National Centre
for the Replacement
Refinement & Reduction
of Animals in Research

2013
Research Review

Pioneering Better Science

The NC3Rs

The National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) leads the discovery and application of new technologies and approaches to minimise the use of animals and improve animal welfare (the 3Rs). It funds research, supports training and development, and stimulates changes in regulations and practice. Primarily funded by Government, the NC3Rs is also supported by the charitable and private sectors. It works with scientists in universities and industry in the UK and internationally.

Further information can be found at **www.nc3rs.org.uk**

Contents

Foreword

Positioning the UK at the forefront of 3Rs research

It is almost ten years since the NC3Rs was established to accelerate the development and application of science and technology to replace, reduce and refine the use of animals for scientific purposes (the 3Rs). Since then we have positioned the UK at the forefront of 3Rs efforts globally. Funding cutting-edge research and world class scientists has been key to this.

The aims of our funding strategy are to:

- **Drive scientific advances** through project, pilot study and strategic grants;
- **Build capacity for training and development** through PhD studentships and the David Sainsbury Fellowship scheme, launched in 2009 and 2012 respectively;
- **Support growth and commercialisation** through the challenge-led competition CRACK IT Challenges*, introduced in 2011;
- **Provide for research infrastructure, networking and equipment** through the Infrastructure for Impact scheme, launched in 2013.

*CRACK IT is not included as part of this portfolio review; it will be covered in a separate review in 2015.

This strategy has allowed us to build up a strong portfolio, in which 3Rs research is embedded in the mainstream scientific community. By focusing on high quality, funding from the NC3Rs has become increasingly valued and sought after. We have demonstrated a track record for high impact science and as a result have been able to attract additional core resource, enabling us to make more awards and to expand the number of funding schemes available.

In this review we showcase 12 examples from the NC3Rs research portfolio, focusing primarily on project grants which have been the mainstay of our funding strategy. We have selected case studies to illustrate the breadth of the science we support and the benefits delivered. High impact projects range from new discoveries in epilepsy and cancer research, changes to international regulations for testing endocrine disrupting chemicals, through to addressing major challenges faced by the pharmaceutical industry. We also highlight some of the multi-disciplinary approaches and innovative technologies that have developed through NC3Rs funding, including unique bioengineering solutions for basic research in neurodegeneration and drug discovery.

At the heart of our mission is the commitment to minimise the use of animals and improve animal welfare. In the review, we illustrate how NC3Rs-funded research is achieving this in practice with examples that apply to rodents, fish and non-human primates. We also provide an estimate of the potential 3Rs impact that will be delivered once the research is fully adopted across the scientific community in the UK and internationally. In some cases NC3Rs research, such as that to refine euthanasia or improve pain assessments, will benefit millions of animals.

We begin the review with an analysis of the NC3Rs research portfolio including the amount committed by funding scheme and the breakdown of awards by scientific discipline. Using the 3Rs evaluation framework that we published in 2012, a summary of the impact of NC3Rs research is provided. This includes our first analysis of the citation of peer-reviewed publications arising from NC3Rs research.

Delivering genuine and sustained progress in the 3Rs is primarily dependent upon scientific discovery and technological innovation. Research funding is essential.

Dr Vicky Robinson, Chief Executive
Professor Stephen Holgate CBE,
Board Chairman

1 Portfolio and impact analysis

Here we provide an analysis of the NC3Rs research portfolio, focusing on awards by year and institution, scientific category and 'R'; the key technologies and approaches used; and the outputs and impacts delivered, including publications.

Portfolio analysis

Awards by year and institution

The NC3Rs is the main funder of 3Rs research in the UK. Since launching in September 2004 we have made 167 awards totalling £35.1 million. This includes:

- £29 million for project, pilot study and strategic grants;
- £4.8 million for PhD studentships and early career fellowships;
- £1.3 million for Infrastructure for Impact grants.

since 2004 | 167 awards | totalling £35.1 million

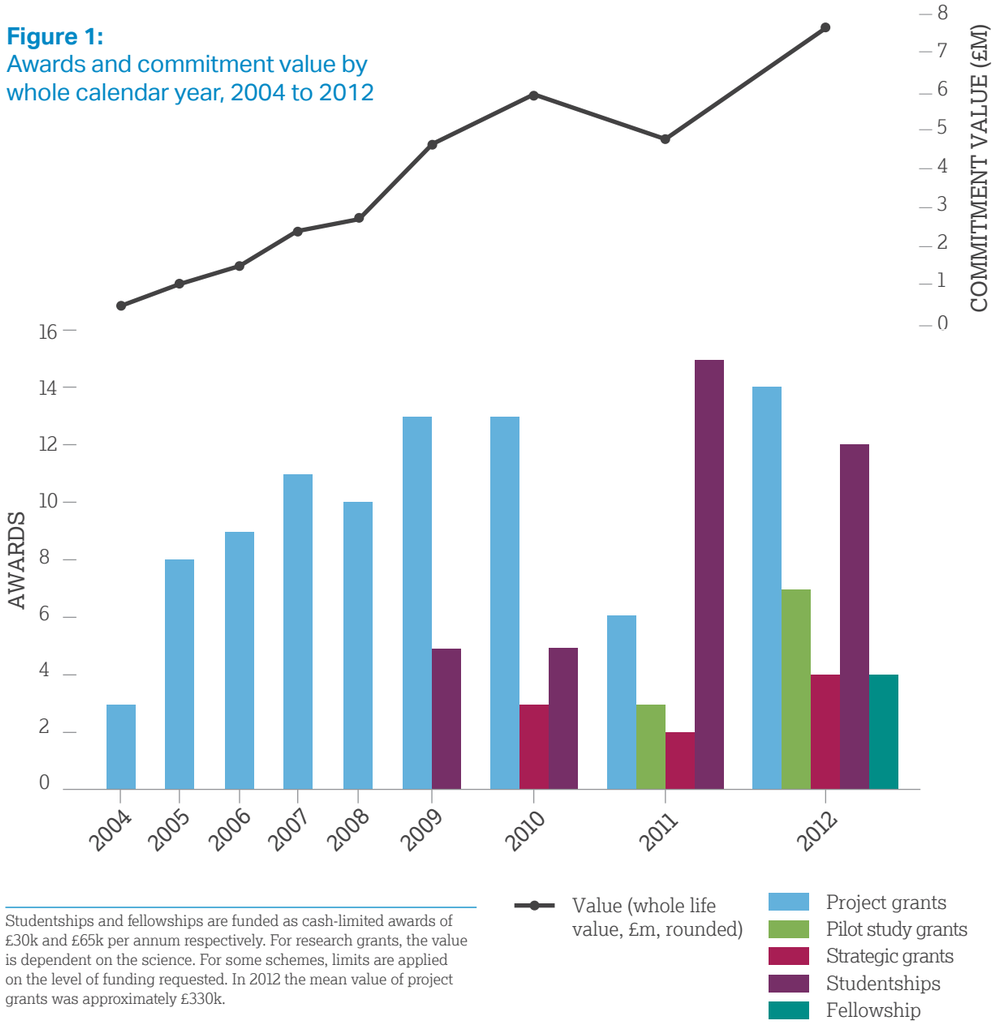
Figure 1 shows the number of awards made per year by funding scheme, and the yearly commitment on awards, for the period 2004 to 2012.

The total number of awards has increased from three in 2004 to 41 in 2012. We initially focused on supporting project grants and this has subsequently allowed us to evolve our portfolio to include a greater focus on training and development with the introduction of funding schemes for early career scientists in 2009 and 2012. Annual commitment (in terms of whole life value of awards) has increased from £0.5 million in 2004 to £7.7 million in 2012. The apparent decrease in 2011 is due to allocation of funds to support the first CRACK IT Challenges competition.

To date we have made awards to 152 principal investigators in 60 research organisations across the UK. Approximately 89% of the awards are to universities, of which 75% are members of the Russell Group of research intensive universities. The top ten research organisations in terms of amount awarded are shown in Figure 2. Collectively, these universities account for over 50% of our total commitment.

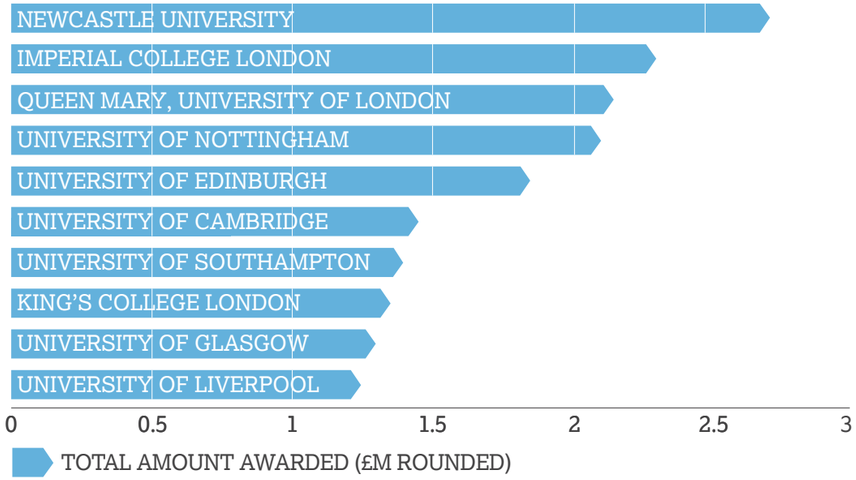
We use the same well-established peer review processes as the major bioscience funding bodies and an equivalent quality threshold for funding. Applications for NC3Rs funding are assessed using two key criteria: the quality of the science proposed and its potential 3Rs impact. The average success rate across our funding schemes is 19%, which is broadly similar to other UK bioscience funders. Table 1 summarises the success rates by scheme since 2009.

Figure 1:
Awards and commitment value by whole calendar year, 2004 to 2012



Studentships and fellowships are funded as cash-limited awards of £30k and £65k per annum respectively. For research grants, the value is dependent on the science. For some schemes, limits are applied on the level of funding requested. In 2012 the mean value of project grants was approximately £330k.

Figure 2:
Top ten recipient institutions of NC3Rs funding, 2004 to 2013*



*Up to 1 September 2013.

Table 1: Success rates* (%, rounded) of research award applications by financial year and funding scheme, 2009 to 2013

Funding scheme	2009/10	2010/11	2011/12	2012/13	2013/14	Mean
Project grants	18%	25%	12%	18%	11%	17%
Pilot study grants	–	–	20%	18%	22%	20%
Strategic grants	–	17%	33%	25%	–	25%
Studentships	10%	7%	17%	23%	–	14%
Fellowships	–	–	–	13%	13%	13%
Infrastructure for Impact grants	–	–	–	–	25%	25%
Mean	14%	16%	20%	19%	18%	19%

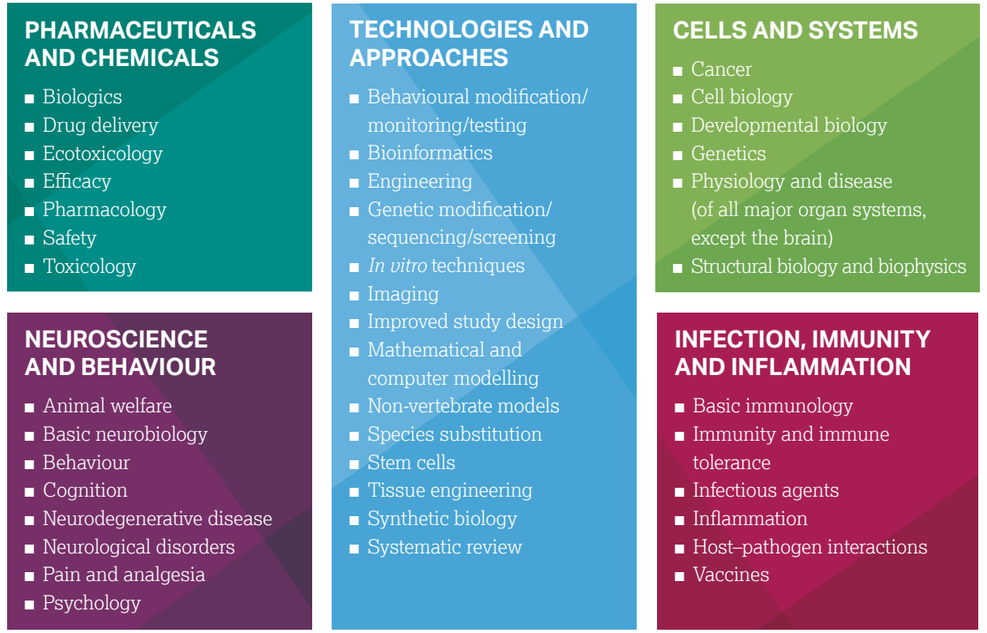
Classification of awards by scientific category and 'R'

The NC3Rs funds research across the biosciences. To monitor this we classify awards by scientific discipline and the technology or approach used as shown in Figure 3. Figure 4 shows the breakdown of our total research commitment (including the number and value of awards) by broad scientific category – cells and systems; infection, immunity and inflammation; neuroscience and behaviour; pharmaceuticals and chemicals.

The largest number of awards are in the categories 'neuroscience and behaviour' and 'cells and systems'.

