Home Office

NON-TECHNICAL SUMMARY

Research into resistance to infectious fish disease using genome altered fish

Project duration

5 years 0 months

Project purpose

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

Key words

infectious fish disease, genome editing, disease resistance, sustainable food production, improved welfare

Animal types	Life stages
Rainbow Trout (Oncorhynchus mykiss)	Embryo and egg, Neonate, Juvenile, Adult
Salmon (Salmo salar)	Embryo and egg, Neonate, Juvenile, Adult
Common carp (Cyprinus carpio)	Embryo and egg, Neonate, Juvenile, Adult
Tilapia (Oreochromis spp)	Embryo and egg, Neonate, Juvenile, Adult

Retrospective assessment

> The Secretary of State has determined that a retrospective assessment of this licence is required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

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?

The overall aim of this project is to investigate the genetic basis of resistance to infectious diseases of fish - to identify genes and mutations affecting resistance in cultured species. Specifically we aim to compare both wild type and genome altered fish to experimentally inform the various genes and mechanisms responsible for observed resistance differences in a range of serious diseases of farmed fish.

A retrospective assessment of these aims will be due by 13 July 2030

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?

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?

Food from water is a critical element to satisfy the protein requirements of a growing global population. As capture fisheries production has largely remained flat since 1990 aquatic food production is transitioning from wild capture to farming: aquaculture’s contribution to the supply of fish for human consumption exceeded that of wild-caught fish for the first time in 2014 (FAO, 2016). To meet this growing global demand for fish, it is estimated that aquaculture production will need to further double in the period to 2050. Despite the successes to date, aquaculture still faces many problems. Infectious

disease of viral, bacterial, protozoan and parasitic origin is the most significant constraint, causing lost production, increased costs, wasted resources and major problems of animal welfare.

A wide diversity in individual response to infectious disease is an obseved and well known phenomenon. Traditional controlled breeding programs for key production species have enabled enhancement of key traits, including disease resistance, in stocks over the long term. In the last two decades advances in genome sequencing allowing cost effective individual and family typing have enabled enhanced breeding programs to somewhat refine and speed up this process by using marker assisted selection. Understanding the mechanisms and genes involved underpinning the observed variation in resistance or susceptibility to diseases is now key to enabling and implementing more specific and rapid future control strategies based on selective breeding. Most recently advances in techniques for targeted specific genome editing, in particular relatively accessible gene editing based on CRISPR/Cas9 methods is transforming our ability to interrogate key genes and pathways found within the regions of genomes close to markers identified in earlier studies.

This technique allows precise edits to be induced into the genomic DNA of a given species. It consists of two key components: Cas9, an enzyme that can cut a double DNA strand at a very precise point; and CRISPR, a short strand of RNA, that guides the Cas9 enzyme to bind with a specific sequence on the genome to make a specific cut. This enables us to splice out part of a gene to disrupt its function, insert a new sequence into the genome, or to effectively swap one allele for another containing a different sequence. It also allows us to insert synthetic gene promoters or inhibitors to drive gene expression in a targeted manner. The advances in genome editing using CRISPR/Cas9 means that high efficiency, targeted changes to the genomes of fish (and other organisms) is now possible via delivery of genome editing reagents directly to the developing zygote shortly after fertilisation, typically via microinjection. CRISPR/Cas9 has been successfully applied in Atlantic salmon embryos.

Thus, a combination of powerful genome wide assessment of individuals of varying phenotype, functional assessment *in vitro* ("in the test tube") of the impact of gene edits of identified target genes in cell cultures followed by assessment and confirmation through *in vivo* (in live animals) editing and controlled disease challenge offers a massively faster route to demonstrate key genes which can then be selected for by traditional methods.

As such future selective breeding programs based on gene associated selection, informed by small scale empirical studies with genome edited animals such as those proposed within this licence have the potential to support the sustainable increased production of food from water with acceptable environmental impact, reduced chemical use and immense welfare improvements in aquaculture in a timely manner.

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

The outputs will include fundamental knowledge on the mechanisms of genetic resistance to infectious diseases which will be disseminated in high impact publications and international symposia. Outcomes also include future targets for selective breeding programs and novel strategies to combat diseases impacting on aquaculture so industry focused publications, meetings and conferences will also be targets for dissemination.

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

In the short term academia will benefit from empirical data driven publications. In the short to medium term the aquaculture industry will benefit from knowledge on future relevant targets to include in traditional selective breeding programs enhancing resistance to disease in target species. In the medium to long term the outputs potentially benefit rural economies engaged in aquaculture at the national and international levels offering reduced production losses, food security, employment and associated benefits. Our previous work on identifying markers associated with resistance to infectious pancreatic necrosis virus in Atlantic salmon which are now used in traditional selective breeding strategies is estimated to have a value of approximately £26m per annum to the UK industry alone. As breeding programs are introduced for new targets developed under this licence and become effective there will be further massive welfare improvements for future cultured fish experiencing reduced impact of disease.

