Working Group on Sex in Experimental Design of Animal Research

Meeting Report
6 September 2021

UKRI Medical Research Council

NC3Rs National Centre for the Replacement Refinement & Reduction of Animals in Research

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Contents

Cover image credit: Brown mice at the MRC Toxicology Unit

Aims

The aim of this meeting was to agree the key features of a policy on the inclusion of sex in experimental design for animal, tissue and cell research that would be feasible, enforceable and improve MRC-funded research quality, and to explore how the policy might be implemented. Experts in animal, tissue and cell research, experimental design and statistics were brought together to address the following objectives:

(i) **Community Perceptions:** Discuss the recent community survey conducted by MRC on benefits and challenges of addressing sex in research questions, experimental design, and analysis.
(ii) **Current Practice:** review MRC grant and fellowship applications to understand how applicants currently address sex in research questions, experimental design, and analysis.
(iii) **Implementation and evaluation:** Discuss implementation of the policy, including identifying the barriers to adoption and how they might be overcome, and what evaluation might be needed to review the policy's impact.
(iv) **Recommendations:** Considering the above, discuss draft recommendations for an MRC policy on the inclusion of sex in experimental design for animal, tissue and cell studies.

Membership

Working Group Chair
Mandy MacLean, University of Strathclyde, Populations and Systems Medicine Board (PSMB) Member

Working Group Members
Elizabeth Fisher, UCL, Neurosciences and Mental Health Board (NHMB) Member

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Observers

Stephanie Masefield – Senior Policy Manager, BBSRC

Summary of recommendations

The Working Group recommendations for MRC policy are summarised as follows:

- MRC should require sex to be specified and justified in the experimental design of grant applications involving animals, and human and animal tissues and cells.

- MRC should require applicants to specify sex for human or animal tissues and cells used in experiments, and if not known, for it to be determined.

- MRC should expect both sexes to be used in experiments involving animals, human or animal tissues or cells, unless there is robust justification for not doing so.

- MRC should expect applicants to plan their statistical analysis to take sex into consideration.

- MRC should encourage but not require that these principles be applied to immortalised cell lines.

- The choice of model, genetic background, and age need to be clearly justified, even if both sexes are used.

- Pre-defined situations justifying the use of only one sex should be: cases of acutely scarce resources (e.g. human tissue samples from rare diseases), purely molecular studies (such as protein-protein interactions) and/or sex specific conditions or phenomena (e.g. ovarian cancer). Other exceptions may be considered on a case-by-case basis.

- Variability due to the oestrous cycle should only be accepted as a justification in limited circumstances.

- Use of only one sex in previous work from the researchers or others is not sufficient as justification for further single sex experiments.

- Evidence of the absence of a sex difference (for example, in the biological pathway being studied), is not sufficient to justify using only one sex, but may be considered in support of other reasons during the peer-review process. The lack of data regarding sex differences does not indicate there are none, and thus is not the same as good evidence that there are no sex differences.

- MRC communications should emphasise:

    - Existing MRC position on costs
    - NC3Rs support and alignment on policy change
    - Countering misconceptions on female variability
    - Countering misconceptions on sample size
    - Promoting resources to help applicants with experimental design
    - Promoting resources to help applicants with data analysis
    - Global context of other funder policies

Summary of discussion

(i) Definitions and background

The Working Group were asked to consider whether MRC should pursue the integration of sex into its existing experimental design framework.

- **Sex** refers to the biological attributes of humans and animals that differentiate male, female and intersex, including chromosomes, gene expression, hormone levels and function, and reproductive organs. There is variation in the presentation of different biological components of sex.
- **Gender** is distinct from sex and refers to the attribution of behaviours, expectations, and roles to different sexes in humans, therefore may vary over time and by social and cultural context. There is diversity in how individuals and groups experience and express gender.

This report (and the Working Group discussion) was limited to sex. The Working Group was encouraged to consider appropriate policy within the scope determined by research types for which sex was the relevant biological variable: in basic and pre-clinical research involving animals, animal and human tissues, and cell lines. Research involving human participation was excluded as the policy considerations are different.

(ii) Community perceptions

The Working Group considered the results of a recent MRC survey of 800 health and medical science researchers and clinicians on their perspectives on diversity in

research. 33% of respondents performing animal research used only one sex of animal. Only 30% of these are researching a single sex disease or mechanism. The other most common justifications for using only one sex of animal were to reduce costs (22%) and to reduce animal numbers (35%).