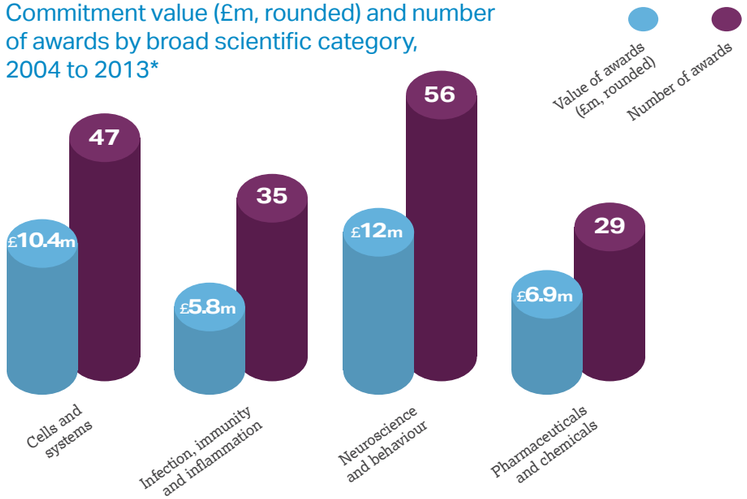
*Number of awards/number of applications. 2013 figures are up to 1 September 2013; strategic awards and studentships for 2013/2014 will not be decided until 2014.

Figure 3: The scientific classification scheme for NC3Rs awards



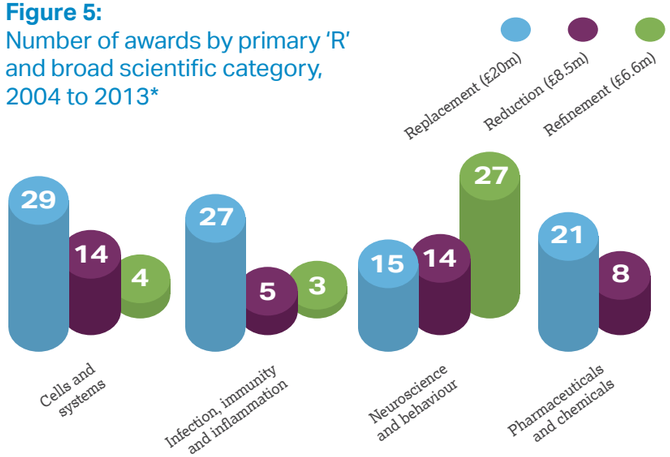
We also categorise awards according to whether the primary focus is on replacement, reduction or refinement. In some instances awards may focus on more than one 'R'. Figure 5 shows the number of awards by primary 'R' and broad scientific category. Around 55% of awards are directed primarily at replacement, 25% at reduction and 20% at refinement. This distribution largely reflects the fact that the majority of the applications received have been for replacement. There are no refinement awards within the category 'pharmaceuticals and chemicals'. We do, however, recognise the importance of this area and have an extensive collaborative programme focusing on improving the welfare of animals used in toxicology studies.

Figure 4:
Commitment value (£m, rounded) and number of awards by broad scientific category, 2004 to 2013*



*Up to 1 September 2013.

Figure 5:
Number of awards by primary 'R' and broad scientific category, 2004 to 2013*

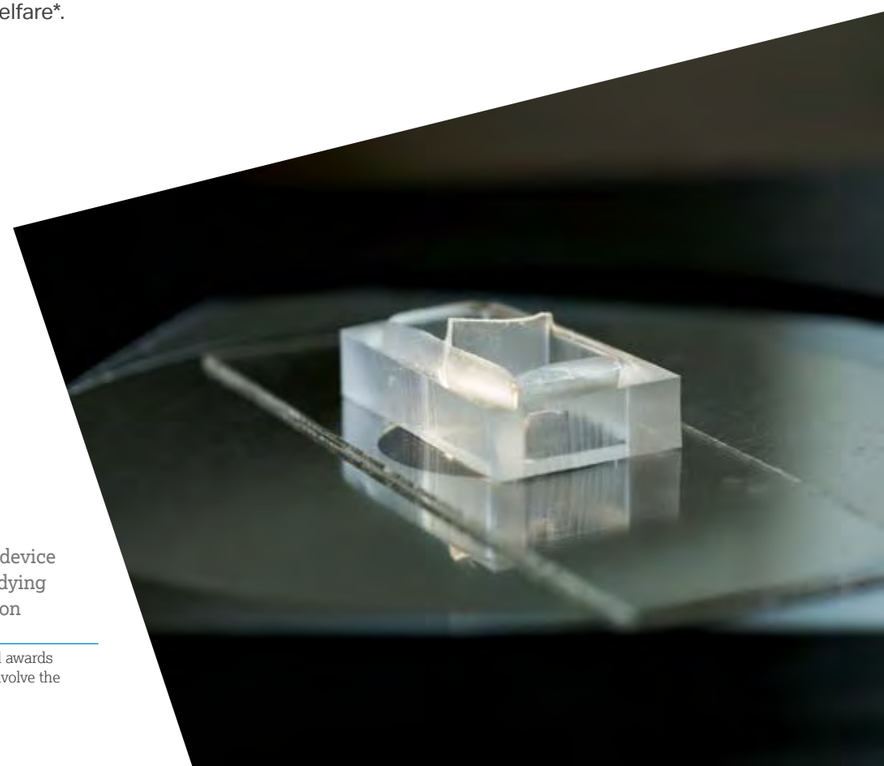


*Up to 1 September 2013.

Key technologies and approaches

The NC3Rs supports a broad range of technologies and approaches, as shown in Figure 6. In order to address specific concerns or exploit the 3Rs potential of new technologies we have targeted investment through strategic calls and highlight notices. This has resulted in an increase in awards for tissue engineering and stem cells, mathematical and computational modelling and behaviour-based approaches for assessing welfare*.

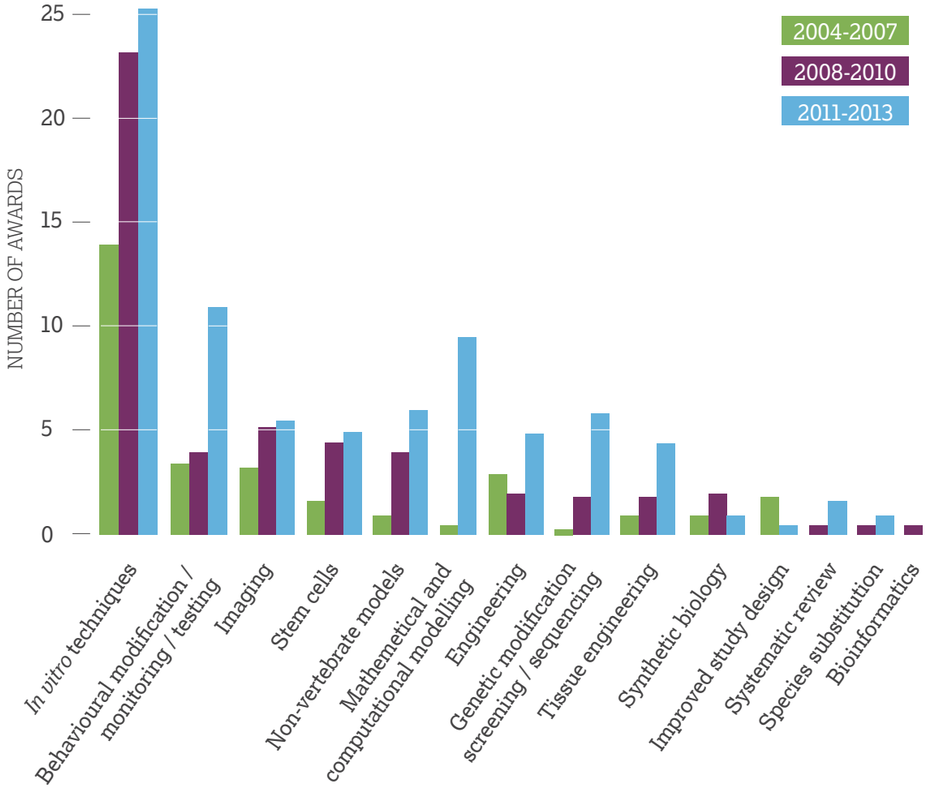
Table 2 shows the priority areas we have targeted and the strategic partners we have worked with to help raise awareness within specific communities or to increase the available budget.



A multi-chamber device developed for studying neural degeneration

*Around one third of all awards made by the NC3Rs involve the use of animals.

Figure 6:
Technologies and approaches developed through NC3Rs awards*, 2004 to 2013



*Awards may involve more than one technology or approach, in which case the weighted average has been used.

- *In vitro* techniques – awards utilising novel *in vitro* techniques but excluding those involving stem cells and tissue engineering.
- Non-vertebrate models – awards using invertebrates, or eukaryotic non-animal models such as *Dictyostelium discoideum,* in place of vertebrates.
- Synthetic biology – awards aiming to develop new models, or improve existing models, by applying molecular genetic techniques.
- Improved study design – awards aiming to improve an existing methodology but excluding all other categories of technologies and approaches.
- Species selection – awards aiming to replace the use of a 'higher' vertebrate species with one of lower neurophysiological sensitivity.

Table 2: Strategic awards, highlight notices and partnerships

Title	Year	NC3Rs awards		Partner
		Value	Number of awards	
Strategic awards				
Euthanasia of laboratory rodents	2009	£295,595	1	–
3Rs in asthma research	2010	£999,211	2	MRC: workshop co-sponsor
Human cell-based carcinogenicity assays	2011	£879,042	2	UKEMS: workshop co-sponsor
Mathematical modelling in toxicology	2012	£724,603	4	EPSRC: co-funder
Imaging technology development for the 3Rs*	2013	–	–	ESP KTN: workshop co-sponsor
Highlight notices†				
Tissue engineering solutions for replacing animal experiments	2007	£1,262,315	4	BBSRC: joint highlight notice
Refinement of procedures of substantial severity	2007	£380,588	3	–
3Rs and fish	2008	£1,165,772	3	–
Refinement in rodent husbandry, care and procedures	2008	£572,912	2	–
Replacing animals protected under the Animals (Scientific Procedures) Act 1986 with invertebrate models	2009	£914,804	2	BBSRC: joint highlight notice
Animal welfare measures and assessment	2012	£2,355,305	8	BBSRC: joint highlight notice

*Awards will not be made until 2014. †Highlight notices are used to stimulate applications in specific areas under the annual project grant scheme.

Impact analysis

In 2012 we published the first 3Rs evaluation framework which sets out the metrics we use to assess the value and impact of NC3Rs research (www.nc3rs.org.uk/evaluationreport). To complement this, we have used the online RCUK Research Outcomes System as the mechanism by which award holders provide continuous feedback on the outcomes of their research. Up to 1 September 2013, more than 97% of NC3Rs award holders had reported data into the system. This includes*:

- 131 peer-reviewed articles and reviews, and 40 other publications (e.g. book chapters, guidelines)
- 238 conference presentations, lectures, seminars and workshops
- 18 public engagement events (e.g. presentations to schools, students and parliamentarians)
- 50 new research materials (e.g. animal models, cell lines, DNA products, assays, databases, software)
- 73 unique collaborations
- 33 instances (£12.3 million) of further funding (excluding NC3Rs funding) – £3.5 million from the charitable sector, £2.8 million from research councils (MRC and BBSRC) and £0.9 million from industry
- 19 research prizes and appointments (e.g. to learned societies)
- Five citations on policy and regulatory change at national and international levels

More detail is given in the case studies featured in this Research Review.

*The number of awards made by the NC3Rs has increased in recent years. This means that over half of the NC3Rs research portfolio (55%) comprises active awards. The full outputs and impacts from these awards will be reported at a later date.

Publications by NC3Rs-funded scientists

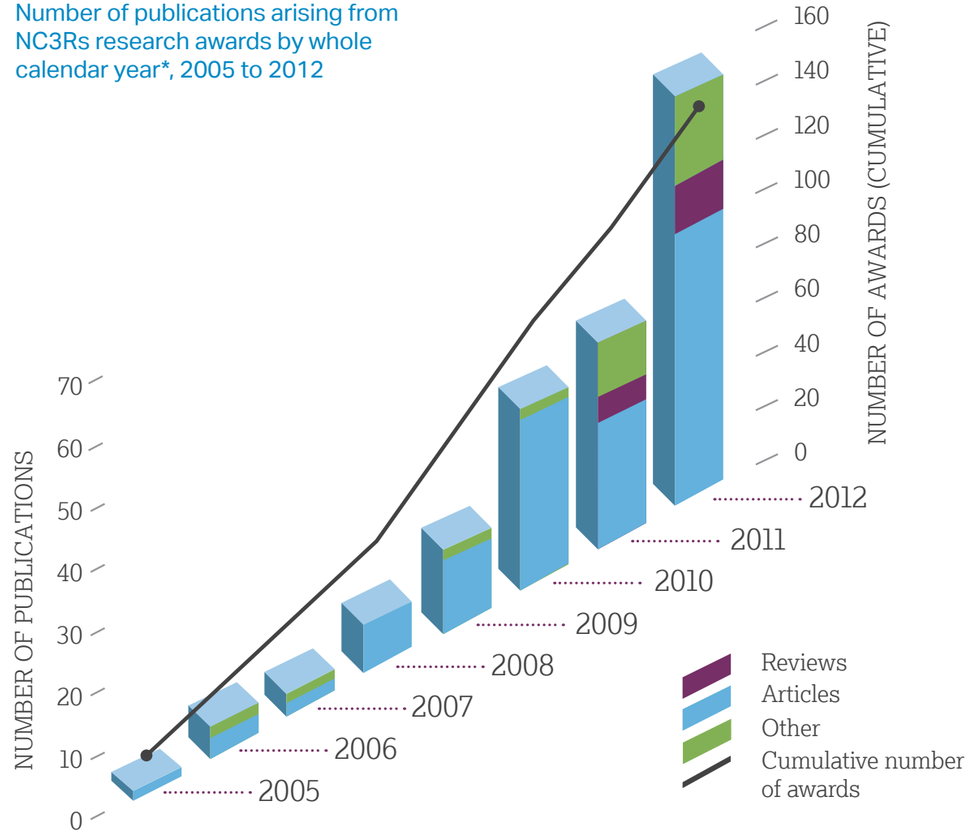
Changing practice and delivering 3Rs benefits requires the new methodologies, approaches and technologies arising from the science we fund to be scrutinised and accepted by the scientific community. Publishing is key to this. Figure 7 shows the number of publications arising from NC3Rs research awards by calendar year. Publication output has grown in line with the number of awards.