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

The work is collaborative across academic and industry partners, as such dissemination will be wide and active via all partners in each project. Publications in well respected journals (high impact factor) as well as informative workshops and solutions for industry to take forward into breeding programs are expected.

Species and numbers of animals expected to be used

- Rainbow Trout (Oncorhynchus mykiss): 5000
- Salmon (Salmo salar): 5000
- Other fish:
    - Common carp (Cyprinus carpio): 5000
    - Tilapia (Oreochromis spp): 5000

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 be using commonly cultured production fish species such as trout, salmon, carp and tilapia as appropriate to the disease and pathogen under investigation.

We will utilise both genome edited and wild type (unedited) fish and controlled disease challenges as a means to compare and interrogate the function of target genes potentially involved in disease resistance.

At some point animals at most life stages will be used from embryos, through juveniles to adult. The choice of life stage will be appropriate for the disease under investigation. For example, *in vivo* gene editing is undertaken at the 1-4 cell stage in embryos, the impact of some viral diseases can be

assessed in life stages as early as recently hatched first feeding fry whereas work on marine sea lice in salmon requires adult life stages of salmon in seawater (post smolts).

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

Newly fertilised eggs will be edited *in vivo* by microinjection. Initially this genome editing will be undertaken at other establishments with demonstrated expertise in the process under their PPL licence. Groups of wild type sibling embryos which have not been edited will also be reared for controlled comparison. Later in the project we hope to transfer the technology and expertise for genome editing to our laboratory.

Eggs edited at other establishments will be held for development through to eyed egg stage and then transferred to our facilities. Eggs will be further held for rearing through hatch, first feeding fry, parr and post smolt stages as required.

At the relevant life stage edited and sibling unedited fish will be exposed to infectious agents in controlled disease challenges by a variety of methods including immersion in water containing the infectious agent, direct application to body surface (via patch), direct injection of the infectious agent (intraperitoneal or intramuscular) and cohabitation either directly with infected individuals or indirectly via water.

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

The main adverse effects in edited embryos will be unwanted physical impacts due to microinjection of embryos and unintended impacts of the genome edits on the development of the embryo. These are likely to manifest early in the development prior to first feeding stages and will be apparent during daily monitoring during which dead and dying embryos will be removed. Experience gained by researchers at our project partner institute over recent years means current post injection survival rates in edited Atlantic salmon and Rainbow trout are now approximately 80% to eyed egg stage.

Expected impacts of the chosen edits on late developing embryos and fish may range from none to significant depending on the gene target chosen and type of edit. Where the edit involves swapping from one allele type to another, both of which are observed in normal variation within the healthy population, the impact is expected to be non existent or negligible. Where the edit involves disruption of a gene target predicted to be involved in disease resistance (gene knockout) the impact may be more significant. The functions of these taget genes may not always be known although selection will be informed by evidence from genome wide polymorphism studies, *in silico* (on the computer) mining of genomic databases, literature on gene function and host/pathogen interactions for similar pathogens in other species and *in vitro* studies in cell cultures. Due diligence will be taken to, where possible, inform from literature possible adverse consequences of editing certain genes to avoid creation of knockouts with expected deleterious health or welfare phenotypes. Embryos and fish showing more subtle unexpected effects will be identified by close monitoring with fish showing symptoms of developmental abnormality, poor growth, disease or abnormal behaviour removed and humanely killed.

The impacts on fish undergoing disease challenge are expected to be significant for a large proportion of the animals, because the animals may develop clinical signs of disease. These may include marked darkening, lethargy, loss of appetite for extended periods, loss of condition, equilibrium, hypo or hyper

ventilation, irregular swimming patterns, protruding eyes (exopthalmia), lesion, haemorrhages, intermittent loss of balance which, if left unattended, may progress to total loss of buoyancy, total loss of equilibrium, no response to stimuli and eventually death. The actual adverse effects are managed by defined humane endpoints implemented by intensive monitoring which involves both direct visual checks and desktop observations of live videos from in-tank underwater cameras where feasible. Severity experienced will be mitigated by timely removal and humane termination a maximum of less than twelve hours after reaching defined thresholds.

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 maximum severity of animals during development is mild for allele swap edits and moderate for gene disruption or knockout edits.

