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NON-TECHNICAL SUMMARY

The genetic and developmental basis of morphological and behavioural variation in cichlid fishes

Project duration

5 years 0 months

Project purpose

- (a) Basic research
- (d) Protection of the natural environment in the interests of the health or welfare of man or animals

Key words

evolution, organismal trait diversity, genetics, development, cichlid fishes

Animal types	Life stages
Cichlid fishes	embryo, neonate, juvenile, adult

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 the genetic and developmental mechanisms underlying organismal diversification (how organisms evolve and become different species) using the morphological (physical characteristics) and behavioural variation present in cichlid fishes as a model system.

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?

One of the enduring challenges in evolutionary biology is understanding how organisms adapt and diversify in their surrounding environments. This issue has been recognised as a significant scientific challenge of the 21st century, particularly in light of ongoing environmental changes. Yet, we do not fully understand how new species form and how organisms can rapidly adapt to and cope with novel challenges.

To understand the mechanisms by which organisms diversify and adapt, we need to study which genetic and developmental changes encode adaptation to novel environmental variables. For example, changes in the light environment (e.g., water turbidity) might affect colour attributes and how animals see each other; or changes in food availability, might affect feeding ecology and craniofacial attributes (variable mouth morphologies). So what are the genetic and developmental mechanisms that make just rapid morphological changes possible?

In this project, we study cichlid fishes, a group of animals known for their rapid speciation, diversification and adaptation patterns. These fishes show a lot of diversity in pigmentation patterns and feeding morphologies that evolved as a response to changes in their surrounding environments (such as variable light conditions and novel feeding grounds). Here, we will identify which genetic mutations and developmental mechanisms encode such rapid adaptations. Understanding the genetic underpinnings of adaptation is important so we can understand the patterns of rapid adaptation which will help predict how organisms cope with environmental challenges.

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

The proposed research project addresses fundamental questions regarding the genomic and developmental basis of adaptation and organismal diversification and also in vertebrate evolutionary biology, therefore the main benefits will be to fundamental scientific knowledge and will generate several publications reviewed by other scientists. We list the main expected findings below:

1) Identification of genes underlying morphological and behavioural variation (such as variation in head shape, colouration patterns and courtship behaviours).

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2) Identification of cells and developmental process (e.g., pigment cell migration) that generate the variable adult pigmentation and craniofacial morphologies.

3) Determination of how certain hormones (such as thyroid and sex steroid hormones) control the development of morphological traits and whether these have an impact on the courtship and breeding behaviour of these fishes.

Taken together, these three major findings will uncover fundamental insights into the genetic and developmental basis of rapid morphological change as a response to adaptation to the surrounding environment. For example; what are the type of genetic mutations that encode adaptation - do these mutation involve gene regulation or gene function; are there many genetic/developmental changes required for morphological changes to occur, or does adaptation proceed through a small number of mutations which results in drastic changes in developmental processes with a large morphological effect; how sensible are these genetic and developmental systems to hormonal manipulation which can be easily disrupted by environmental variables?; etc. The answers to these questions will uncover mechanisms of adaptation and diversification and help predict how rapidly organisms respond to changes to their surrouding environments.

Furthermore, given the focus on embryonic developmental mechanisms in vertebrates (e.g. pigmentation, lateral line and craniofacial development), this work will also contribute toward the understanding of how vertebrate organisms function and develop with important implications to all vertebrates including humans.

Data generated will be useful to other researchers and will lead to more collaborative work. We will publish in open access journals and deposit our data in publicly available, free repositories, that can be re-used by other researchers (reduction measure). We will publish both scientific findings as well as protocol development and optimisation.

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

Short term benefits are further protocol optimisations, improvement of rearing practices and sharing of tools with the community. We are contributing to the development of cichlids as an emerging model system that can be used not only for evolutionary biology but also for developmental biology studies (see our recent review about the potential of the model system: (https://doi.org/10.1186/s13227-022-00205-5).