The citation of papers by other research publications can be used as a way of comparing research productivity and quality. By normalising for variations between scientific fields and year of publication, measures of citation impact can indicate the extent to which the research has been used by others.

Figure 8 shows an impact profile for 112 NC3Rs papers reported in the Research Outcomes System and published between 2005 and 2012, using 'normalised' citation scores taken at the end of 2012. The distribution of papers arising from NC3Rs funding is shifted towards the right; this shows that these papers performed better than the UK average for the fields of biological sciences research and clinical/health and medically-related research. A higher percentage of NC3Rs-funded research (48.0%) is at least above the world average (NCI ≥ 1.0) in comparison with UK biological (46.2%) and medical (42.0%) research. A similar share of NC3Rs-funded research (8.1%) is highly-cited in comparison with these fields (8.8% and 7.8% respectively).

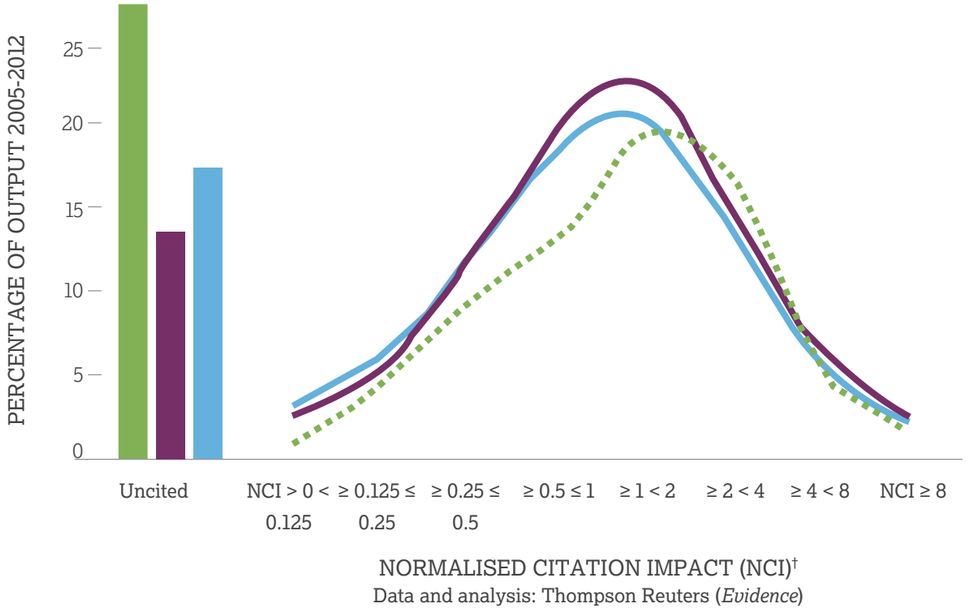
The profile also shows that NC3Rs award holders have published substantially more research that is uncited (27.7%) in comparison with UK biological (17.1%) and medical (21.4%) research. The most likely explanation for this is the fact that it takes time for new technologies and approaches to be adopted and therefore cited. We work closely with the scientists we fund to assist with dissemination and uptake across their institutes and disciplines. Our interactions with the major funding bodies and regulatory authorities also provide a platform for promoting wider acceptance. We recognise however that there is more to do to demonstrate the utility of new 3Rs technologies and approaches and to encourage confidence in shifting from standard practices. We will be placing greater emphasis on this in the future.

Figure 7:
Number of publications arising from NC3Rs research awards by whole calendar year*, 2005 to 2012



*All reviews and articles are peer reviewed. Others include peer reviewed and non-peer reviewed conference papers, technical reports and editorials.

Figure 8:
Impact profile for research published by NC3Rs award holders*, when compared with UK biological sciences research and UK clinical/health and medically-related research



*Articles and reviews only; excludes those papers published in journals not abstracted by Thompson Reuters, or not listed in bibliographic databases.

† Normalised Citation Impact measures the performance of a set of papers normalised against the average performance of the world for the field in which the papers are published and their year of publication. Normalised citation scores above 1.0 indicate papers cited more often than the world average.

3Rs impacts

The ultimate goal of NC3Rs research funding is to deliver high quality scientific and technological innovations which reduce animal use and improve animal welfare. The NC3Rs evaluation framework provides a tool to measure the actual impact of our science on the 3Rs. For many awards, it will take some time for the full impact to be realised both nationally and internationally. To provide a guide to the potential impact of these awards we have introduced a '3Rs label'. This gives a measure of impact based on current animal use from information that we are able to obtain from the scientific literature, published statistics and discussions with award holders and other stakeholders. Depending on the information available we provide estimates for the UK only, Europe or worldwide. 3Rs labels are provided for all of the case studies in this review.

POTENTIAL 3Rs IMPACT	
NO. OF ANIMALS	70,000/year in the UK
SPECIES	Mice
SECTOR	Government, Pharma
AREA	Pharmaceuticals & Chemicals – Biologics
'R'	Replacement

POTENTIAL 3Rs IMPACT	
NO. OF ANIMALS	40,000/year in the UK
SPECIES	Mice
SECTOR	Pharma
AREA	Cells & Systems – Cancer
'R'	Replacement, Reduction

2 Cells and systems

3D micro-cancers to reduce animal use in drug development

Principal Investigator:
Dr Louis Chesler, Team Leader

Organisation:
The Institute of Cancer Research

Award:
£291,488, in 2010, over 24 months

Title: Replacement of animals in cancer drug development by using 3D *in vitro* functional assays for increased predictive power

POTENTIAL 3Rs IMPACT

NO. OF ANIMALS	40,000/year in the UK
SPECIES	Mice
SECTOR	Pharma
AREA	Cells & Systems – Cancer
'R'	Reduction, Replacement

Principal Investigator Dr Louis Chesler with Co-Investigator Professor Suzanne Eccles

"Our 3D tumour models are enhancing the clinical relevance of early preclinical cancer studies and reducing the number of animals required to bring each new drug to the clinic."

There is a need to improve the cancer drug development pathway

Paediatric solid tumours (such as glioblastoma and neuroblastoma) remain among the most deadly and difficult to treat of all childhood cancers. Disease relapse is frequently characterised by metastasis and resistance to conventional treatments. There is a major clinical need to develop new drugs for these and other types of cancers, including those found in adults. Drug attrition rates for cancer are higher than for many other therapeutic areas and a number of factors contribute to this. One key reason is the failure of preclinical strategies to accurately select appropriate targets and evaluate novel therapeutics. Another problem in paediatric cancer, and especially in solid tumours, is the difficulty in obtaining tumour tissue to study in order to increase understanding about drug responses.

Preclinical testing typically involves applying compounds to simple 2D tumour cell culture systems to assess for inhibition of proliferation before selecting the best candidates for evaluation in animal tumour models. The 2D assays fail to reflect the complexity of tumours within the body and particularly the limited drug access to solid tumours that is observed in patients. Attention has therefore focused on developing 3D cultures which more closely mimic the *in vivo* tumour microenvironment, including hypoxic regions and necrotic cores. The true value of such models is dependent on the ability to generate reproducible tumour spheroids (micro-cancers), combined with tools that allow automated analysis. In 2010, Dr Louis Chesler and Professor Suzanne Eccles at The Institute of Cancer Research (ICR) were awarded NC3Rs funding to develop such a system.

Micro-cancers to study tumour growth, invasion and angiogenesis

Initial experiments using 40 different cancer cell lines demonstrated the ability of the cells to form spheroids or micro-cancers spontaneously in culture. Micro-cancers from cell lines representing hard to treat diseases (glioblastoma, oral squamous cell carcinoma and triple negative breast carcinoma) were subsequently selected for development of functional assays for target validation and drug evaluation.

The micro-cancers show the key cancer hallmarks of cell migration, invasion and angiogenesis. By growing them singly in 96-well, round-bottomed microplates and utilising advanced high content imaging, the system provides for the first time a high throughput and quantitative screening tool for the development of novel cancer drugs.

Clinically important discoveries using the micro-cancers

The micro-cancers have been used to test a range of compounds, identifying those with enhanced potency against cell migration and invasion compared with proliferation, suggesting a particular use for invasive and/or metastatic cancers. In addition, using genetic knockdown, it has been possible to identify key roles of signalling molecules (such as the protein kinase, MAP4K4) in tumour cell motility and invasion, not detectable in standard 2D assays.

Micro-cancers have also been generated for prostate, colon and lung cancer and particularly those in which local invasion is a major clinical problem, such as glioblastoma and head and neck squamous cell carcinoma (HNSCC). The 3D assays revealed *in vitro* phenotypes in HNSCC not evident in standard 2D assays (such as enhanced growth and invasion, mimicking their behaviour *in vivo)* and these are being exploited to streamline the discovery of novel biomarkers and therapies to overcome drug resistance.

Reducing animal use by more effective selection of compounds

Use of the micro-cancers in drug discovery projects has enabled researchers at the ICR to select only those compounds which are predicted to have optimal efficacy *in vivo.* As a result, in one project in particular, approximately 30% of compounds that previously would have progressed to *in vivo* tumour testing have not advanced into animal studies.

Promoting the uptake of micro-cancers

To date the research has led to three publications and a number of presentations at international conferences. The methodology article published in *BMC Biology* achieved a status of 'highly accessed' - in the top ten in just its first year with over 16,000 readers to date. Ten collaborations have been formed with ICR colleagues, national and international institutions and a pharmaceutical company to exploit the new assays. Dr Chesler and Professor Eccles are also collaborating with companies to further miniaturise the assay and to refine and extend the software. This will enable the assays to be used to quantify additional features of malignancy and to increase throughput such that screening for inhibitors of tumour cell proliferation, invasion and also angiogenic potential in 3D is a practical reality.

2 Cells and systems

Oncology research using the fruit fly

Principal Investigator:
Owen Sansom, Professor of Molecular Oncology

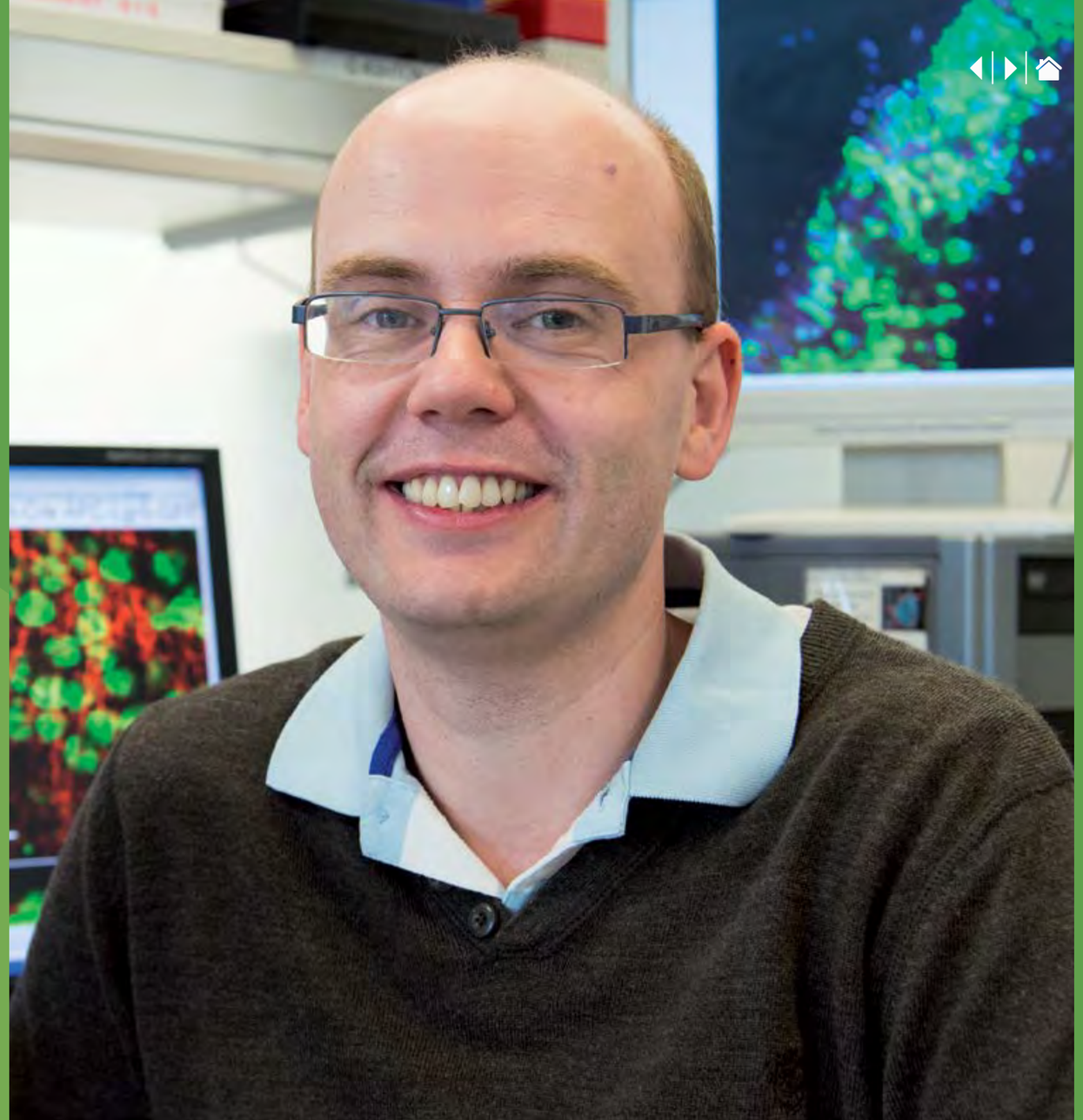
Organisation:
The Beatson Institute for Cancer Research