95% of animal users and 88% of cell users saw benefits to considering sex and other aspects of diversity in their experimental design. The most commonly identified benefits were translatability, reproducibility, and novelty of results. However, most respondents could also see drawbacks (92% and 82%, for animals and cells respectively). Cost and complexity of experimental design were the most common perceived drawbacks of considering sex. Animal researchers were concerned about the larger number of animals needed, while researchers using cells were concerned about the ability of commercial suppliers to provide information about sex.

99% of respondents felt there was a need to develop guidance about sex in animal research, and there was a high level of support (74%) for funders taking on this role. Most respondents (79%) wanted any new policy to be supported by written guidance for peer reviewers and applicants.

The Working Group were encouraged by the widespread understanding in the community that considering sex has an impact on reproducibility and translatability of research. The group agreed that the results supported MRC taking a leading role, as other funders outside the UK have adopted such policies and these have been widely accepted. However, they emphasised that careful messaging and other MRC support would be required to combat the widespread perception of drawbacks.

(iii) Current and Future Practice

Evaluation of Current Practice

The Working Group considered the results of an evaluation exercise performed by the office, which analysed applications involving animal research that were submitted to MRC boards and panels in May-June of 2020. Applications were assessed as to whether they specified the sex of animals to be used, and whether that choice was explained. Several in-detail case studies of example applications were also provided to the group for consideration.

MRC's current Guidance to Applicants encourages the inclusion of relevant information about the animals that applicants plan to use (e.g. species, strain, sex, developmental stage, weight) in the Je-S application form section on animal species. When analysis was conducted, however, it was found that only 44% of grant applications submitted to the MRC specify what sex of animal they are using. Accepted grants were no more likely to specify sex than grants that had been rejected, and less likely to justify their choice (26% vs 49%). 34% of those that reported sex information planned to use a

single sex of animal. Members expressed concern that applications had been funded without sex information being present, and without the issue being raised at triage. Information on sex is not currently requested from applications involving the use of cells.

It was noted that NC3Rs' update to the ARRIVE Guidelines in 2020 involved a shift from the original guidance that sex must be reported for all animal experiments, to now requesting 'species appropriate' details for the animals used. The new guidance might not require sex information be reported for embryos or juvenile fish and invertebrates.

Working Group recommendations about future policy

The Working Group advised that **it was always appropriate to include consideration of sex in grant applications involving animals** and that the current practice was not acceptable. It was agreed that an explicit policy was necessary to drive change. While good experimental design might take account of many variables, the Working Group agreed that as females account for 50% of the population, this variable particularly needs to be addressed. Sex is also significantly easier to explore than other non-binary variables such as age or genetic background. The importance of ensuring MRC funded research is relevant to as much of the population as possible was emphasised. Members commented that the literature precedent of performing single sex studies was a factor in holding up change.[1]

The Working Group agreed that the choice of what sex of animal to use is dependent on the research question. However, the **default position should be to use both sexes of animal unless there is a reason not to**. In either case, the choice should be appropriately justified and clearly explained.

Sex should be considered in research at the cellular and tissue level as well as at the level of the whole organism, and the biological considerations that apply for human cells and tissues are similar to those in animal research. While additional factors (e.g. socio-economic status, gender) might be relevant for studies involving humans, it is less likely that these would be generally applicable or known (given the limited information available from a tissue supplier) for all studies involving human cells or tissues. It was therefore agreed that **the principles for animal studies should also apply to human or animal tissues and cells, particularly primary cell cultures, organoids, embryonic and induced pluripotent stem (IPS) cells, with exception of immortalised cell lines**, but not to human participant studies. If unknown, **the sex of cells should be determined**. The sex of most commonly used immortalised cell lines is already known and should be stated.[2] If unknown, these should be determined (for

1. Karp, N, Reavey, N, Sex bias in preclinical research and an exploration of how to change the status quo https://bpspubs.onlinelibrary.wiley.com/doi/full/10.1111/bph.14539

[2] Shah K, McCormack CE, Bradbury NA. Do you know the sex of your cells?. *Am J Physiol Cell Physiol*. 2014;306(1):C3-C18. doi:10.1152/ajpcell.00281.2013

example by PCR-based methodology or karyotyping). Researchers should be encouraged but not mandated to use immortalised cells of both sexes.

Accounting for sex throughout the research

Working Group members discussed that early accounting for sex should become the default position when considering research question and subsequent study design. It should be considered during data collection, analysis of results and reporting of findings. The intention is not to mandate use of any specific experimental design or a defined statistical analysis but to encourage the application of appropriate design and statistical analyses based on the research question and the scientific context.

For example, factorial statistical design allows examination of both the primary intervention variable (e.g. a genetic knock-out or an environmental change) and sex, and tests whether the effect of the intervention depends on sex (the interaction). With this design, if the interaction is a large effect it can be detected without the need to increase the sample size. This approach will allow the researcher to assess the generalisability of the findings. For this assessment, the sample size would generally be similar to that needed for a single sex study.

