Home Office

NON-TECHNICAL SUMMARY

Tuberculosis pathogenesis and treatment in zebrafish and medaka.

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

Tuberculosis, Mycobacteria, Fish tuberculosis infection models

Animal types	Life stages
Zebra fish (Danio rerio)	Embryo and egg, Neonate, Juvenile, Adult, Aged animal
Medaka (Oryzias latipes)	Embryo and egg, Neonate, Juvenile, Adult, Aged animal

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?

Our overall aim is to advance our understanding of how mycobacteria produce tuberculous disease and how the host responds to mycobacterial infection.

A retrospective assessment of these aims will be due by 22 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?

Our research focuses on understanding tuberculosis (TB), an infectious disease that is caused by a germ (bacterium) called *Mycobacterium tuberculosis*. According to the latest World Health Organization (WHO) report, in 2022, TB made about 10 million people sick and killed 1.3 million people worldwide (https://www.who.int/teams/global-tuberculosis-programme/tb-reports/global-tuberculosis-report-2023). TB has also caused social problems for those who have it, as they often can't work or take care of themselves, and they might be rejected or isolated by others, including their families.

Diseased people can spread the disease by coughing and breathing. Therefore, TB remains a major problem globally, particularly in poorer countries. Medical solutions like vaccines and drugs are very important to try to fight this disease. Unfortunately, the current TB vaccine, Bacillus Calmette-Guérin (BCG), which has been used for over 100 years, only works well for infants and very young children but doesn't stop TB in adults. The medicines that do work need to be taken for many months, which is difficult for people in poorer countries without good access to health facilities. This can lead to TB not being fully cured or becoming resistant to drugs. There are over half a million cases of drug-resistant TB every year, and it is more deadly and much harder to treat.

TB bacteria have been around for thousands of years, infecting people and making them sick. Over time, TB bacteria have learned how to hide from the body's defences to make people ill. After over a

century of study, scientists still don't know all the details about how the bacteria cause disease. Our work aims to find new ways to treat and prevent TB by understanding better how the TB germs interact with the body to cause the disease.

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

This project will help us learn more about how TB bacteria cause tuberculosis and how they become resistant to antibiotics. Over the next five years, we will keep studying the disease-causing genes of TB bacteria. We want to find out how these genes cause disease. We hope to use this new knowledge to find new medicines to fight TB.

We also want to find out which genes in people protect them against TB by making the TB bacteria weaker and ultimately killing them. In the past, studying just one genetic change in people that made them more likely to die from TB, helped us find many new drugs that strengthen our bodies' defences to fight off TB bacteria. Doctors are starting to try some of these drugs in humans now. We think that by continuing this work, we will find new drugs to help fight TB by strengthening other parts of our bodies' defences.

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

Some of our discoveries are being tested in people with TB. Over the last few years, we found a new way that the TB bacterium uses to cause disease in certain people. This helped us find two groups of cheap and easy-to-get drugs that might be used to treat TB. We believe more of our discoveries will be tested on people in the next part of our project.

One of our big efforts to test our findings from fish on TB patients took five years of work and just finished, so we are looking at the results now. This means TB patients will get help from our research in the future. Because these efforts to move our findings to humans take a long time, not all of them will happen while our project is still going on. However, we expect that some tests on people will start before our project ends.

Our discoveries will be published in medical or scientific journals. Other scientists or the wider community will be able to read them and acquire further research ideas and better understanding of TB.

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

We will share what we learn by writing articles and giving talks at conferences and other places. We will keep working with other experts, both scientists and doctors, to make sure that our new findings can give the most benefit.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 727000
- Medaka (Oryzias latipes): 22500

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 use zebrafish and medaka fish to study tuberculosis by infecting them with bacteria called *Mycobacterium marinum*, which are similar to the bacteria that cause tuberculosis in humans. This bacterium naturally infects these fish in nature and makes them sick in a way that's similar to how tuberculosis affects people. By studying these fish, we can learn more about tuberculosis and how it works.

These fish have some big advantages. Since *M. marinum* naturally infects them, their disease is more similar to human tuberculosis than using human tuberculosis bacteria in animals, for example mice, that don't normally get sick from it. Also, because both young zebrafish and medaka are see-through up to the first 3-4 weeks of age, we can watch the infection process and disease happen in real-time in whole live animals. This is not possible in other animals like mice, guinea pigs or rabbits. We can also easily change particular genes in the fish and see how different genes affect how sick they get. We also can test new medicines on these fish to find out what might work against tuberculosis. Medaka fish are naturally more resistant to the disease, which helps us understand how some people might be better at fighting tuberculosis.

The clear bodies of the young fish let us see different stages of the infection over the course of just a few days. For some experiments, we use adult fish with fully developed immune systems more similar to the people who get TB, to make sure our findings in young fish also apply to older fish.