The expected severity of fish under disease challenge is severe, this will be reduced to moderate or less by intensive monitoring and removal at defined humane endpoints. Because in certain challenges, disease progression is rapid from no apparent or mild clinical signs to death (often observed in viral challenges with small fish), mortalities may occur despite frequent checks. In all challenges, as soon as clinical signs appear we will increase the monitoring frequency to an appropriate level that avoids or minimises deaths between checks, so that no animal will be subjected to prolonged suffering. We estimate that up to approximately 20% of the fish may be classed as severe, this includes fish that die between observation periods and animals that exist in a moribund state for an extended period (i.e. overnight). A further estimated 40% of animals may reach the moderate severity (i.e. exist in a moribund state for less than 12 hours) due to limited signs prior to moribundity or where progression from apparently healthy to moribund can be rapid (e.g. large numbers of larval or juvenile fish stages). Finally, the remaining 40% of fish used are likely to be classified as mild (or subthreshold) severity. These will either be necessary negative controls, fish that did not develop clinical signs of disease or fish that display one or more clinical or behavioural signs of disease (e.g. darkening, loss of appetite) below a threshold (defined later) but no other behavioural signs of distress. Protocols are accompanied with guidance on monitoring and identification of when the identified humane end-point(s) have been reached. The most severe intended end-point is moribundity (or near moribundity) and not mortality.

What will happen to animals used in this project?

- Killed

A retrospective assessment of these predicted harms will be due by 13 July 2030

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?

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?

The research aims to investigate resistance to diseases in whole animal models. The infectivity and response to infection require complex interrelated metabolic, anatomical and immunological mechanisms involving multiple cell and tissue types that cannot be currently modelled except in a protected animal. Where appropriate, replacement technologies such as tissue culture will be employed to test the edits and investigate their effect on (e.g.) viral replication *in vitro*, and this will inform the choice of edits to be used for the animal experiments. However, data derived from *in vitro* studies are not typically accurately reflective of the whole animal response. The animals used are the appropriate species for the investigation, i.e. the natural host species, the most relevant farmed species or a potential resistant species. *In vitro* methods (cell culture) are used to produce viral inoculums for disease challenge.

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

Non-animal models *in silico* and *in vitro* models based on cell culture will be used in the project to inform choice of edits to be tested in animal experiments. Analysis of genomic data from earlier genome wide association studies and mining existing data from online databases will be used to inform candidate resistance genes and mutations. High priority targets will be tested in cell culture models, this will include genome editing of target loci in the cells, enrichment for edited cells and exposure to pathogen. Measures of cell survival and pathogen replication will inform the likelihood of an edit having an effect on resistance *in vivo.* These cell cultures are necessarily derived from fish species, of which there are relatively few compared to terrestrial animal and human models.

Why were they not suitable?

Cell cultures derived from humans or terrestrial animals are not appropriate for fish disease research. In most cases the pathogens of poikilothermic (cold blooded) fish do not replicate at the temperatures terrestrial animal cell culture models are held. Protein similarity (homology) between pathogen receptors in host cells is also rarely sufficient to allow binding and entry. The immune system of invertebrates differs considerably from that of fish, preventing direct comparisons in pathogen challenge. Fish cell culture models though informative to a certain degree are not solely sufficient since infection barriers and the immune system have many different elements and span various organs and tissues, thus whole animal models remain essential to this research area.

A retrospective assessment of replacement will be due by 13 July 2030

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your experience?

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?

We have estimated the number of animals based on performing two studies for each species over the lifetime of the project. For each study it is likely that up to 5 candidate target resistance genes will be edited as separate groups and then subsequently challenged by exposure to pathogen alongside unedited siblings. Pathogen challenge studies are planned to include tank duplication, which will ensure robustness of the findings and resilience of the studies, and up to 50 fish per tank at the challenge stage. Accepting that the reproduction strategy of fish involves high production and relatively high losses under normal conditions and that genome editing has additional impact on survival in pre first feeding stages; to ensure successful production of sufficient fish to challenge at fry stages or later we anticipate that up to approximately 500 eggs may be edited per group per experiment, equivalent to 2500 per experiment per species and 5000 per species over the life of the project at the editing stage. From this the number of protected stages that will eventually be exposed to pathogen in disease challenges is likely to be 1000 per species over the life of the project. The numbers proposed includes some margin for variance.

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

The experimental design for assessment of the impact of selected gene edits on disease resistance by exposure to pathogen *in vivo* has been kept relatively simple and is based on a long history of successful disease challenge experience at our facility. The number of potential candidate target resistance genes for *in vivo* analysis is refined and minimised by preliminary *in silico* and *in vitro* analysis.