This policy does not mandate that researchers design experiments to test for the interaction between the intervention/treatment and sex. When testing an interaction, the sample size will usually be larger. Where there is already evidence of a biologically significant interaction between sex and the intervention, it might be a priority to explore it, but it was agreed it should remain a question for applicants and peer reviewers to determine if this is appropriate.

It is particularly important that, when using both sexes, researchers do not pool the data across the sexes but include sex in the analysis. Pooling data can lead to increased unexplained variance and reduce the statistical power of the experiment, while missing the primary benefit of using both sexes (determining if there is difference in the studied effect between sexes). **MRC should therefore expect that studies be designed to allow for collection of data by sex and analysed taking sex into account.** This is in line with NIH policy which asks researchers to treat sex as a biological variable (to plan their experiments with the aim to, at a minimum, include both sexes and collect data which can be analysed by sex).[3]

Animal numbers, cost and 3Rs implications

Using both sexes of animals, tissues and cells may raise logistical, ethical and cost issues. For animals, more complex housing and care arrangements might be required, increasing costs beyond a simple proportionate increase in animal numbers. Singly-housing animals, as might be required for instance for some adult male animals, might have ethical (increased suffering to the animal) and cost implications. It is appropriate to

[3] Consideration of Sex as a Biological Variable in NIH-funded Research Guidance https://orwh.od.nih.gov/sites/orwh/files/docs/NOT-OD-15-102_Guidance.pdf

take these issues into account when considering the benefit of using both sexes of animals in an individual grant.

The Working Group suggested that there may be concerns about aggressive behaviours in mature male mice.[4] Resources to help researchers potentially reduce this issue are available. This may include strategies to maintain stable social groups after maturity, such as the transfer of odour cues from the nesting area during cage cleaning or applying nesting material as environmental enrichment.

Current MRC policy does not cap the cost of individual grants. The emphasis is on justifying the expense and demonstrating value for money. However, there is a common perception among applicants that more expensive grants are less likely to be funded, which would need to be addressed through **targeted communication to applicants, peer reviewers and board and panel members at the roll out of the new policy.**

It is important to note that the use of both sexes of animal is not in conflict with 3Rs principles even if additional animals are required, as in that case additional information would have been gathered by the experiment and the results could be more widely applicable. The current community perception that 'reducing' animal use means using the absolute lowest number of animals in a given experiment is inaccurate. The 'reduce' principle is defined as using the lowest number of animals, to give an appropriately designed and analysed animal experiment that is robust and reproducible, and truly adds to the knowledge base.[5] **It is in accordance with 3Rs principles to use additional animals if, when using both sexes, more widely applicable and informative experimental data are acquired. The scientific community should be reassured of these principles.**

As discussed above, there might be ethical issues involved in using both sexes in a particular model, or for a particular experiment (i.e. long durations of single housing), that would make 3Rs principles a relevant consideration for not using both sexes. This would need to be explained in the application in accordance with the policy on justifications given below.

Potential Justifications for not using both sexes

The Working Group agreed that there were several notable situations, for example as mentioned in the NIH policy, that would justify the use of only one sex. **They include: cases of acutely scarce resources (e.g. human tissue samples from rare diseases), purely molecular studies (such as protein-protein interactions), and/or sex specific conditions or phenomena (e.g. ovarian cancer).** The Working Group noted that the mere lack of evidence regarding sex differences (i.e. where there are no

[4] Lidster, K., Owen, K., Browne, W.J. *et al.* Cage aggression in group-housed laboratory male mice: an international data crowdsourcing project. *Sci Rep* **9,** 15211 (2019). https://doi.org/10.1038/s41598-019-51674-z

[5] https://nc3rs.org.uk/the-3rs

data to suggest sex differences are unlikely) is not a good enough justification. Previous work from the researcher or others having been performed in one sex is not sufficient as justification. Evidence of the absence of a sex difference (for example, in the biological pathway being studied), is not sufficient to justify using only one sex, but may be considered in support of other reasons during the peer-review process. **Where other strong justification is provided, this should be considered,** with an awareness that the conclusions from the study would be limited by the inclusion of only one sex**.**

Variability due to oestrous cycle is often inappropriately used to justify using only male animals.[6] MRC's community survey showed that this was given as a reason for only using one sex of animal by 18% of those that did so. Recent large-scale evidence suggests no greater variability in female than male mice for a variety of metabolic and behavioural endpoints of wide interest.[7,8] The Working Group advised that **variability due to the oestrous cycle should only be accepted as a justification in limited circumstances**, such as where there is a known molecular interaction which influences the research endpoint or where supporting data are submitted.