Long term, this will generate knowledge on how organisms adapt and how they diversify and become different species as a response to factors in their surrounding environment. More specifically, we will uncover the genomic and developmental basis of these processes which will help predict how genomic, developmental and behavioural variation influences rapid organismal adaptation. This will be beneficial to the field of evolutionary biology and also conservation biology. Further, we believe that our results will benefit several field of biological sciences (e.g., evolution, developmental biology, molecular biology, and genetics) because our project integrates across biological scales (genomics, cellular and developmental biology and behaviour). We will publish and disseminate important insights into the genes and developmental processes that generate vertebrate morphological diversity in nature and in the laboratory. Using cichlids, we will be able to connect natural variation to developmental processes

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that are important to the ontogenesis (period of development from embryo to adult) of all vertebrates, including humans.

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

We will present our results in local and international conferences and we will publish in international open access journals (as we did and are currently doing in relation to our current regulated work) and we will share our data in online repositories to be freely used by other researchers. Conferences and publications enable us to communicate our results and experimental methods to a broad scientific community. For example, we have organised a Cichlid Genome Editing workshop bringing together a group of experts in cichlid developmental genetics. As a result of this workshop we wrote a review on the use of the method in different species. Moreover, the cichlid community in the UK is highly collaborative and meets regularly to present research updates, which increases data sharing and also increases protocol optimisation. Finally, we will contribute to public outreach activities organised by our host institution to show case our work on cichlid morphological evolution and its importance to society.

Species and numbers of animals expected to be used

- Other fish: 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 are using embryos, juveniles and adults of East African cichlids of the African Great Lakes due to their unique and fascinating diversity of craniofacial and body shapes, pigmentation patterns and behaviours. Although they vary in morphologies, they are genetically very similar. The combination of these features makes them a great system to identify the genes underlying cichlid evolution. As such, there is a large scientific community that build important and useful resources (e.g. genomes and genome editing tools). They represent a model system where integrative approaches are possible (e.g. merging evolutionary biology with cell and developmental biology) thereby reducing the need to use different model system to answer related questions.

The species we proposed to use are large, robust and thrive in an aquaria environment and will withstand the proposed regulated procedures with the minimum of stress. In order to continuously minimise animal suffering we will always use up to date technology (e.g. use skin swabs instead of fin clipping when possible) and refine the housing environment. Moreover, we are up to date with current research and have refined methods of Schedule 1 killing, anaesthesia and analgesia.

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

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Creation of genetic alterations: Fish eggs will be collected and placed in a petri dish with aquaria water. Then, they will be gently held and injected so as not to damage the egg. Immediately after, the eggs will be returned to aquaria. Injection of a batch of 50 eggs takes roughly 30 minutes. Injections will be performed to create genetic alterations, meaning the addition or deletion of genetic material.

Confirmation of genetic alterations: After the creation of genetic alterations, fish eggs will be grown until they reach their juvenile stage. At this point, we will confirm the presence of genetic alterations by cutting a little portion of a fin, or by taking a swab from the fish and sequence their DNA. The cichlid fish we are using are large and robust, typically about 5-10cm in length. Therefore, this procedure will be performed without anaesthesia, as this brings a higher risk of adverse effects. Melafix - a substance that fights infection and promotes fin regrowth - may be used. We will monitor for abnormal behaviour post fin clipping that might indicate pain, and if required isolate the fish and use analgesia treatments (following Named Veterinary Surgeon advice). In the past we have never witness such abnormal behaviour, so we tend to avoid analgesia as the harms of isolation would outweigh the benefits of its use. The amount of fin removed does not compromise swimming or any other aspects of normal fish physiology or behaviour, taking the sample takes less than a minute.

Repeated imaging through time: Fish showing morphological modifications (e.g., colour pattern modifications) due to the genetic alterations will be imaged throughout time, while the morphological trait (e.g., colour pattern) is developing. To improve the imaging, individuals will be anaesthetised and subjected to a substance that contracts the pigmented cells that make up the colour pattern. In these species, the development of colour patterns is highly dynamic and changes as the fish grows, therefore it is not possible to use such pigmentation markings to keep track of individuals. Thus, the fish for this experiment, may be housed singly (for a maximum period of 150 days) in order to keep track of individual identities, and while this is not the ideal situation for cichlid fishes, it will increase the value of the longitudinal imaging data while having little impact on fish growth and morphological trait formation. An added reason for the fish to be housed singly for this experiment, is that pigmentation formation is inhibited in most individuals if they are kept all together, as the presence of a dominant male will supress pigmentation development in subordinate individuals. Thus, to synchronise pigmentation pattern development between individuals (and increase replicates), each fish has to be given their own territory. We will control for signs of stress by comparing growth rates between single housed animals and animals kept in a group. Our preliminary data shows that juvenile fish kept in single housing grow at normal rates and gain colour quite rapidly.