Award:
£350,528, in 2010, over 36 months

Title:
Using the *Drosophila* fly intestine to investigate Wnt targets *in vivo*

POTENTIAL 3Rs IMPACT

NO. OF ANIMALS	10,000/year in the UK
SPECIES	Mice
SECTOR	Academia, Pharma
AREA	Cells & Systems – Cancer
'R'	Replacement, Reduction



"It is possible to robustly model colorectal cancer in *Drosophila* and as a result I have been able to use the fly as an initial screen to investigate genes involved in carcinogenesis, selecting only those of real importance for study in the mouse."

Colorectal cancers are highly prevalent

Colorectal cancer is the third most common cancer worldwide. Inactivating mutations in the Adenomatous Polyposis Coli *(Apc)* gene occur in around 80% of colorectal cancers and appear to be associated with the very earliest stages of malignancy. APC is part of the WNT signal transduction pathway which drives the transcription of a number of key oncogenes such as Myc and Cyclin D2.

Inactivation of *Apc* affects the critical control point in the cell cycle, the G1 to S transition, resulting in dysregulation of cell growth in intestinal epithelial cells and the formation of intestinal polyps. It is rarely mutated in other cancers and a key challenge is to understand APC function within the intestinal epithelium and to identify why it is such a potent tumour suppressor in this tissue.

Genetic experiments for colorectal cancer research use large numbers of mice

Mouse models of colorectal cancer were first used in 1928 and since then numerous genetically altered, chemically induced and xenograft models have been used to study pathogenesis and test potential therapeutics. Complex genetic experiments, generating double knockouts and conditional mutations, are increasingly required to study the role of APC and other downstream molecular targets of the WNT pathway. Such experiments use large numbers of mice to generate relatively few animals with the appropriate genotype. Compounding this, single knockout mice in the WNT pathway are often sterile or have reproductive problems (for example, Cyclin D1 mutant mice do not lactate). Moreover due to genetic redundancy, other family members in the mammalian system may mask phenotypes.

In 2010, Professor Owen Sansom, The Beatson Institute for Cancer Research, was awarded NC3Rs funding to validate the use of the fruit fly, *Drosophila melanogaster,* as an alternative colorectal cancer model to the mouse.

A fly model of colorectal cancer

The adult *Drosophila* midgut is remarkably similar to the vertebrate intestine in that the fly intestinal epithelium undergoes constant self-renewal and is replenished by stem cells. WNT signalling regulates the behaviour of *Drosophila* intestinal stem cells. Professor Sansom has shown previously that *Apc1* deletion from the fly intestine results in a phenotype similar to the mouse with an expansion in the number of intestinal progenitors and increased proliferation and thickening of the intestinal epithelium.

With NC3Rs funding, Professor Sansom has demonstrated that the *Drosophila* model also has the key molecular hallmarks seen in the mouse. This includes upregulation of dMyc following loss of *Apc1,* and the prevention of intestinal stem cell hyperprofileration in *Apc1* mutants by knocking down *myc* expression. Important functional roles for the JAK/STAT and SRC signalling pathways have also been identified, which had previously been suggested in mammalian cells but not characterised.

Drosophila melanogaster

A screen to minimise the use of mice

Professor Sansom has demonstrated that the *Drosophila* colorectal cancer model can be used to investigate molecular targets downstream of APC, and that the fly can be used as a screen to decide which genes to test in the mouse intestine. This selective approach allows the number of mice used to be minimised. For example, Professor Sansom has shown previously in the mouse that the G protein RAC1 is required for intestinal hyperproliferation following *Apc* loss. To test whether RAC1 overexpression is sufficient to cause intestinal stem cell proliferation and colorectal cancer initiation, the gene was overexpressed in the fly rather than the mouse. This replaced the use of around 400 animals which would be required for the generation and characterisation of RAC1 overexpressing mice.

A similar number of mice were replaced by SRC overexpression and loss of function studies in the fly midgut. SRC is a tyrosine kinase which is amplified or mutated in human colorectal cancer although the *in vivo* relevance of this is unclear. The fly studies demonstrated that SRC drives intestinal stem cell proliferation and is required for tissue regeneration. Overexpression of SRC causes hyperproliferation. Based on this information it was considered that the mouse overexpression studies were unnecessary.

Extending a local reduction in mouse use to the colorectal cancer research community

The selective use of mice based on evidence derived from the fly has allowed Professor Sansom's laboratory to use around 2,000 fewer mice a year. The model has the potential to be adopted more widely to minimise the use of mice and is already being used by groups in Edinburgh, Cambridge and New York. To date the work has been disseminated through seven publications, one of which was further highlighted in *Nature Reviews Cancer.* There has also been dissemination at various scientific events including a symposium 'Cold blooded cancer: non-mammalian models for oncology research' at the 2012 National Cancer Research Institute meeting. In 2012, Professor Sansom was awarded the CRUK Future Leaders in Cancer Research Prize in recognition of his work to understand the early changes associated with intestinal cancers.

3 Infection, immunity and inflammation

A bioreactor to predict the efficacy of antifungal therapies

Principal Investigator:
William Hope, Professor of Therapeutics and Infectious Diseases

Organisation:
University of Liverpool

Award:
£210,664, in 2007, over 24 months

Title: An *in vitro* model of the human alveolus to predict the efficacy of systemic antifungal therapy

POTENTIAL 3Rs IMPACT

NO. OF ANIMALS	100,000/year globally
SPECIES	Mice
SECTOR	Academia, Pharma
AREA	Infection, Immunity & Inflammation – Infectious agents
'R'	Replacement

"Our dynamic *in vitro* model of the human alveolus can be used to study the pharmacokinetics and pharmacodynamics of antifungal agents. It has reduced mouse use in my laboratory alone by 2,000 animals per year."

Atmospheric *Aspergillus* causes lung infections in immunocompromised patients

Fungal spores are present in the atmosphere and are normally not detrimental to health. They can, however, cause fatal infections in immunocompromised patients, such as those receiving organ transplants or with haematological cancers. Each year in the UK there are an estimated 4,000 cases of lung infection caused by the fungus *Aspergillus fumigatus*. Approximately half of these patients will die. Providing improved or new therapeutics is essential.

Determining the clinical severity of fungal infections

Clinical breakpoints are terms used to categorise microorganisms, including *Aspergillus*, as clinically susceptible, intermediate or resistant to antimicrobial agents. This information is used by clinicians to select appropriate therapeutics and doses for patients and is important in preventing drug resistance.

Breakpoints are determined using information on the pharmacokinetics (PK) and pharmacodynamics (PD) of the drug. This requires a model of infection which allows the efficacy of the drug to be assessed and the PK/PD relationship to be measured. Studies are typically performed in animals but the infection and response to drugs can differ from humans. This can make it challenging to establish safe and effective dose regimens for managing infection in patients because if a patient has drug levels below the clinical breakpoint they may not respond to treatment.

Large numbers of animals are used to establish the efficacious dose of antifungal drugs

Up to 2,000 mice are used to characterise the PK/PD relationship of each antifungal drug. For lung infections, *in vitro* models of the human alveolus have been developed but these are static and not suitable for PK/PD analysis of drugs. In 2007, Professor William Hope, University of Liverpool (previously University of Manchester), was awarded an NC3Rs grant to develop a dynamic *in vitro* model to study invasive pulmonary aspergillosis and to measure the PK/PD relationship of antifungal drugs.

A microfluidic *in vitro* lung model of *Aspergillus* infection

Professor Hope has designed and constructed a bioreactor to house a cellular bilayer consisting of human alveolar epithelial cells and pulmonary endothelial cells grown on a semipermeable, polyester membrane. The bilayer delineates an upper compartment (representative of the air space of the alveolus) and a media-filled lower compartment (representative of the pulmonary capillary) connected to a central reservoir (representing the blood).

Aspergillus spores are introduced into the alveolar compartment, where they germinate to form hyphae (the invasive forms of the fungus) which quickly invade the cellular bilayer. Fresh cell culture media is pumped into the reservoir while spent media is removed at the same rate. Antifungal drugs can then be injected into the circuit, mimicking systemic drug administration. By sampling the media it is possible to determine first-order PK.

The model also incorporates the biomarker galactomannan for assessing PD. This biomarker is a component of the *Aspergillus* cell wall which is released during growth. Its presence in blood is used to diagnose invasive aspergillosis in patients. The bioreactor can be sampled repeatedly for galactomannan allowing the generation of a rich data set that if performed *in vivo* would require large numbers of animals and serial blood sampling.

Translation to benefit patients

Voriconazole is an antifungicide widely used as a first-line therapy for the treatment of invasive pulmonary aspergillosis. A detailed understanding of its PK/PD relationship has been difficult to establish in animals. Professor Hope has been able to use the new model to investigate the PK/PD relationship of voriconazole, establishing its clinical breakpoint. This was published in the scientific literature and on the website of the European Committee on Antimicrobial Susceptibility Testing, which is responsible for setting clinical breakpoints.

Professor Hope has secured additional funding from Astellas Pharma to use the model to define breakpoints for the novel antifungal compound, isavuconazole. The drug is now in Phase III clinical trials as a treatment against a range of medically important fungal pathogens. The model is also being used as part of collaborations with Canadian investigators, and for characterising novel antifungal compounds with the UK biotechnology company F2G. Its use could potentially be extended to other experimental contexts such as the immunopathology of infection.

There is one journal article describing the new model and one technical note arising from this award. Another five articles report the use of the model to evaluate different drugs and combination therapies.

Bioreactor used to model *Aspergillus* infection

3 Infection, immunity and inflammation

Minimising fish use in immune studies

Principal Investigator:
Dr Bertrand Collet, Senior Research Scientist

Organisation:
Marine Scotland Science

Award:
£435,700, in 2011, over 24 months

Title: Development of a non-lethal sampling method to monitor immune response and disease progression in salmonid fish

POTENTIAL 3Rs IMPACT

NO. OF ANIMALS	30,000/year in the UK
SPECIES	Salmonid fish
SECTOR	Academia, Government, Animal Health
AREA	Infection, Immunity & Inflammation – Infectious agents
'R'	Reduction, Refinement

“Our new sampling protocol reduces the number of fish needed for vaccine and immunology studies by 86%.”

Growth of the salmon farming industry is driving an increase in fish disease research

In recent years the use of fish in scientific procedures has increased substantially. This is partly due to the increased investment in research to understand the fish immune system and to develop vaccines to support the fish farming industry. Each year in the UK, approximately 300,000 fish are used for fundamental or applied research, 10% of which are used in vaccine and immunology studies.

Sampling methods currently use large groups of fish and are limited scientifically

Evaluating pathogen virulence and testing the efficacy of vaccines is traditionally carried out in infection challenge experiments. These involve infection of groups of fish with the pathogen of interest, with cohorts of fish killed at regular time-points for tissue collection in order to measure pathogen load and/or immune parameters as the infection progresses.

There is significant inter-fish variability as the animals are sourced from farms and are genetically diverse. Variability is also inherent in immune studies as the animals never get infected at the same time and this means that the expression of cytokines, key regulators of the immune response, is never synchronised. In order to minimise statistical ‘noise’ arising from the inter-fish variability large numbers of animals are used. The studies provide basic information on gene expression but cannot be used to address specific questions about whether vaccines are able to increase or decrease cytokine levels for example, which are essential for designing control measures against fish diseases.

A new sampling protocol to reduce fish use by 86%

In 2011 Dr Bertrand Collet at Marine Scotland Science was awarded NC3Rs funding to develop a method which would enable the immune response to various pathogens to be studied in individual fish longitudinally.