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

We create genetically modified zebrafish and medaka by manipulating their eggs to stop some genes from working properly. We can also add in proteins that are fluorescent and shine under a microscope; this allows us to better see the infecting bacteria and the cells they infect. We collect tiny fin samples from juvenile or adult fish to check their genetic makeup after putting them under with anaesthesia. Fish regrow their fins, so this process does not harm them long-term. We might need to breed these modified fish with normal fish a few times to remove any additional unintended changes that occurred in other genes. This way we can establish a line of fish with only the modifications we want to study. To obtain eggs, we use natural spawning by letting males and females mate. We use the eggs from these new fish for various experiments. Sometimes we freeze sperm to later fertilise eggs in a dish and grow the fertilized eggs to adulthood to get fish lines. Fish used for breeding will be kept up to 30 months of age and if they show any abnormal signs, such as an injury or a wrong swimming pattern, they are immediately and humanely killed.

We test different substances in uninfected fish, for example drugs, to determine the best concentration and when to add them. We then do infection experiments using young fish before 14 days of age. All animals are humanely killed at the end of the experiment.

For the most part, we perform infection experiments. We infect both young (larvae, up to 14 days of age) and adult fish with bacteria that are close cousins of human TB bacteria that are the cause of fish tuberculosis. These bacteria, called *Mycobacterium marinum*, naturally infect fish in the wild but in order to perform reliable and reproducible experiments and get meaningful and consistent results, we need to make sure that each fish gets infected with the same number of bacteria into the same location and at around the same time. Bacteria are grown outside of the animal and kept frozen until the infection experiment to ensure that high-quality bacteria are used for each experiment. For infecting adult fish, we administer bacteria by injecting bacteria into a blood vessel, the body cavity or the muscle. These infection experiments are performed under anaesthesia. Chemicals, for example drugs, may be given to the infected fish to test their effects on infection. Mostly, we simply add the chemical to the water in which the fish are kept. Some drugs or substances may need to be injected in the fish daily for maximum a week. Infection experiments with adult fish typically last up to two months, after which the fish are humanely killed. When infected adult fish show disease signs, such as slow swimming and skin disease structures, they are immediately and humanely killed.

For larval fish infections, we administer bacteria by injecting them into one of three parts of the fish: the main blood vessel, the muscle, or the space just nearby to the brain called the hind brain ventricle. We administer substances such as drugs usually by putting them into the water in which the fish swim. Sometimes we use other methods to get the drugs into the fish. These methods include injecting them in the muscle or putting them directly into the gut of the fish. After infection, we generally examine fish under a microscope. Food may not be provided until 14 days of age because they have enough nutrition from their own yolk and adding additional food into the water may cause water pollution, which is harmful for the fish. Most young fish are humanely killed by 14 days of age. In rare cases, they might be kept until 45 days, in which case they are housed in tanks in a special fish husbandry rack with food provided. All young fish in the infection experiments are humanely killed at the end of the experiment. When infected fish show any disease signs, such as slow swimming and no response to touching them gently with a plastic pipette, they are immediately and humanely killed.

In most cases, fish are humanely killed by giving them a drug that makes them unconscious and die. This type of drug is called an anaesthetic. In experiments where we need to preserve parts of the animal or the entire animal for future analysis, fish are killed by keeping them in very cold water for a long time so they become unconscious and die. This is a humane way to kill fish without giving them an anaesthetic.

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

Most of the infected fish will survive the injection, typically into the body cavity, but we expect that up to 20% of adult fish might die within 24 hours despite our best efforts to find them right after the procedure.

As the disease develops, the adult fish will show signs similar to tuberculosis, such as swimming slowly and having tiny blood spots on their skin. Over time, these symptoms will get worse, and the fish will become sicker and die from the infection, typically within two months. This can happen in a few weeks or months, depending on how many bacteria they got infected with. We check the adult fish twice a day including weekends and all holidays, and will humanely kill them as soon as we see signs of illness, to avoid having them suffer. Even with our careful checking, up to a further 10% of adult fish might die suddenly from the infection without showing obvious signs of illness before they die.

For larvae, about 10% might die within 24 hours due to the injection procedure, even though we try to spot these fish damaged by injection right after and kill them immediately and humanely. Since larvae don't show clear signs of being sick, it is hard to tell whether they are sick and how bad their sickness is. To reduce their suffering, we will humanely kill any larvae that stop moving when gently touched with a plastic pipette. Even with checking them at least once a day, up to a further 20% of larvae might die without obvious symptoms. This can happen to both genetically modified and normal larvae. So, it is not due to being extra sensitive because of their genetic modifications.

In most cases, fish will be humanely killed using an anaesthetic drug. In experiments where we need to preserve biological samples or the entire animal for future analysis, fish will be killed by keeping them in cold water for a long time so they become unconscious and then die. This is a humane way to kill fish without an anaesthetic drug.