Hormonal manipulations: Fish embryos and early juveniles will be subjected to hormonal manipulations, where certain hormonal (e.g., thyroid and sex steroids) levels will either be increased or decreased. Substances that manipulate hormonal levels will be administered by dilution in the aquaria water.

Behavioural assays: to determine the role of the genetic and morphological alteration in the reproductive and courtship behaviour of these fishes, we will perform mate-choice, male-male competition and exploratory behaviour of these species. This will allow us to measure the fitness effects of the induced genetic alterations and hormonal manipulations. We will conduct three types of behavioural assays:

1. For male competition, we will use long aquaria (150cm), that will be divided into five compartments. Every compartment will hold a male with different morphological attributes, the

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dividers will have holes where females can go through. Mating success will be measured by identifying who fathered most progeny.

2. For mate choice, we will have tanks with three compartments, where a female will be in the middle compartment and there will be a male on each side compartment. Mate choice will be measured as time spend with each male.

3. For exploratory behaviour, we will use a very large tank (150cm x 50cm x 50cm). The individual will be placed at the end of this tank, and will be allowed to roam for 15 minutes. Then its movements will be tracked using tracking software to see where and how did this individual explored it's surrounding environment.

Only fish showing no signs of suffering and distress will be used in these behavioural trials. As these behavioural trials mimic the semi-natural conditions we rear our cichlids in we do not expect this protocol to induce any suffering, harm or distress.

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

Genetic alterations and hormonal manipulations: What we propose to do to the animals will minimally impact on their health and welfare as the genetic alterations and hormonal manipulations suggested are unlikely to lead to any malformations or adverse effects during regulated stages. We will inject and treat fertilised eggs with particles that modify specific gene portions and administer substances that modify hormonal profiles. These modifications might also result in harmful embryonic growth malformations (e.g. fish embryos that do not grow, embryos that lack organs, hatchlings that swim and behave abnormally, etc.). If the latter occurs affected animals will be humanely killed as soon as the malformation is detected before they hit the independent feeding stage.

Repeated imaging: Fish showing colour modifications will be imaged throughout time, while the colour pattern is developing. To improve the imaging, individuals will be anaesthetised and subjected to a substance that contracts the pigmented cells that make up the colour pattern. The repeated exposure to this substance and to anaesthetic can lead to a 5% rate of non-recovery from the anaesthetic. Imaging will first be performed every five days to limit exposure to the anaesthetic and to the substance that causes pigment cells to contract. We will only shorten the interval between imaging sessions if the morphological trait develops too fast for differences to be detected with larger intervals. Animals that are not fully recovered at the end of this procedure will be killed humanely. Further, the fish for this experiment, may be housed singly in order to keep track of individual identities, and while this is not the ideal situation for social cichlid fishes, this will increase the value of the longitudinal imaging data (e.g., we will be able to follow individual cells) while having little impact on fish growth and morphological trait formation. The pigmentation traits we are interested in are related to dominance and their development/appearance is inhibited by conspecifics, only showing up in the most dominant fish. Therefore to synchronise trait development across all individuals we need to isolate them so that each has their own territory.

Behavioural trials: We expect no adverse effects. This protocol is considered to be regulated because we will be using genetically altered animals and animals that underwent hormonal manipulations. Only health fish showing no signs of suffering and distress will be used in these behavioural trials. As these behavioural trials mimic the semi-natural conditions we rear our cichlids in

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we do not expect this protocol to induce any suffering, harm or distress. Nonetheless, we will closely monitor the fish to detect any signs of suffering and stress during the procedure and in 24 hours following the procedure.

East African cichlids are large and robust species which will withstand well our proposed procedures. After any procedure, animals will be monitored for an adequate amount of time to ensure recovery. Animals are expected to remain healthy and normal and, should this not be the case, they will be humanely killed immediately to prevent any pain, suffering, or distress.

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)?

Mild Severity - 100%

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

- Kept alive
- Killed
- Used in other projects

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?

Our research focuses on understanding how vertebrate diversity is generated at the genetic and developmental level. Correct development depends on cell and tissue interactions present in the embryo. Therefore, the study of development cannot be replaced by in vitro or ex vivo models, since the interaction with the live and intact tissue is needed.