Studies were carried out using Atlantic salmon *(Salmo salar)* or Rainbow trout *(Oncorhynchus mykiss)* and a variety of pathogens causing or threatening significant economic loses to the UK fish farming industry, including salmon anaemia virus, salmon alphavirus and the bacterium *Yersinia ruckeri.* The fish were electronically tagged for identification purposes and pathogen load and immune markers were measured using small volumes of blood (around 100 microlitres) taken under general anaesthesia from the same individuals at various time-points throughout the infection cycle.

Using the traditional approach of pooling tissue from a group of fish requires 84 animals for a study looking at seven time-points. The equivalent study using the new sampling method uses 12 fish, representing an 86% reduction.

Repeat blood sampling does not affect fish welfare

To minimise the potential stress of repeated sampling, Dr Collet has developed an ‘in-tank’ general anaesthesia procedure which avoids having to ‘net’ the fish. Observation of fish behaviour after anaesthesia and sampling has shown no adverse effects. In a typical infection study, blood samples are taken every four days for up to 28 days. Analysis of the haematocrit, the volume percentage of erythrocytes in the blood and a physiological marker of stress, also shows no effect from repeated sampling, with animals being able to fully recover between time-points.

Innovation in bioanalytical tools for studying the fish immune response

Unlike in mammals, there is a dearth of reagents against fish immune molecules. A key challenge of using the blood sampling approach was the requirement for new analytical techniques which enable the use of small volumes. To address this, Dr Collet has developed for the first time a cell-based functional assay for cytokine analysis in fish. The assay uses a trout cell line expressing the luciferase gene under the control of the interferon-induced MX gene. Other assays are under development, which are likely to have even greater sensitivity. These include transgenic fish cell lines expressing fluorescent molecules, under the control of various interferon regulator factor genes, which translocate into the nucleus upon activation.

Adoption of the new protocol by other research groups

The new method using significantly fewer fish has been adopted by Dr Collet's collaborators at the Universities of Aberdeen and Stirling and is also being modified for the study of fish parasites. The work has also been disseminated at the international workshops of the European Veterinary Immunology Group and the Danish Fish Immunology Research Network. They will also be used in the European Commission funded project TARGETFISH, which aims to develop targeted disease prophylactics for use in European fish farming.

There is one paper arising from this grant to date.

Further NC3Rs funding to develop fish cell lines

Infection and immunology experiments are currently dependent on the use of live fish. There are few well-defined fish cell lines available and most of these are not susceptible to the virus strains responsible for fish diseases. In 2013, Dr Collet was awarded an NC3Rs pilot study grant to develop cell lines from the Atlantic salmon gill, heart, kidney and spleen.

4 Neuroscience and behaviour

Social amoebae for epilepsy research

Principal Investigator:
Robin Williams, Professor of Molecular Cell Biology

Organisation:
Royal Holloway, University of London

Award:
£415,248, in 2009, over 36 months

Title: Replacing, refining and reducing animal usage in epilepsy research using a non-sentient model

POTENTIAL 3Rs IMPACT

NO. OF ANIMALS	500,000/year globally
SPECIES	Mice, rats
SECTOR	Academia, Pharma
AREA	Neuroscience & Behaviour – Neurological disorders
'R'	Replacement

“Our *Dictyostelium* model has dramatically reduced rodent use for screening compounds for seizure control.”

Around 50 million people worldwide have epilepsy

Epilepsy is a chronic neurological condition characterised by repeated seizures. Around 50 million people worldwide have epilepsy. Anti-epileptic drugs are the mainstay of treatment but current therapies are poorly effective in around one third of people (approximately 160,000 people in the UK alone).

Epilepsy research is currently dependent on the use of animals

Investigating the changes that occur in neural activity in the brain during an epileptic seizure almost exclusively uses animals, either as a source of primary cells or brain slices for *in vitro* experiments or for *in vivo* studies. Worldwide an estimated 500,000 rodents are used annually for epilepsy research. Some of the procedures can be distressing for the animals involved and in the UK they are classified as causing moderate or severe suffering.

Studies, mainly in mice and rats, led in 1963 to the accidental discovery that the short-chain fatty acid sodium valproate is an effective drug in seizure control. Today, sodium valproate is globally the most widely prescribed drug for epilepsy treatment, accounting for 52% of prescriptions. It is, however, associated with a number of side effects, including hepatoxicity and teratogenicity. Work to provide improved treatments with fewer side effects have been hampered by a lack of understanding of the mechanism of action of sodium valproate.

Using amoebae to identify potential anti-epileptic drugs

In 2009, Professor Robin Williams, Royal Holloway, University of London, was awarded NC3Rs funding to develop *Dictyostelium discoideum* as a model system for studying the molecular pharmacology of sodium valproate, providing a basis for discovering potentially new anti-epileptic drugs.

Dictyostelium discoideum is a social amoeba found in forests. It can easily be grown in the laboratory and is commonly used for studies on cell movement and signalling. At times of starvation, the amoebae aggregate to form multicellular fruiting bodies. Professor Williams has demonstrated that sodium valproate inhibits chemotactic cell movement and therefore fruiting body formation in *Dictyostelium* by attenuating phosphoinositide turnover.

Using this biochemical pathway, a wide range of compounds that are chemically similar to sodium valproate have been screened in the *Dictyostelium* model system. This has identified new fatty acids and fatty acid derivatives with potential anti-epileptic activity. Working with Professor Matthew Walker, Head of the Department of Clinical and Experimental Epilepsy, University College London, the fatty acids have been subsequently tested in rat *ex vivo* hippocampal slice models of seizure-like activity and *in vitro* hepatotoxicity and teratogenicity assays. The most potent were then investigated for efficacy *in vivo* in a rat model of epilepsy.

Anti-epileptic drugs are typically tested for efficacy in two animal models at five doses and with around eight animals per dose. By using *Dictyostelium* as a pre-screen, Professors Williams and Walker used 100 rats to test 60 compounds; the standard approach would have used 4,800 animals. This represents a 98% reduction.

A potentially improved diet to treat drug resistant epilepsy

In children with severe drug resistant epilepsy a ketogenic diet is often prescribed. Initially introduced in the 1920’s, it is essentially a ‘semi-starvation’ diet which involves heavily limited carbohydrates and lots of proteins. The diet was improved in the 1990’s to include medium-chain triglyceride (MCT) oil and to allow some carbohydrate consumption. One of the most potent anti-epileptics found in Professor Williams’ study was decanoic acid, which is a major constituent of MCT oil. This provides a mechanistic basis for how the diet works and opens the door to making a more palatable and improved diet for the control of epilepsy. A licence agreement has been signed with Nestlé’s Health Science company, Vitaflo.

To date there have been nine papers arising from this grant.

4 Neuroscience and behaviour

A multi-chamber device for studying neural degeneration

Principal Investigator:
V. Hugh Perry, Professor of Experimental Neuropathology

Organisation:
University of Southampton

Awards:
£181,068, in 2006, over 24 months; £210,884, in 2009, over 24 months

Titles: *In vitro* multi-chamber systems for studying neural degeneration processes;
A compartmentalised chamber for the *in vitro* study and manipulation of axon degeneration

POTENTIAL 3Rs IMPACT

NO. OF ANIMALS	100,000/year globally
SPECIES	Mice, rats
SECTOR	Academia, Pharma
AREA	Neuroscience & Behaviour – Neurodegenerative disease
'R'	Reduction

Principal Investigator Professor Hugh Perry with Co-Investigator Dr Tracey Newman

"Our multi-chambered *in vitro* culture system provides a novel bioengineering solution that will enable the use of rodents to be reduced in neurodegeneration studies."

Neurodegeneration occurs in many diseases affecting the central nervous system

Neurodegeneration is a component of many neurological disorders including multiple sclerosis, stroke and Alzheimer's and Parkinson's diseases. Understanding how to protect neurons from injury and death is a key area of research for new therapies. Neurons can be divided into three sub-cellular compartments – the cell body, axon and dendrites, each residing in a different microenvironment. Recent research has shown that degeneration of the cell body, axon and dendrites occurs by different mechanisms.

Better *in vitro* systems would reduce the use of animals in neurodegeneration research

Studying the cellular and molecular changes that occur during neurodegeneration often involves the use of rats and mice; it is estimated that worldwide 120,000 animals are used in invasive studies and 10,000 as a source of primary neurons for *in vitro* models. The utility of *in vitro* systems is limited by the size and polarity of neurons and the difficulty in mimicking the different microenvironments for each compartment.

Reducing the number of animals that are used requires better *in vitro* systems that enable the different compartments of the neuron to be manipulated and studied independently. In 2006, Professor Hugh Perry and Dr Tracey Newman, University of Southampton, were awarded NC3Rs funding to develop such a system, with additional funding provided in 2009 to support scale-up and testing.

A bioengineering solution for *in vitro* studies

A microfluidic device comprised of two chambers has been developed. The cell bodies of the neurons reside in one chamber and the axons in the other. Axon growth can be further controlled by using thin stripes of the substrate laminin separated by polyethylene glycol so that the axons form bundles or fascicles as they would in the central nervous system. Fluids do not readily pass between the two chambers and this means that the cell body and axon can be exposed to different environments, for example, by the inclusion of other cell types or the addition of drugs to one or other chamber.

The device is the first high-throughput system that can be imaged using conventional microscopy, and is amenable to electrophysiology, transfection, and other manipulations where direct contact with the neuron is required. Neurons can be maintained for up to four weeks and are phenotypically similar to those grown using conventional *in vitro* techniques.

Large scale production is essential to maximise use of the device and ensure that it is commercially viable. In 2009, additional funding was provided to simplify the device construction, moving away from high technology microfabrication to a more standard tissue culture device to enable future scale-up using conventional plastic injection moulding. The detailed fabrication protocol has been made widely available. There has been one publication arising from the grant.

Identifying the earliest events after neuron injury

Professor Perry and Dr Newman are using the device to study events that occur during neuronal injury or transection. When an axon is cut or crushed a sequence of events is initiated, termed Wallerian degeneration, which include breakdown of the axonal cytoskeleton and myelin degradation. The early molecular events that underpin this are poorly understood although studies in mutant mice suggest that it may be possible to slow and modulate neurodegeneration. Using proteomic analyses, Professor Perry and Dr Newman have shown that remodelling of the actin cytoskeleton is one of the earliest detectable changes after axon injury, and that this change may be controlled intrinsically by the axon. The new device is being used to investigate this further, including by pharmacological modulation.

4 Neuroscience and behaviour

Reducing infection risk in monkeys used to study the control of movement

Principal Investigator:
Stuart Baker, Professor of Movement Neuroscience

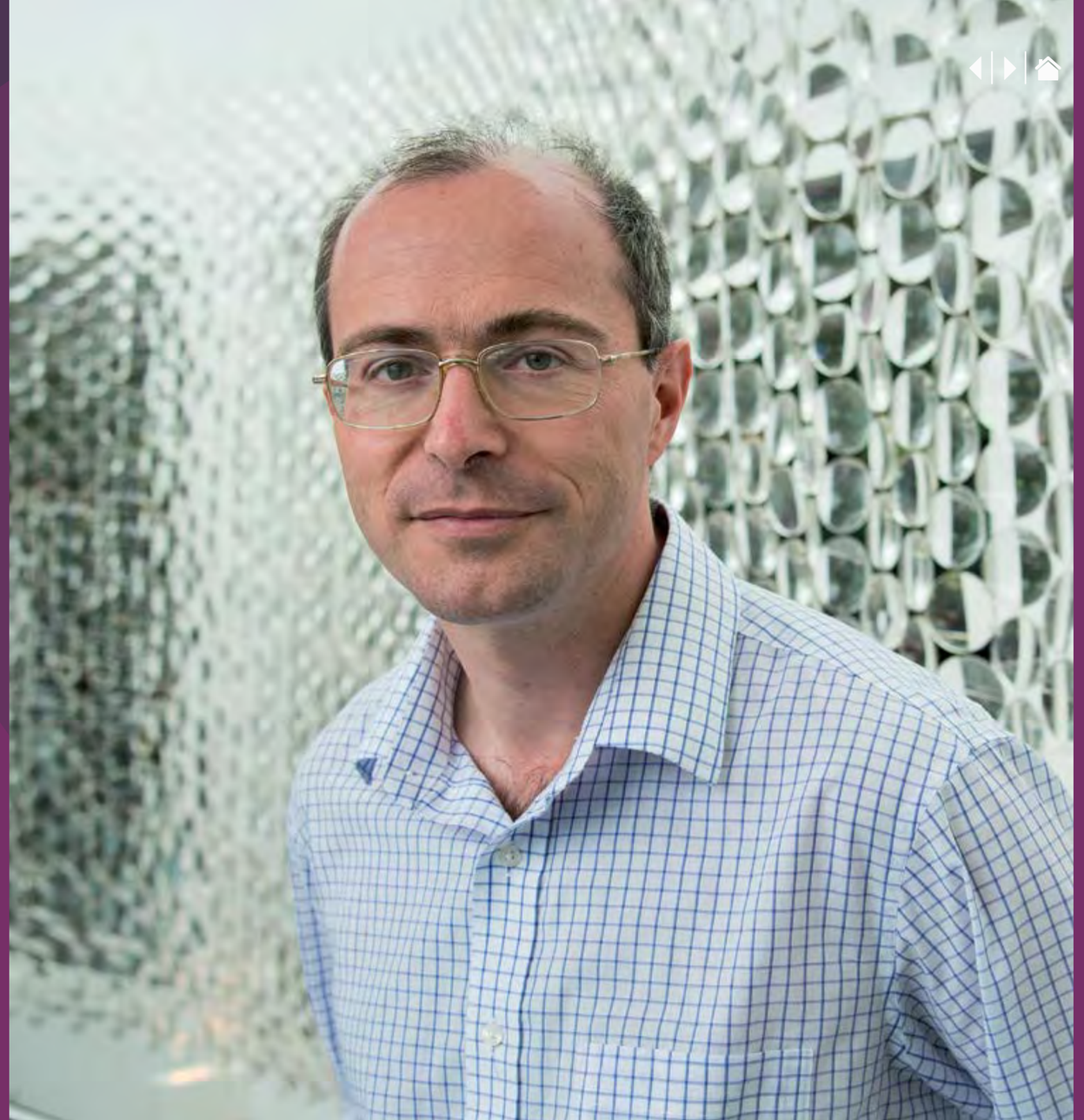
Organisation:
Newcastle University

Awards:
£149,176, in 2006, over 24 months; £71,994, in 2011, over 18 months

Titles: Transcutaneous signal transmission without breaching the skin's natural barrier to infection; Wireless high bandwidth transcutaneous signal transmission

