevant and possible) are sufficient to obtain robust results. The design of quantitative experiments generally follows the ARRIVE guidelines and sample sizes may be set using power analysis. Any exceptions are where there is a degree of variability beyond our control (for example, where minor fluctuations in conditions together with threshold effects require more animals to be examined in order to have statistically significant results). We generally use a significance level of 5% and a power of 80%, estimating standard deviation from pilot experiments. We may include advice taken from local statisticians as well as make use of online tools, such as the NC3Rs' Experimental Design Assistant. For some important questions that we wish to address there can be a choice between using a mild procedure but many animals because the measurable effect is weak, or a moderately severe procedure with few animals because the effect is robust. Our choice will depend on the specific question and available resources, but it will most often be to use fewer animals.

(ii) Genetically altered chicken embryos depleted of their own germ cells will be used whenever possible as surrogate hosts of exogenous avian germ cells to produce chicken chimera founders. The transmission rate in these GA hosts is 100% allowing to reduce both the number of chimera founders

and the number of offspring for the establishment of the line. This strategy is more efficient than using host surrogate embryos treated with chemotherapeutic reagent to reduce the number of the host germ cells and improved transmission of injected germ cells. Moreover, these germ cell chimeras can be used not only as line founders, but also as regular providers of eggs for experimental studies and may be the method of choice to maintain a line with a harmful phenotype or a phenotype that leads to sterility.

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

We try to keep as few chickens as possible by carefully monitoring our flocks and by good practice.

Whenever possible and when there are no harmful phenotypes (or infertility) we maintain GA lines as homozygotes to reduce the numbers of animals required for experiments and to reduce the need to genotype.

In case of infertility or harmful phenotype we may maintain the line via the germline chimera founders.

We may also make use of fluorescence reporters that can (in some circumstances) avoid the need of genotyping.

The ability to generate transgenic lines using primordial germ cells which can be cultured and frozen down means that lines of chickens would no longer need to be held and bred indefinitely. Frozen germ cells can be used many years later when a particular breed of chickens would be again needed by researchers.

Whenever possible, we pre-screen substances (including molecules to induce gene expression, cell death, mutagens, etc.) and agents such as viruses, in the egg (*in ovo*), or in cell culture (*in vitro*) to determine approximate doses required. We also test genome editing components *in vitro* (e.g. in primordial germ cells), prior to the generation of genetically altered animals.

To maximise information gained from single animals, we use live imaging when feasible (e.g for embryo work), obtain data on as many tests of behaviour as possible on single animals, and obtain relevant tissue samples from multiple sites after killing, where more than one project involves the study of an animal with a particular genotype; for example as *Foxl2* is relevant to studies of the gonad, pituitary and eyelid, we often collect multiple tissues from single animals.

Similarly, when designing new genetic tools, and maintaining animals derived with these, we will, wherever possible, do so in a way to allow them to be shared amongst as many people as possible, including making use of the Institute sharing platform. This efficient use of animals minimizes the number used.

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.

-Cultured germ cells that can be genetically modified to disrupt gene function and used to produce chimera founders is unique to the chicken. The germ cell transplantation to form chimeras are carried out at early embryonic developmental stages (unregulated stages) and in an animal model that does not entail a surgical operation on the mother, or her death, to provide access to the embryos.

Moreover our use of genetically modified surrogate hosts for the generation of germline chimeras results in 100% transmission of injected germ cells, reducing the number of chicklings to be screened and making these chimeras also good providers of fertilised eggs for experiments. The latest refinement of the protocol, which allows to generate chimera founders (G0 generation) from both sexes of GA surrogates, may eliminate the necessity of breeding the siblings (G1 generation) for line maintenance. This is particularly important if the genetic modification causes infertility or if it adversely affects multiple tissues.

-Sex determination is an embryonic process so many experiments can be carried out in the egg.

-We choose well-established protocols, known to have minimal harmful effects, whenever possible. Many of the genetic and physiological manipulations, as well as the administration of substances, including gene inducers and repressors, viruses, cells and grafting of tissues, are standard and previous refinements from the literature will be used, if possible. For novel types of manipulation, or where insufficient information is available, small-scale pilot experiments are conducted in order to determine the best conditions to obtain a sufficiently robust and meaningful response from the minimum dose, exposure time, or treatment. These pilot experiments help to minimize any potential suffering.

-Although it is not always possible to predict the nature or severity of any defect that arises from a newly generated genetic alteration, we take steps to minimise unwanted phenotypes and/or the number of animals exhibiting these. For example, we may make use of tissue-specific regulatory elements and whenever practical, we may make genetic alterations that are inducible, so that the animals do not show a phenotype until expression of the candidate gene or a deletion is induced.

-When the experiment is predicted to lead to harmful effects outside the body system under study, we will provide treatments designed to alleviate these; for example the eyelid phenotype present in chickens mutated at the *Foxl2* locus is constantly monitored and regular ointment treatment is provided.

-To minimise stress during breeding and maintenance we follow best practice guidelines, institute refinements and, for some strains, our own specific procedures of husbandry. (e.g. the *Foxl2* knock-out line is in pens layered with horse shavings to minimise dust). In the case of any new strain of animal or application of any new procedure or refinement we pay special attention by increased observation and monitoring starting with the embryo and then in the hatched chicken, until we have become familiar with the phenotype and/or the consequences. If welfare implications are identified they will be acted upon and refinements considered in consultation with the NVS, NACWO and animal technicians.

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

We use the chicken as a model complementary to the mouse, to study sex determination/differentiation, partly for evolutionary comparisons, partly because some aspects of the process may be more similar between chicken and human than mouse and human (e.g some features of fetal ovary differentiation) and partly because certain embryological techniques are feasible *in ovo*, but not in utero. A significant fraction of our research involves studies on chicken embryos *in ovo* prior to two-thirds through embryogenesis.

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

For all manipulations, we will adhere to the relevant guidelines that aim to minimise suffering. We examine the animals for signs of pain and discomfort (such as prolonged lethargy) and monitor body condition, killing the animals if the distress is likely to be more than temporary. Many of the genetic and physiological manipulations, as well as the administration of substances, including gene inducers and repressors, viruses, cells and grafting of tissues, are standard and previous refinements from the literature will be used and added to if possible.

For novel types of manipulation, or where insufficient information is available, small-scale pilot experiments are conducted in order to determine the best conditions to obtain a sufficiently robust and meaningful response from the minimum dose, exposure time or treatment. These pilot experiments help to minimize any potential suffering.

Appropriate aseptic surgical techniques, temperature, and fluid therapy, will be applied as necessary.

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

These will include publications from the NC3Rs, the Institute for Animal Technology and the BVAAWF/FRAME/RSPCA/UFAW Joint working group, but also relevant articles in scientific journals.

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

We stay up to date via regular communication with animal care staff at the Institute, other scientists in our fields, via e-mail and other updates and publications from, and occasional attendance at meetings held by, the NC3Rs, the Institute for Animal Technology, and the International Society for Transgenic Technology, and through regular visits to their websites: https://www.nc3rs.org.uk/3rs-resources https://www.transtechsociety.org/ https://www.iat.org.uk