The genetically altered lines generated and tissues collected will be made openly available and shared with the scientific community (with necessary permissions) which will replace/reduce animal use in the collaborator’s laboratory.

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

We have considered mathematical models and cell cultures.

Why were they not suitable?

At the moment there are no alternatives that faithfully replace animal development from a single cell to its adult form. To fully understand how genetic mutations and hormones impact animal development, we need to observe how animals develop from embryos to adults in a natural context. Cell cultures and mathematical models are not able to recreate complex interactions that occur at the organismal level between cells and tissues. Furthermore, part of our project also focuses on the impact of genetic and developmental changes in behavioural profiles of these fishes. This integrative approach would be impossible without using animals.

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 plan to generate up to 15 lines of genetically altered animals. From past experience we estimate that for their generation and breeding we will need approximately 1500 animals. This is roughly 50 injected eggs per gene that will grow to sexual maturity and then crossed to generate a stock of 50 F1s per line.

We will then repeatedly image the embryonic development of such lines. We estimate (based on previous studies) that we will need at least 12 individuals (6 females and 6 males) per group/treatment. Here we will use these lines to:

1. Compare the development of wild type and genetically altered individuals. This will require 12 individual per group (wild type and GA) and per line (15 lines), thus we will require a maximum of 360 individuals.

2. Compare the development of wild type and GA animals reared in normal conditions with animals with hormonal levels manipulated. This will require 12 individuals per group and we plan to use a maximum of 5 GA lines. The studied groups will include GA and wild type animals reared under normal condition, treated with hormones and the vehicle substance, and vehicle substance only. We plan to use four types of hormonal manipulations (enhancement and inhibition of thyroid and sex steroid hormones). This will require a maximum of 1440 animals.

Finally, we plan to determine the impact of genetic alterations and hormonal manipulations in the reproductive and exploratory behaviour of these fishes. This will require 15 individuals per group and we plan to use a maximum of 5 GA lines. The studied groups will include GA and wild type animals reared under normal condition, treated with hormones and the vehicle substance, and vehicle substance only. We plan to use four types of hormonal manipulations (enhancement and inhibition of thyroid and sex steroid hormones). This will require a maximum of 1800 animals.

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

Genetic alterations will be performed on a carefully chosen set of genes. At the beginning of this research programme, we used computational methods and available data to confirm that the candidate genes are associated with morphological variation. For example: 1) we will perform comparative transcriptomics experiments to analyse gene expression profiles for the tissues of interest between species, and; 2) we will perform hybrid crosses between species and sequence the genome of hybrids to perform genomic mapping of genes underlying morphological variation. These experiments will yield very specific candidate genes that are expressed in the tissues of interest and that have mutations causally associated with variation in the trait of interest. As such, we will only study the gene function of highly relevant candidate genes.

During our previous licensed work, we have gained experience that allow us to determine the ideal numbers of animals for each of the protocols in this licence. More specifically, we published work on genome editing in cichlids and on the repeated imaging of genetically altered animals and as such could determine the ideal number of animals required to generate interpretable results. We have also conducted a few pilot experiments on hormonal manipulations to determine dosage and numbers required to measure morphological effects. Finally, we have conducted pilot trials and discussed with colleagues in cichlid behavioural ecology who advised on the numbers required to conduct pilot experiments for each of the behavioural trials.

We will strive to use the optimum number of animals and throughout the duration of the project we will continuously consider if the benefits of the number of animals used and procedures performed outweigh the potential harm. We will plan, conduct and report our experiments according to the PREPARE and ARRIVE guidelines and use the NC3Rs Experimental Design Assistant (https://www.nc3rs.org.uk/our-portfolio/experimental-design-assistant-eda).

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

Efficient breeding: For the generation and breeding of cichlid genetically altered strains, only individuals with confirmed gene modifications will be used. Gene modifications are confirmed by collecting tissue biopsies (e.g. by removing a very small piece of fin or collecting skin swabs), which are then used for genetic tests.

Pilot studies: We commit to perform pilot studies for protocol we have less experience with, namely hormonal manipulations and behavioural assay. We will consult the NC3Rs Experimental Design Assistant (see above) to help design and conduct the pilot studies (e.g., https://www.nc3rs.org.uk/3rs-resources/conducting-pilot-study).