POTENTIAL 3Rs IMPACT

NO. OF ANIMALS	20/year globally
SPECIES	Macaques
SECTOR	Academia
AREA	Neuroscience & Behaviour – Basic neurobiology
'R'	Refinement



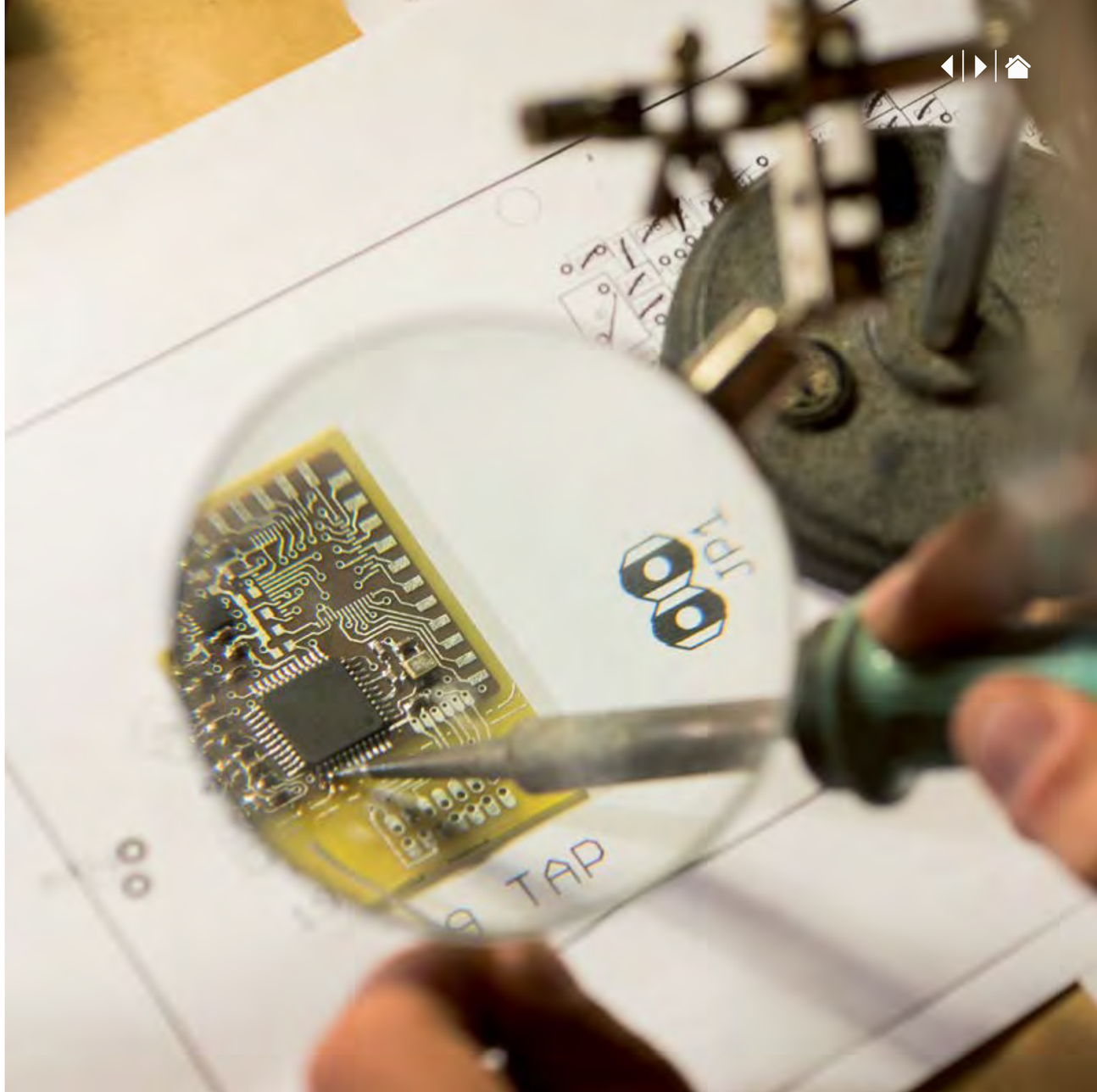
“Our novel telemetry device should eliminate the repeated infections associated with current methods of EMG recording in monkeys.”

Monkeys are used to study muscle and brain activity

Macaque monkeys are used for the study of movement control and coordination. Experiments typically involve evaluating the electrical activity of skeletal muscles using electromyography. Current methods for electromyogram (EMG) recording from the arm and hand involve wires fed subcutaneously from electrodes in the muscles of interest to a connector surgically implanted on the animal’s back or head. A recording device is plugged into this connector to make measurements, which are transferred to a computer for analysis. The electrodes can yield high quality recordings for over one year.

Methods for recording muscle activity can lead to infections

The presence of the connector prevents the skin from healing fully, which means that the animal can be prone to infections that can track down the electrodes and can be difficult to treat. As well as affecting animal welfare, the infections can also compromise the quality of behavioural data obtained, due to the clinical malaise suffered by the monkey. Very severe infections can require the animal to be euthanised, prematurely ending an otherwise productive experiment and requiring another animal to be used to complete the research.



Circuitry of wireless recording device

A telemetry device for EMG recording

Professor Stuart Baker, Newcastle University, was awarded NC3Rs funding in 2006 to develop a radiotransmitter for implantation under the skin, which would be capable of communicating information wirelessly to a receiver outside of the body. An internal device would avoid a permanently open wound, enabling the skin to heal and restore its natural barrier to infection. Similar telemetry devices are already widely used in other types of animal research, but the commercially available systems cannot transmit the large amount of data that is required to enable the effective study of muscle and brain activity.

Pushing telemetry technology to new limits

Working with electronics and bioengineering experts from Newcastle University and University College London, Professor Baker initially developed a prototype device capable of amplifying and digitising up to 16 channels of EMG recording (5kHz sampling rate per channel) and faithfully transmitting rapidly changing signals through the skin by radio. Power is supplied by inductive coupling to an implanted coil antenna, avoiding the need for frequent battery changes and allowing a long implant life without further surgery.

The prototype device has undergone extensive bench testing and trialling in one monkey. It integrated well with tissue, 'powered up' on demand, transmitted data and remained infection free. Although the major engineering challenges of high bandwidth radio transmission, inductive powering, and biocompatible and stable encapsulation and insulation of a complex circuit have been addressed, the signal amplifier integrated circuit did not adequately high-pass filter the recordings, leading to signal drift and saturation. In 2011, Professor Baker was awarded further NC3Rs funding to produce a fully functional telemeter in which the circuit was redesigned using a newly developed amplifier integrated circuit, with high-pass filtering better suited to EMG recording.

The improved device has been tested so far in two animals at collaborating laboratories in London and Fribourg (Switzerland), where it has delivered high quality telemetry data without infection. It has the potential to benefit around 20 monkeys per year globally.

Scale-up to maximise use

Many experiments require signals from inside the body to be transmitted transcutaneously. These are affected by the same problems with infection caused by wired interconnects. The technology therefore has considerable potential to refine experiments, not just in monkeys but also in other species such as rabbits, pigs and cats.

A key aspect of the design is that the radio communication is controlled by a field programmable gate array (FPGA). This allows straightforward reassignment of the available transmission bandwidth according to the requirements of individual experiments. Professor Baker uses the device to transmit 16 channels at 5k samples per second per channel with 12-bit resolution – parameters suitable for EMG recording. By simple changes to the FPGA program, it is possible to transmit four channels at 20k samples per second per channel with 12-bit resolution, which would be suitable for single unit neural recording from the brain. This flexibility greatly increases the possible applications and the aim now is to develop the system commercially.

4 Neuroscience and behaviour

Using facial expressions of pain in animals

Principal Investigator:
Matthew Leach, Lecturer in Animal Science

Organisation:
Newcastle University

Award:
£247,800, in 2012, over 36 months

Title: The assessment of pain using facial expressions in laboratory mice, rats, rabbits and macaques

POTENTIAL 3Rs IMPACT

NO. OF ANIMALS	10 million/year globally
SPECIES	Mouse, rat, rabbit, macaque
SECTOR	All sectors
AREA	Neuroscience & Behaviour – Pain and analgesia
'R'	Refinement

“We are validating the use of facial expressions in rodents and rabbits as a reliable and rapid means of identifying those animals in pain. This could benefit millions of laboratory animals worldwide.”

There is a need for new methods to assess pain in animals

Many research animals undergo painful procedures. In the UK, it is common practice to give analgesia after surgical procedures. The effective alleviation of pain depends on the ability to reliably assess its severity and duration. Traditional methods of pain recognition and assessment that monitor gross behaviour or clinical signs (for example, weight loss) are time consuming to conduct and limited by the fact that they are not specific to pain. They also tend to focus on the animal’s physical reaction to pain (the sensory component of pain), rather than how it makes it ‘feel’ (the emotional component), which is arguably the most critical from an animal welfare perspective.

The grimace scale uses facial expressions for pain assessment

Facial expressions are considered by some to be the ‘gold standard’ method for pain assessment in non-verbal humans, such as newborn babies and people with severe cognitive impairments. Researchers at McGill University (Canada) have identified specific facial expressions in rodents that relate to pain intensity in nociceptive tests. Termed the grimace scale, the facial expressions provide a potential new approach for assessing pain in animals. In 2011, Dr Matthew Leach, Newcastle University, was awarded NC3Rs funding to investigate this further, focusing on rodents, rabbits and macaques.

Validating facial expressions

Facial expressions, such as narrowing of the eyes or fattening of the cheeks, could be easier to use than behavioural indices of post-surgical pain such as back arching, belly pressing and flank twitching, since all of the indicators are focused in one small area – the face. Evidence from Dr Leach's previous work suggests that humans have a tendency to focus on an animal's face when assessing pain.

Validating the grimace scale in mice following surgical and other procedures

Vasectomy is carried out as a routine procedure in most facilities that produce genetically modified mice. Using a combination of high-speed cameras, high-definition video footage, and manual and automated behavioural scoring systems, Dr Leach has validated the Mouse Grimace Scale (MGS) for the assessment of pain following vasectomy via the scrotum in CD1 mice*. This demonstrated that the MGS provides a quick and reliable 'cage-side' means of assessing (or 'scoring') the severity and duration of pain associated with vasectomy with minimal training required. It takes only one hour to complete the scoring of 18 animals pre- and post-operatively using the MGS compared with 18 hours using behavioural indicators.

Importantly, the scale is also sensitive enough to assess the effectiveness of two routinely used analgesics, meloxicam and bupivacaine.

Dr Leach is currently evaluating the MGS for assessing chronic pain in two mouse cancer models – a bladder tumour model in C3H mice, and Ewing's Sarcoma model in Rag-2 immunodeficient mice induced by intravenous implantation, with the aim of developing improved humane endpoints.

A grimace scale for rabbits

Dr Leach has developed the Rabbit Grimace Scale (RbtGS). The scale was produced using data from a study commissioned by the Swedish Board of Agriculture, comparing clamp tattooing of the ear, a procedure commonly used to identify rabbits farmed for meat production in Europe, with and without local anaesthesia. Like the rodent grimace scales, the RbtGS is based on changes in a number of 'facial action units', such as orbital tightening, and bulging or flattening of the cheeks and nose. Changes in these action units reliably indicate acute pain following tattooing without anaesthesia and correlate with physiological signs of stress, such as increased heart rate. The RbtGS is now being validated for scoring pain following ovariohysterectomy.

*Data was collected from mice undergoing vasectomy for routine genetic modification programmes and not specifically for the purpose of this study.

Using facial expressions to assess pain in non-human primates

There is currently no objective method of assessing post-operative pain in non-human primates. Dr Leach is working on a grimace scale for rhesus macaques using an already established research tool for analysing facial movement – the Macaque Facial Action Coding System. This is analogous to a human system used to describe and understand facial expressions and their role in communication.