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

Several postdoctoral scientists at the project partner's institute now have experience of genome editing in salmonids using CRISPR/Cas9 techniques gained from the early world leaders in the field in Norway. In collaboration we have previously tested CRISPR/Cas9 editing on salmon embryos, which were successfully reared to pre-first-feeding alevins in our experimental facility. They have now undertaken several genome editing trials in salmonids and significantly improved post editing survival rates. We utilise gametes from the world's most well established and professional suppliers of aquacultured fish. We utilise professional statisticians at both institutes to ensure animal experiments have adequate statistical power to detect significant impacts of the edit on the target trait (i.e. disease resistance). The edited animals will be compared to unedited controls from the same families, which

will control for background genetic effects. These measures are aimed to ensure the project maximises the chances of success whilst using the minimum number of animals necessary to achieve that result.

A retrospective assessment of reduction will be due by 13 July 2030

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

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.

Genome editing will be performed on the very early (1 to 4 cell stage) of fertilised embryos immediately after fertilisation. These developing embryos will be held through eyed egg stage, hatched and subsequently reared to juvenile and adult stages as required for the pathogen under study (protocol 1). Many of the unintended adverse effects of genome editing are expected to become apparent in developing embryos and then newly hatched developing alevins and fry prior to first feeding and as such will be removed from the studies before they reach the point they are considered sentient and protected.

The assessment of response to disease will be performed by controlled challenge i.e. exposure to pathogens via one of several optional routes (protocol 2). Fish will be raised according to each species preference across each life stage, the specifications of which are obtained from review of relevant literature and expert opinion and detailed in species specific husbandry cards. Features include (not exhaustive) temperature, salinity, pH, dissolved oxygen, excretory product levels, hygiene, light intensity and photoperiod, stocking density, nutrient requirements, recommended feed types, growth rates, and enrichment (e.g., water movement).

Disease challenges for several viral diseases can be performed on fish soon after first feeding whereas disease challenges for sea lice need to be undertaken in the older sub adult seawater stage of the salmon life cycle.

Mortality is not an intended endpoint. Despite our best intentions and efforts to apply early humane endpoints the nature of the work (disease challenges with significant pathogens) means that some fish may reach a severe severity limit. Fish under procedure will be monitored via direct visual checks using standardised in-house record sheets for abnormalities. It is not possible to maintain large numbers of fish under anaesthesia for the duration of the studies which could also interfere with natural transmission. Early and humane end-points will be used to minimise suffering while still providing valid

results. The frequency of direct visual checks will increase when adverse effects are expected or present, with frequency related to the severity and speed of progression of clinical signs. Direct visual checks will be supplemented by video observations using underwater cameras mounted within tanks, when possible, to observe behavioural and morphological changes not easily detectable during a normal direct check.

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

During the wider project we are using *in vitro* editing of established fish cell lines to refine the potential list of candidate resistance genes and to inform potential impact of genome edits. However susceptibility and response to pathogens requires complex interrelated metabolic, anatomical and immunological mechanisms that cannot be fully adequately modelled except in a protected whole animal.

More immature life forms or less sentient species, e.g. fish embryos or invertebrates, are not a suitable replacement due to the great differences between pathogen infection and host interactions affecting species susceptibility and confounding embryonic mortalities (natural and as a result of editing) and sensitivity or survival of the pathogen at different life stages in seawater.

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

Routine monitoring for fish varies by species but is typically two or three times daily for salmonids whilst feed is offered manually. This provides the ability to check the fish as well as assess feeding behaviours ensuring all fish get equal access. Checking will be increased during and immediately post exposure to pathogens and throughout the post exposure holding period of studies if and when clinical signs are observed. Each individual is checked against agreed criteria and observation recorded on score sheets. Visual checks will be supported by additional video surveillance where feasible. Where behavioural changes such as flashing and jumping associated with irritation in parasite challenges are expected, tanks will be netted and will contain minimal furniture to reduce possibility of physical damage to fish as a result of this behaviour.

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

We utilise the planning research and experimental procedures on animals – recommendations for excellence (PREPARE) guidelines and fish specific guidelines from the Norwegian national consensus platform for replacement, reduction and refinement (NORECOPA) and the Experimental Design Assistant for experimental design. We use ASPA codes of practice; Guidelines for the use of Fishes in Research (American Fisheries Society); guidelines on the care and use of fish in research, teaching and testing (Canadian Council on Animal Care); and any relevant news articles from NORECOPA, the Laboratory Animals Science Association (LASA), the Animal Welfare Research Network (AWRN), and the Institute of Animal Technicians (IAT).

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

The project licence holder is part of the local AWERB which facilitates continual professional development via named information and training officers and regular surveillance and dissemination of new 3Rs literature and regular checking of information updates via e.g. NORECOPA and NC3Rs.

A retrospective assessment of refinement will be due by 13 July 2030

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?