Sharing tissues and data: We will share tissues from the fish we cull with collaborators within the University. For every fish culled in our facility we always enquire if tissues are needed, so that animal use is optimised. We also publish our results in open access journals and upload all raw data to data repositories and our group's GitHub page.

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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 will use as a model species East African cichlid fishes. We will generate genetically altered lines, conduct hormonal manipulations, image trait development and conduct behavioural trials. All our proposed procedures are mild.

The species group we study are evolutionary important and there is a large scientific community that build important and useful resources (e.g. genomic and molecular tools). They represent a model system where integrative approaches are possible (e.g. merging evolutionary biology with cell and developmental biology) thereby reducing the need to use different model systems to answer related questions.

The species we proposed to use are large, robust and thrive in an aquaria environment and, we know from our past and current experience, that they will withstand the mild regulated procedures with the minimum of stress. In order to continuously minimise animal suffering we will always use up to date technology (e.g. using skin swabs instead of fin clipping when possible) and refine the housing environment. Moreover, we are up to date with current research and refining methods of Schedule 1 killing, anaesthesia and analgesia.

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

A major component of our research focuses on understanding how vertebrate diversity is generated at the genetic and developmental level. We have a particular interest in the development of tissues derived from neural crest cells, namely colour patterns and craniofacial skeleton, which only exist in the vertebrate group. Our questions are thus centred on vertebrate biology and evolution, as such we cannot use less sentient species.

Correct vertebrate development depends on cell and tissue interactions present in the embryo and juvenile fish. To properly characterise such processes we need to image the same individuals through time - from embryo to juvenile and adult periods - to be able to document how, for example, pigmentation and craniofacial shapes develop. Thus, we cannot solely use immature life stages or animals after they have been terminally anaesthetised.

However, we are committed to reduce animal numbers, and will whenever is possible use imaging data and tissues obtained from immature life stages (before independent feeding stage) and terminally anaesthetised animals.

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

We will incorporate new recommendations on animal rearing and welfare as we have done up to now. Cichlid housing will be continuously improved with rocks, vegetation and hiding places characteristic of their natural environment. This will be achieved by introducing artificial plants, stones, clay pots, and plastic tunnels in the aquaria. Group housing decreases stress and aggression levels in fish. We will maintain adult fish in high-density groups to reduce aggression, thus avoid unnecessary stress or injuries from chasing or fighting. Researchers and animal technicians will pay close attention to the fish in every tank, in order to control stress and aggression levels. If required, fish will be temporarily separated and groups redesigned to avoid further injury and aggression. In case of signs of aggression (e.g., bitten fins or after fin clipping for confirmation of genetic alteration, melafix (substance that promotes fin regeneration and growth) can be added to the aquaria water to promote regrowth of affected fin areas. We will continuously run trials on optimising feeding regimes and control water quality to ensure optimal husbandry. We perform fish health and welfare checks twice a day.

We commit to continuously work on our protocol refinement, for example, under our current project licence, we have: 1) optimised cichlid genome editing protocols ; 2) refined a protocol of repeated anaesthesia and longitudinal imaging of cichlid fish, which we will use in this project; 3) refined housing environment and husbandry techniques (e.g., we maintain and breed cichlid species that rarely do so inn a laboratory setting); 4) applied skin swabs as a DNA sampling method for larger specimens; 5) shared such protocols with the cichlid community. Further, using immature life stages (embryonic stages) we have conducted preliminary hormonal manipulation studies which informed the viable concentration ranges to use in our experiments. These concentrations ranges result in viable late embryos, as such we think it is safe to apply these ranges past the independent feeding stage.

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

We will follow the best practice guidelines available at www.nc3rs.org.uk. Experiments will be planned, documented, and reported according to the PREPARE and ARRIVE guidelines.

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

We stay up-to-date with new information and new resources as they become available. We are signatories of the NC3Rs (National Centre for the Replacement, Refinement, and Reduction of Animals in Research) newsletter and follow their website (www.nc3rs.org.uk). Moreover, named persons in our establishment (i.e. the Named Animal Care and Welfare Officer - NACWO, Named Veterinary Surgeon - NVS, and Named Information Officer - NIO) inform us of relevant new information and resources. My research group is in constant contact with our NACWO, NVS and animal care staff, to discuss and exchange improvements in animal rearing, wellbeing and implementation of new resources.

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