Expanding the use of the grimace scale

There have been two publications to date. Dr Leach has given over 20 lectures on his work, including at the annual congresses of the American Association of Laboratory Animal Science, Australian and New Zealand Laboratory Animal Association, and Chinese Veterinary Medicine Association. New collaborations have been established to develop grimace scales for mice used as models of pancreatic cancer, neurotrauma and multiple sclerosis with scientists at Cancer Research UK, Queen Mary, University of London and the Babraham Institute respectively.

In addition, collaborations are under way to develop grimace scales for other species including lambs (Massey University, New Zealand) and horses (University of Milan, Italy).

4 Neuroscience and behaviour

Improving rodent welfare during euthanasia

Principal Investigator:
Huw Golledge, Senior Research Associate

Organisation:
Newcastle University

Award:
£295,595, in 2009, over 36 months

Title: Assessing the humaneness of gas euthanasia techniques for laboratory rodents

POTENTIAL 3Rs IMPACT

NO. OF ANIMALS	100 million/year globally
SPECIES	Mice, rats
SECTOR	All sectors
AREA	Neuroscience & Behaviour – Animal welfare
'R'	Refinement

“Putting these research findings into practice could reduce the suffering of an estimated 100 million rodents that are euthanised per year globally.”

There is a lack of consensus on which inhalational euthanasia methods are most humane

Worldwide, an estimated 100 million mice and rats are used every year in scientific research. The majority are killed via inhalation euthanasia, with carbon dioxide (CO_2) being the most commonly used agent. A number of studies, however, suggest that CO_2 is aversive to rodents, and may even cause acute pain at high concentrations. In mice CO_2 can directly stimulate the amygdala and induce fear-like behaviours at around 10% concentration. Since rodents must be exposed to approximately 30% CO_2 before consciousness is lost this raises serious welfare concerns.

Whether there is an alternative to CO_2 which is both more humane and practical is unclear. Volatile anaesthetics, such as isoflurane, have been used for rendering animals rapidly unconscious prior to being killed with CO_2 or by a physical method of euthanasia. However, all volatile anaesthetics so far tested appear to also cause aversion in rats and mice. Key questions remain on whether isoflurane is less aversive than CO_2 and if not are there other strategies which allow CO_2 to be used more humanely in rodents. To investigate these questions, Dr Huw Golledge, Newcastle University, was awarded the first NC3Rs strategic grant in 2009.

Apparatus for conditioned place aversion studies

CO_2 and isoflurane are similarly aversive

Dr Golledge has used conditioned place aversion (CPA) to investigate the relative aversiveness of CO_2 and isoflurane. This is the first time CPA has been used to investigate how rodents 'feel' during the euthanasia process. It is possible to test with CPA whether animals avoid areas where they have previously been exposed to CO_2 or isoflurane, thus providing a measure of whether the animals remember their aversion in the absence of the euthanasia agent. Using this approach, Dr Golledge has demonstrated that both CO_2 and isoflurane are aversive, with no statistically significant difference in the level of aversion between the two agents.

Argon is more aversive than either CO_2 or isoflurane

Non-anaesthetic gases such as argon and nitrogen have also been proposed as alternatives to CO_2 and are listed as permissible euthanasia agents for rodents under Directive 2010/63/EU on the protection of animals used for scientific purposes.

Dr Golledge has shown that argon is significantly more aversive to rats than either CO_2 or isoflurane. As a result of this, argon is not in the list of approved methods for euthanasia in Schedule 1 of the amended Animals (Scientific Procedures) Act 1986.

Euthanasia in the home cage with isoflurane may be the best option

The stress associated with euthanasia is likely to arise from a combination of the physiological consequences of exposure to euthanasia agents with procedural factors, such as moving animals to an unfamiliar environment. A reduction in stress may be possible if agents are applied in the animals' home cage, or if euthanasia is timed to occur during periods of minimal ambient stress, such as when the animals are resting or sleeping. This approach would be particularly relevant for animals housed in individually ventilated cages, since these sealed cage units lend themselves to being filled with gaseous agents.

To investigate this, euthanasia was carried out with CO_2 or isoflurane in three scenarios, first in empty cages into which mice were placed immediately prior to euthanasia (this is analogous to the normal procedure), second in the animals' home cage whilst they were sleeping, and third in the home cage whilst the animals were awake. The studies showed that of the different scenarios the use of isoflurane administered to sleeping mice may represent the most humane method as the time spent awake prior to losing consciousness (and exposed to an aversive agent) was shortest, thus minimising the duration of any stress or distress.

Addition of nitrous oxide shortens the time to loss of consciousness

Nitrous oxide, commonly known as 'laughing gas', is used in man to accelerate the induction of anaesthesia with volatile anaesthetic via a mechanism referred to as the 'second gas' effect. Dr Golledge has shown that the addition of nitrous oxide to isoflurane or to a rising concentration of CO_2 reduces the time to loss of the righting reflex in mice by 17.6% and 10.3% respectively, without increasing stress-related behaviours. This provides a potential refinement of the euthanasia process by shortening the time to loss of consciousness.

Promoting a change in euthanasia practice

The research has provided an evidence base for improving animal welfare during euthanasia. Dr Golledge has disseminated his work to an international audience in Europe, the USA, Canada and Australia, including the plenary lecture at the 2012 annual conference of the Australian and New Zealand Laboratory Animal Association.

With NC3Rs sponsorship, Dr Golledge has also organised an international meeting to disseminate the research results to key stakeholders to ensure that they contribute as rapidly as possible to improvements in practice. The meeting also provided an opportunity for sharing data on euthanasia methods for neonatal rodents and fish. There have been three publications arising from the grant to date and three new collaborations established with research groups at Newcastle University, Fera (an executive agency of Defra) and the University of British Columbia (Canada).

5 Pharmaceuticals and chemicals

Better genotoxicity assays to reduce animal use

Principal Investigator:
Richard Walmsley, Professor of Genetics

Organisation:
University of Manchester and Gentronix Limited

Award:
£133,012, in 2006, over 12 months

Title: Development of a new human cell genotoxicity assay to reduce the use of live animals in drug development

POTENTIAL 3Rs IMPACT

NO. OF ANIMALS	500,000/year globally
SPECIES	Rats, mice
SECTOR	Pharma, Chemicals, Consumer Products
AREA	Pharmaceuticals & Chemicals – Toxicology
'R'	Reduction

“Over 100 companies worldwide are now using our human cell-based assay to reduce the number of animals used to distinguish safe compounds from genotoxic ones.”

Genotoxicity assays have a high incidence of false positive results

It is a legal requirement that new substances such as pharmaceuticals, chemicals and consumer products are screened for their potential to cause cancer by genotoxic and non-genotoxic mechanisms. The standard regulatory test battery includes an assessment of genotoxicity in bacterial and mammalian cells *in vitro*. The assays are highly sensitive but have poor specificity and consequently many substances (historically over 50%) are erroneously identified as potential carcinogens (so-called false or misleading positives).

A positive result in an *in vitro* test can trigger extensive mechanistic studies, usually in rats and mice, to confirm the genotoxic potential of the substance and assess the relevance of the result in terms of exposure. False positives therefore drive the use of animals. In 2006, Professor Richard Walmsley, University of Manchester, was awarded NC3Rs funding to support the development of a mammalian cell-based genotoxicity assay with high specificity and a low rate of false positive findings.

Development of a new human cell-based assay

Professor Walmsley had previously developed the GreenScreen HC assay. This is a human cell-based reporter assay which exploits elements of the human GADD45a gene coupled to the gene encoding green fluorescent protein (GFP). Expression of GADD45a is up-regulated in response to genotoxic stress, and DNA damage therefore leads to increased cellular fluorescence through the production of GFP, which can be detected spectrophotometrically.

With NC3Rs funding, Professor Walmsley has been able to further develop and test the GreenScreen HC assay. This has included validation of a flow cytometric method, which allowed the development of the S9 version of the assay that tests for compounds requiring metabolic activation to become genotoxic. There are now published GreenScreen HC validation data for 161 compounds with *in vivo* genotoxicity data and 129 compounds with carcinogenicity data.

A high specificity genotoxicity assay reduces the use of animals

The GreenScreen HC assay is unique in two respects. Firstly, it detects all mechanistic classes of genotoxicants, including those which cause mutations, chromosome breakages and abnormal chromosome numbers. It therefore provides a single test for genotoxic hazard screening. This is in contrast to current regulatory assays, such as the Ames test and mouse lymphoma assay, which only identify mutagens and hence the requirement for a test battery approach. Secondly, in addition to its high sensitivity (89%) to genotoxic carcinogens, GreenScreen HC also has a high specificity (95%) – a much lower false positive rate than other routinely used tests.

For each positive identified in a genotoxicity assay, up to 200 animals are used for follow-up investigation. The improved specificity of the GreenScreen HC assay has had a significant impact with around 500,000 fewer animals used globally each year for genotoxicity studies in the pharmaceutical and chemical sectors.

The assay has a 96-well microplate format suitable for high-throughput screening. Four compounds are tested at nine serial dilutions per plate, and results are available within 48 hours. Small amounts of compound are required and this allows the assay to be used to make internal decisions early in development, thus preventing animals being used unnecessarily for other studies. To realise the full potential of GreenScreen HC, however, particularly in terms of reducing animal use, it needs to be approved for regulatory purposes. Formal validation and acceptance by regulatory authorities is essential. Currently the only genotoxicity assays that have this status are the bacterial and mammalian assays, which have lower specificity than GreenScreen HC. In April 2013 Gentronix submitted a case for retrospective validation to the European Centre for the Validation of Alternative Methods.

Global uptake across the pharmaceutical and chemical sectors

The GreenScreen HC assay has been commercialised through the spin-out company Gentronix, which was founded by Professor Walmsley with the support of the University of Manchester. The assay has been used by around 100 companies worldwide in diverse sectors including pharmaceuticals, agrochemicals and consumer products. Close to 12,000 compounds have now been assayed. Gentronix has also introduced the BlueScreen HC, with the GADD45a gene coupled to the *Gaussia* luciferase reporter gene. Using the Gentronix assay as part of an *in vitro* screening cascade, GlaxoSmithKline has effectively removed attrition due to candidate drug genotoxicity, saving approximately 300 animals per year.

There have been five publications from the NC3Rs-funded research.

5 Pharmaceuticals and chemicals

Testing chemicals for endocrine disruption using fewer fish

Principal Investigator:
Ioanna Katsiadaki, Science Leader – Aquatic Animal Health

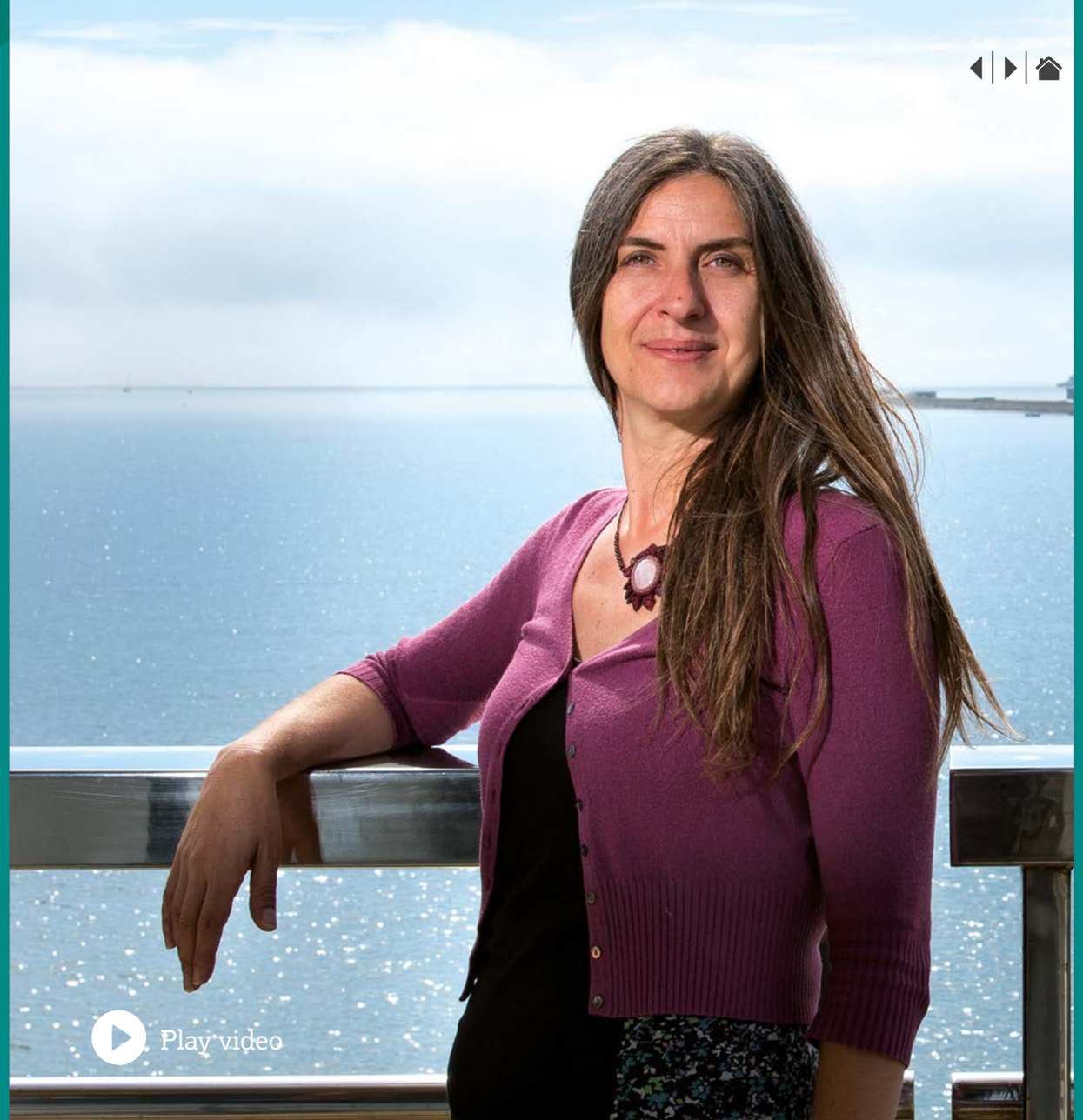
Organisation:
Centre for Environment, Fisheries and Aquaculture Science (Cefas)

Award:
£398,680, in 2008, over 36 months

Title: Validating a sexual development test using the 3-spined stickleback for addressing the 3Rs in fish toxicity testing

POTENTIAL 3Rs IMPACT

NO. OF ANIMALS	20,000/year in Europe
SPECIES	Fish
SECTOR	Government, Chemicals
AREA	Pharmaceuticals & Chemicals – Ecotoxicology
'R'	Reduction



"The use of the stickleback instead of other fish species will result in a 50% reduction in the number of fish used for testing the effects of chemicals on sexual development."