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)?

Zebrafish: sub-threshold 25%; mild 4%; moderate 64%; severe 7%.

Medaka: sub-threshold 43%; mild 24%; moderate 28%; severe 4%.

What will happen to animals used in this project?

- Killed
- Kept alive at a licensed establishment for non-regulated purposes or possible reuse
- Used in other projects

A retrospective assessment of these predicted harms will be due by 22 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?

Tuberculosis is a complicated disease because it involves bacteria interacting with many different types of immune cells and other cells in an infected animal. The disease process includes several steps: immune cells move around, try to eat the bacteria, and sometimes die along with nearby cells. The interactions between these cells also play a role in how the disease progresses.

A key feature of tuberculosis is the tubercle, a special structure where both infected and uninfected cells gather to create a place where the bacteria live. This tubercle forms because of the interaction between various types of cells. Additionally, tuberculosis can affect different organs and tissues in the body, making it even more complex. Because of all these factors, it is impossible to recreate this process in a lab outside of the animal's body.

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

We considered two non-animal alternatives in this project:

1. Use of cultured cell lines (the founder cells of which were got from human, mouse, zebrafish, medaka, non-human primate, insect and Chinese hamster cell lines).

2. Studies in TB patients.

Why were they not suitable?

1. Cells in culture do not always behave as they do within the tissues in the infected animal and they cannot recreate the complex disease processes, especially because in TB, the immune cells move around and the way they move and how much they move often determines whether the disease will become severe. This is impossible to recreate in cultured cells in a dish.

2. We have only limited information about genes that change human TB severity. In humans we cannot change genes to check their effects, the way we can in fish. We must look for the few people who exist with the changed genes and try to find their effects. This usually works better when we start with the fish and identify an important gene. We can then look for people with these same gene changes and see if they have the effect on TB that we expect from our findings in the fish.

A retrospective assessment of replacement will be due by 22 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 estimated the number of animals to be used based on our previous records of fish breeding and experiments. We currently maintain about 1000 tanks, each contain mostly 3-60 adults for breeding. For the zebrafish infection experiments with larvae, we expect to use 100,000 larvae per year (roughly 10 researchers under this licence perform 2 experiments per week with 100 larvae at a time). For the zebrafish infection experiments that specifically assess survival after infection, we expect to use 2,000 fish per year (roughly 3 experiments per month with 50 fish, either adults or larvae, at a time). For the pilot experiment to determine the dose of the drug, we expect to use 2000 zebrafish larvae per year (roughly 40 compounds to be tested with 50 larvae each per year). Because we mainly use zebrafish, the estimated number of medaka to be used is much smaller.

We also maintain breeding animals until 30 months of age instead of 18 months of age. This is because in our facilities fish stay healthy and reproduce normally until 30 months of age. This greatly contributes to the reduction of animals to be used. For example, assuming half of the breeding zebrafish will be expired due to their age, this will reduce more than 60,000 breeding zebrafish in the period of this licence.

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

We use the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) Experimental Design Assistant along with multiple strategies to identify key genes and pathways likely to be involved in TB. We rely as much as possible on publicly available data from experiments that have already been done. This saves us from using animals to do the same experiments ourselves. We can instead progress directly to the next steps, thus reducing animal use.

Pilot studies using small numbers of animals helps us understand how genetic modifications or drugs affect bacteria or factors in the infected animals. This helps us determine the number of animals needed for larger studies. For example, if we see a bigger difference between the experimental group and the control group in a pilot study, we can use fewer animals in the main experiments.

Control animals (such as normal animals used alongside genetically modified animals, or animals not treated with drugs alongside animals treated with drugs) are always included, even in pilot studies. When possible, we use sibling fish obtained from the same parents to make results as consistent as possible and reduce the number of animals needed. We also randomly put animals from the same clutch into the different experimental groups so that we do not accidentally select animals with certain traits for the different groups. For instance, we might accidentally leave out all the faster moving animals as they might swim away faster when approached with a pipette, so these might accidentally form the last group. In studies with genetically modified fish, we breed them to produce both normal and modified siblings. We only check their genes at the end of the experiment to avoid any bias resulting from our expectations about how genetic modifications should affect infection.

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

We follow special guidelines called Planning Research and Experimental Procedures on Animals: Recommendations for Excellence (PREPARE) and Animal Research: Reporting of In Vivo Experiments (ARRIVE) to help us plan our experiments and report our discoveries. We always try to use the

smallest number of animals needed. For new experiments, we start with small test studies to see how different conditions affect the animals and how much these effects vary. This helps us figure out the minimum number of animals required for the main study.

When we need to preserve the whole animal or its tissues for future research, we make sure to share these samples with other researchers. This prevents us from having to repeat the same experiment as long as there's enough tissue available.

A retrospective assessment of reduction will be due by 22 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.