Pilot studies

The Working Group discussed how to treat preliminary or pilot data obtained in both sexes included in the application in an attempt to demonstrate that a single sex is sufficient for future studies. Data showing there is no sex difference is not sufficient as a justification for performing future studies in one sex. In addition, pilot studies are often conducted with inappropriately small sample numbers, and so encouraging their use could lead to missing sex differences with all but very large effect sizes. Requiring additional information from researchers before they have received funding would also disadvantage more junior PIs. It was agreed that this should not be encouraged as a practice.

Messaging and communications

As noted above, community concerns about increases in cost and compliance with 3Rs principles should be addressed as part of the communications strategy. **The misconception that researchers always need to double the numbers of animals in order to use both sexes should be clearly countered***,* with appropriate experimental design and data analysis resources signposted.

MRC should emphasise that considering variables other than sex, such as age and genetic background, is good practice, but that sex is particularly important, as it is a

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[6] Prendergast BJ, Onishi KG, Zucker I. Female mice liberated for inclusion in neuroscience and biomedical research. *Neurosci Biobehav Rev.* 2014;40:1–5

[7] Becker JB, Prendergast BJ, Liang JW. Female rats are not more variable than male rats: a meta-analysis of neuroscience studies. *Biol Sex Differ.* 2016;7:34

[8] Corrigan JK, *et al*. A big-data approach to understanding metabolic rate and response to obesity in laboratory mice. *Elife*. 2020;9:e53560.

binary difference in the population. **Misconceptions about the impact of oestrous cycle variability in some fields of research should be countered.**

There are many existing resources which MRC could encourage researchers to use, including NC3Rs Experimental Design Assistant (EDA) and other 3Rs resources, the Canadian Institutes of Health Research training programme[9], as well as NIH Sex as a Biological Variable resources.[10] It will be particularly important to encourage use of such resources to help applicants decide appropriate experimental designs and select appropriate power calculations. If applicants assume they need to test for an interaction (which, as discussed above is not always necessary), power calculators may suggest that high sample numbers are necessary in order to include both sexes.

When communicating with more junior researchers, MRC should emphasise support for designing experiments and analysing more complex data. Direct support from MRC in funding statisticians (as opposed to costing them in grants as is currently encouraged) was discussed but is likely not possible.

The Working Group agreed that **while the same principles would apply across animals, tissues and cells, the requirements should be explained separately for each**, with resources such as case studies and guidance for each individually.

(iv) Implementation and Evaluation

Recommended changes to MRC application forms and resources

Applicants are currently prompted to provide information about animals in the Animal Species section of the application form, with additional space for methodological details in the Reproducibility and experimental design annex. The Working Group supported **the inclusion of information about sex as part of the justification of the model in the Case for Support, or in the associated annex on Reproducibility and experimental design** (currently 1 page). It is not necessary to provide additional space for this information. Applicants should be encouraged to include the information on sex as part of a robust justification of their choice of model, alongside genetic background, reproductive status, age and numbers, and their experimental and statistical design.

The Working Group advised **that peer reviewers and board members assessing grants would need detailed instruction**, including a potential change to the reviewer form, adding in an explicit request for comments on how well the proposal addresses sex. In addition, the Working Group suggested MRC should pass on feedback to rejected grants about the improvement needed in this area.

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[10] https://cihr-irsc.gc.ca/e/50836.html

[10] https://orwh.od.nih.gov/sex-gender/nih-policy-sex-biological-variable

The MRC should develop case studies to aid applicants in addressing the new requirements. MRC should work with NC3Rs and MRC-funded researchers to develop resources such as case studies. A possible board member induction workshop in February 2022 was discussed.

The MRC should consider providing or signposting resources on how to analyse data appropriately.

Timeline

A policy launch in spring of 2022 is planned, with changes coming into effect for 2023 Boards and Panels. **A soft launch** was considered to allow the community to adapt to the new requirements. This would consist **of a first year during which applications would be returned for amendments if they are missing required information.** After the 12-month period of the policy launch, applications would be rejected if insufficient information provided.

The Working Group fully supported the adaption of the current experimental design guidance to include sex but advised that **there should be a clear announcement of the new policy**, to avoid confusion. It was noted that the proposed timeline of a 12-month period was short for culture change and roll out may need additional time. The MRC should seek to engage with other funders both within UKRI and externally during the soft roll out period, to work to align policy.

The Working Group encouraged MRC to give advance warning to the community where possible, which has now been completed within the autumn 2021 round of meetings of boards and panels, as well as recent HEI updates.