Endocrine disrupting chemicals can affect sexual development

Endocrine disruptors are chemicals that when absorbed into the body either mimic or block hormones. Many chemicals, including pharmaceuticals, pesticides and plasticisers are endocrine disruptors. There is concern that exposure to endocrine disrupting chemicals (EDCs) in the environment affects the sexual development of humans and wildlife. EDCs are of major concern for fish, as the aquatic environment is an important sink for chemicals and sewage waste. Steroidal oestrogens such as ethinyl-oestradiol – the active ingredient of the human contraceptive pill – are implicated, albeit not exclusively, as causing the widely observed sexual disruption in wild fish.

Sexual development in fish is used as a screen for endocrine disruptors

Sexual development in fish is easily altered by endocrine disruptors and consequently they are used for screening chemicals for this purpose. Between 2007 and 2011, under the management of the Organisation for Economic Co-operation and Development (OECD), the Fish Sexual Development Test (FSDT) was assessed as a potential test guideline for detecting early life-stage effects and potential adverse consequences of putative EDCs on sexual development.

In the test, fish are exposed to the test chemical, from the fertilised egg stage until the completion of sexual differentiation. The fish are then euthanised. Two core endpoints are measured post-mortem as indicators of endocrine-associated developmental aberrations. This includes the concentration of vitellogenin (a precursor protein of egg yolk) and the proportions of each sex determined via histological examination of the gonads (the phenotypic sex ratio). Whether the chemical causes full or partial sex reversal is determined by comparing the sex ratios in groups of fish exposed to the chemical with control groups.

Phenotypic sex ratios in fish can be highly variable

In the FSDT if the genetic (actual) sex of the fish is unknown it is assumed that the normal sex ratio of males to females is approximately 50:50, with any deviation being a result of the test chemical. However, sex ratio in fish is naturally highly flexible and subject to modification by external factors, including temperature. It is not unusual to have a sex ratio of 70:30 males to females or *vice versa*, and this means that a large number of fish must be tested to provide conclusive information to attribute any change to chemical exposure rather than random variation.

The availability of a genetic marker of sex would allow fewer animals to be used by providing unequivocal information on the actual sex ratio and therefore an improved basis to determine whether any change in ratio is a result of the test chemical. It would also have the advantage of allowing better definition of the mode of action of the chemical as an oestrogen or an androgen for example, since any differences between the genetic sex and the phenotypic (developed) sex can be attributed to chemical exposure.

Around 90% of fish do not have a known genetic marker for sexual determination. This includes the zebrafish – the original species of choice for validation of the FSDT. A sex determining region has been mapped to linkage group 19 in the three-spined stickleback and in 2007, Dr Ioanna Katsiadaki, Cefas, was awarded an NC3Rs grant to validate this species for the FSDT.

Validation of three-spined stickleback as a suitable test species

In order to establish that the three-spined stickleback could be used for the FSDT, Dr Katsiadaki, with collaborators from Brunel University and Leicester University, tested a series of endocrine-active reference chemicals including oestrogens, androgens and anti-androgens. The stickleback test showed high reproducibility between laboratories and comparable sensitivity in detecting compounds with endocrine activity to other fish species using the FSDT. The majority of chemicals tested in the three-spined stickleback caused gonadal intersex (that is the presence of both male and female gametes in a single gonad). This was not observed in the control fish, increasing the overall reliability of the test compared to species where gonadal intersex is more commonly seen in controls.

International regulatory acceptance with reduced fish use

The FSDT was published as an official OECD test guideline (TG234) in 2011. The three-spined stickleback is one of the three recommended species, together with the zebrafish and Japanese medaka.

The study design for the OECD validation exercise tested five concentrations of each chemical, with four replicates of 40 fish for each concentration. Each study therefore used 800 fish. Based on experience of the test during validation, Dr Katsiadaki was able to provide a case to reduce the number of fish regardless of species by 25% to 600 per chemical and this is incorporated in the final test guideline. The use of the three-spined stickleback allows the number to be reduced by a further 25% per chemical.

The full impact of the FSDT will be realised with the REACH chemicals legislation which is driving the testing of chemicals for endocrine disruption. As of September 2013, 10,000 chemicals were registered with the European Chemicals Agency for the purposes of REACH. If only 0.5% of these are tested, the FSDT will avoid the use of around 10,000 fish (and 20,000 if the stickleback is used).

The results from this grant have been published in the OECD validation report for the FSDT.

Three-spined stickleback eggs *in vitro*

5 Pharmaceuticals and chemicals

New assays for botulinum toxin testing

Principal Investigator:
Dorothea Sesardic, Principal Scientist

Organisation: National Institute for Biological Standards and Control (a centre of the Medicines and Healthcare Products Regulatory Agency)

Award:
£337,708, in 2010, over 36 months

Title: Development of cell-based assays as replacement assays for botulinum toxins and antitoxins

POTENTIAL 3Rs IMPACT

NO. OF ANIMALS	70,000/year in the UK
SPECIES	Mice
SECTOR	Government, Pharma
AREA	Pharmaceuticals & Chemicals – Biologics
'R'	Replacement

"Our new cell-based assays for the detection of botulinum toxins could replace the use of around 70,000 mice each year in the UK."

Botulinum toxins are increasingly used as medical and cosmetic products

Botulinum toxin, one of the most acutely poisonous proteins known, is a neurotoxin produced by the bacterium *Clostridium botulinum*. There are eight serotypes which cause muscle paralysis by blocking the release of the neurotransmitter, acetylcholine.

In very small quantities, botulinum toxins can be used therapeutically to prevent muscle spasms and are increasingly available as licensed drugs for the treatment of a variety of disorders, such as dystonia (a neurological movement disorder), blepharospasm (involuntary eye muscle contractions) and hyperhidrosis (excessive sweating). 'Botox' injections are also used for cosmetic purposes to temporarily paralyse facial muscles to smooth the skin.

Batch testing involves thousands of mice

Before they can be used in humans, each batch of botulinum toxin is tested for safety, potency and stability, because the toxin is a biological product with inherent variability. These tests are required at a number of stages of the production process in order to meet regulatory requirements before marketing authorisation is granted. Typically, each batch is tested by both the manufacturer and the national competent authority.

Worldwide, the most commonly used test is the mouse lethal dose 50 (LD50) assay. This assay involves injecting groups of mice with dilutions of the botulinum toxin to determine the dose that will kill half of the animals at a defined time-point. The test causes severe suffering as the toxin induces paralysis of the respiratory muscles. In the UK, around 70,000 mice are used each year for botulinum toxin testing; globally the figure is estimated to be more than 600,000.

Current *in vitro* alternatives have limitations

The UK's National Institute for Biological Standards and Control (NIBSC), which is a centre of the Medicines and Healthcare Products Regulatory Agency, has been part of a global effort to develop alternatives for *in vitro* botulinum safety and potency testing. Although good progress has been made many of the current tests are limited because they fail to mimic all of the mechanisms that contribute to the toxin's action *in vivo*. In 2010, Dr Dorothea Sesardic, NIBSC, was awarded NC3Rs funding to develop improved cell-based assays for botulinum toxin serotype A, which is licensed for use in therapies.

Improved functional assays for botulinum toxin testing

Mouse embryonic stem cells (E14Tg2a), human neuroblastoma cells (SH-SY5Y) and commercially sourced neurons derived from human induced pluripotent stem cells have been differentiated into mature neuronal cells and fully characterised for their response to various concentrations of botulinum toxin using a range of functional assays such as electrical activity, synaptic vesicle trafficking and specific immunodetection of intracellular target proteins.

These assays offer the potential for the complete replacement of the mouse studies because their mode of action involves all of the key hallmarks of botulinum neurotoxicity.

Botulinum toxins contain enzymes that breakdown proteins essential for neurotransmitter release. The new assays include a highly sensitive measure of the cleavage of one of these, the presynaptic plasma membrane protein SNAP-25, which is selectively cleaved by botulinum neurotoxin (BoNTs) serotypes A, C and E. Dr Sesardic has demonstrated that incubation of the E14Tg2a-derived neurons with extremely low levels of purified BoNT/A in the presence of high concentrations of stabilising proteins and other excipients that are found in therapeutic products, results in dose-dependent breakdown of SNAP-25. The assay is specific to BoNT/A and the next step is to establish if the assay can be adapted for toxin neutralisation studies.

Dissemination, collaborations and further funding

There have been three publications arising from this grant to date. Collaborations have been established for sharing botulinum toxin sub-serotypes, cells and expertise, with the MRC Centre for Developmental Neurobiology, MRC Laboratory of Molecular Biology, Wellcome Trust Centre for Stem Cell Research, and members of an EU-funded consortium (called AntiBotABE) aimed at developing neutralising antibodies against botulinum toxins A, B and E.

The success of the initial NC3Rs-funded work has led to internal funding from NIBSC for a pilot project to initiate development of a cell-based assay for tetanus toxin. In addition, Dr Sesardic is leading a team to apply the 3Rs to the control of biological medicines, funded as part of a £5 million grant-in-aid to the NIBSC Regulatory Research Unit from the UK Department of Health.

6 Appendices

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6 Appendices

Summary of NC3Rs funding schemes

Project grants
Response mode scheme for hypothesis driven and applied research

Duration:	Up to 36 months
Amount:	Dependent on the science; typically in the region of £330k
Key dates:	Call opens in September; deadline in February; decisions in July
Further information:	www.nc3rs.org.uk/projectgrants

Pilot study grants
Response mode scheme for proof-of-concept studies

Duration:	Up to 12 months
Amount:	Up to £75k
Key dates:	Call opens in September; deadline in February; decisions in July
Further information:	www.nc3rs.org.uk/pilotstudygrants

Strategic grants
Targeted calls in specific research areas

Duration:	Up to 36 months
Amount:	Dependent on the science; typical budget of £500k per call
Key dates:	Usually one call per year with varying deadlines
Further information:	www.nc3rs.org.uk/strategicawards

Infrastructure for Impact grants
Non-research scheme for infrastructure, equipment and networking

Duration:	Up to 36 months (exceptionally up to 60 months)
Amount:	Up to £500k (£250k for equipment only grants)
Key dates:	Usually one call per year with varying deadlines
Further information:	www.nc3rs.org.uk/infrastructure

PhD Studentships
To embed the 3Rs in the training of graduate scientists

Duration:	36 months
Amount:	Cash-limited award of £30k per annum
Key dates:	Call opens in April; deadline in July; decisions in December
Further information:	www.nc3rs.org.uk/studentships

David Sainsbury Fellowships
To support exceptional early career scientists with the transition to independent researcher

Duration:	36 months
Amount:	Cash-limited award of £65k per annum
Key dates:	Call opens in September; deadline in November; decisions in April
Further information:	www.nc3rs.org.uk/fellowships

CRACK IT Challenges

To solve scientific and business problems with a 3Rs theme identified with industry partners

Duration:	Up to 36 months
Amount:	Dependent on the science; from £50k to £1m
Key dates:	Call opens in September; deadline in November; decisions in December
Further information:	www.crackit.org.uk/crack

CRACK IT Solutions

To connect technology developers with new partners, users and markets

Duration:	Up to 12 months
Amount:	Up to £30k
Key dates:	Usually four calls per year with varying deadlines; decisions within one month
Further information:	www.crackit.org.uk/share

Acronyms:

3Rs:	Replacement, reduction and refinement of animals in research
AntiBotABE	Neutralising antibodies against Botulinum toxins A,B,E
BBSRC:	Biotechnology and Biological Sciences Research Council
CRUK:	Cancer Research UK
EPSRC:	Engineering and Physical Sciences Research Council
ESP KTN:	Electronics, Sensors, Photonics Knowledge Transfer Network
MRC:	Medical Research Council
RCUK:	Research Councils UK
TARGETFISH:	Targeted disease prophylaxis in European fish farming
UKEMS:	UK Environmental Mutagen Society

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