We use different stages of zebrafish and medaka — larvae (young fish up to 14 days of age), juveniles (young fish up to 45 days of age), and adults — in our studies. Whenever possible, we prefer to use larvae instead of adult fish and keep the studies short, usually lasting only a few days. This approach helps us see how the fish respond to infections before they show any clear signs of being sick. We continually improve our methods to shorten the time we need to keep infected animals.

We only use adults for important confirmation experiments because adults have more complex immune systems than the larvae, which more closely mimics the case in humans who get TB. Both adults and larvae are handled carefully; we first put them to sleep by placing them in water mixed with an anaesthetic. After completing the experiment, we use the most humane method available to kill them right away. In experiments where we need to preserve biological samples or the entire animal for future analysis, fish will be killed by keeping in cold water for a long time and this is a humane way to kill fish without anaesthetic drug.

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

We don't use fruit flies or worms in our research because they lack the immune cells and pathways that are crucial for fighting tuberculosis in humans. Fish, on the other hand, have similar immune cells and pathways, which makes them a better model for studying tuberculosis. We have demonstrated that these cells and pathways are important in fish, as like human, with TB.

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

To reduce the impact on the animals' well-being during mating and egg production, we place the fish tanks on shelves with a black background. The tanks themselves are transparent, allowing light to enter, while the black shelves help reduce stress from light reflections. This mimics the natural environment in the rivers where they live where light comes from above and the bottom is dark. This arrangement improves the number and quality of eggs produced.

Whenever possible, fish are housed in groups and given a suitable diet to support their survival, development, and breeding. When only a few fish are housed together, we add artificial plants to provide hiding spots and reduce aggression, which can occur when the animals are kept in small groups. In addition to giving them flake food, the fish are fed live newly hatched brine shrimp, which encourages natural behaviour as they chase the food.

For experiments involving new drugs or other chemicals, we determine the way to use them first. Sometimes, these have already been tested in the zebrafish or medaka and we can use their findings to guide us. For new chemicals, we conduct pilot experiments with a small number of animals (5-10 per one condition) to test first for any harmful effects on the animals. We then use the highest dose that does not harm the animals to test to see if it has an effect on TB. We then find the lowest effective dose to minimize any side effects.

We wait for a fixed number of days to look for an effect and we end the experiment if there are no effects seen. If we see the effects of the drugs (either harmful or beneficial) before our planned end point, we stop the experiment earlier. Any animals that show disease signs, such as slow swimming and blood spots on their skin are humanely killed immediately using a regulated method.

Larvae do not need to be fed for the first 14 days post-fertilization during experiments because they get all necessary nutrients from their yolk. This practice benefits their health because not feeding the larvae reduces the risk of getting infected by other bacteria that are present in the food and by their own waste products that increase if they are fed.

Animal survival studies are crucial for understanding tuberculosis, as they reveal insights into how the bacteria cause disease and our immune responses. When conducting survival studies, we use statistical analysis to minimize the number of animals needed. Up to 10% of animals may show disease signs, such as slow swimming and disease marks on their skin, and any such animals are immediately humanly killed by a regulated method. All infected animals in the survival studies are checked twice daily by trained technicians or researchers to quickly detect any signs of illness or distress. Any action taken is recorded and reviewed to ensure proper management and to keep track of the health status of the animals. If an unexpected number of animals show signs of disease, the researcher and NACWO are notified right away.

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

The design for our experiments largely involves larvae under 14 days of age, with fish over this age typically being used for breeding. These will be housed in dedicated centralised aquatic facilities,

where they will be looked after by full time trained animal technicians, who will ensure their welfare, working to the standards defined within the ‘Code of practice for the housing and care of animals bred, supplied or used for scientific purposes’ (https://www.gov.uk/government/publications/code-of-practice-for-the-housing-and-care-of-animals-bred-supplied-or-used-for-scientific-purposes).

There are also several resources to inform us about the current research on refinement of procedures, such as the Norecopa Fish as Research Animals page (https://norecopa.no/species/fish/), the NC3Rs Zebrafish welfare page (https://nc3rs.org.uk/3rs-resources/zebrafish-welfare) and the Laboratory Animal Science Association Resources and Publication page (https://www.lasa.co.uk/current_publications/). We will take these resources into account when deciding on the most appropriate method for procedures.

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

We will be closely in contact with the Named Animal Care and Welfare Officer (NACWO), Named Information Officer (NIO) and Named Veterinary Surgeon (NVS) to be informed of any new publications or reports regarding 3Rs. The NACWO, the PPL holder and research staff working under this licence will attend relevant continued professional development training as well as zebrafish husbandry meetings as with the previous license. We will also ensure that we are informed by searching the scientific literature regularly and attending 3Rs-related meetings internally or externally.

A retrospective assessment of refinement will be due by 22 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?