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EDITORIAL

Sex: A change in our guidelines to authors to ensure that this is no longer an ignored experimental variable

1 | BACKGROUND TO THE PROBLEM

Precision medicine is a buzz word mentioned frequently in the research world currently. Indeed, initiatives such as the UK's 100,000 genomes project are based on the premise that a better understanding of an individual's biology and genomic profile enables clinicians to better serve their patients. In some patients, this approach has led to diagnosis of a rare disease and an effective treatment that was not obvious without the genetic information. In light of these technological advances, it seems unconscionable that "sex," which is probably the most obvious distinguishing characteristic in humans, has been largely ignored in biomedical research.

Hitherto, blame for the frequent failure to translate research findings in the biomedical sciences into any human benefit has focused on issues of subjective bias, flawed experimental design, and inappropriate statistical analysis, as causal factors. By contrast, little consideration has been given to sex differences in either the efficacy of existing therapeutics or the implications for their refinement. Yet it is increasingly apparent that the long-established practice of studying predominantly one sex (typically males) could contribute to an apparent failure to translate. To help rectify this problem, this editorial outlines the rationale for a policy that requires sex to be considered as a categorical variable in all studies submitted for publication in the *British Journal of Pharmacology*. We also offer advice on how to incorporate this variable into an optimised experimental design.

To be clear, in this article, "sex" is defined by the genetic status of the subject, whereas "gender" refers to the social and cultural context. Conceptually, experimental interventions in animals, such as castration/ovariectomy, with and without hormone replacement/substitution, have already provided substantial information about the importance of sex hormones on anatomical and physiological development, as well as ensuing behavioural traits. The study of gender in experimental animals is not possible and at present remains a clinical experimental endeavour.

2 | EVIDENCE FOR SEX DIFFERENCES THAT AFFECT PHARMACOLOGY

Interest in sex differences in response to disease harks back as far as Hippocrates, who noted a fever outbreak in which women were less affected in terms of symptoms and mortality (Hippocrates of Kos, ca. 400 BCE). The discovery of sex differences in systematic comparisons of animals' responses to an experimental challenge is also not new. For instance, evidence that the learning behaviour of female rats was more variable than that of males was reported in the 1930s (McNemar & Stone, 1932). Subsequent studies also found higher variability in the behaviour of female rats (see, e.g., Broadhurst, 1957). A recent study has extended these findings by studying processes involved in fear conditioning, which clearly differ in males and females, especially during extinction (Clark, Drummond, Hoyer, & Jacobson, 2019).

Likewise, in 1947, Boynton and Todd reported that both systolic and diastolic blood pressures, measured in a large cohort, were higher in young men than in young women—a finding that was later replicated in dogs (Van Liere, Stickney, & Marsh, 1949).

Despite the accumulating evidence that sex leads to differences in biology, the sex of animals used in research was rarely reported. For instance, in Volume 1 of the *British Journal of Pharmacology (and Chemotherapy)* (1946), there were 27 research papers, of which 21 did not specify the sex. In the majority of cases, both sexes were probably used, given the large numbers of animals that were studied. Of the remaining six papers, only one specified the use of both males and females, three used males only, but two of these involved a study of the testis. The remaining two papers did not specify sex but included experiments on uterine tissue.

The higher variability of responses to experimental challenges in females provided a perceived justification for studying only male animals, even by groups who had already noted overall sex differences (e.g., Broadhurst, Sinha, & Singh, 1959). By 1960, attempts to reduce variance in experimental data were driving investigators to use a single sex, and this strategy was endorsed by a prominent textbook of Pharmacology (Lewis, 1960). A section of that book, dealing with variability amongst animals, included the recommendation that "[in experiments,] animals from the same strain, of similar age, weight and sex should be used." Meanwhile, in the clinical world, evidence was emerging that thalidomide causes congenital abnormalities in humans. This led to the preferential study of males in order to avoid the risk of exposing young women to novel drugs in clinical trials.

By the late 1970s, it was clear that a wide range of physiological systems were affected by sex differences (circadian rhythms, feeding, the effects of localised lesion of brain regions, sensory systems, e.g., Beatty, 1979). There was further evidence that the profile of these

sex differences depends on the species of the animal and the stage of development. Despite these reports, a strong rationale for studying males, but not females, was entrenched in both clinical and preclinical experiments, albeit for different reasons. Exceptions to this practice included studies using large animals, especially non-human primates, where data from both sexes were pooled (e.g., Yanagita & Takahashi, 1970). However, that approach was probably motivated by the need to avoid unacceptable waste of animals, as much as to study the drug response in a group of "typical" subjects sampled from a population.

Sex differences have now been described in all major fields of pathology, physiology, and pharmacology in humans and experimental animals. For instance, it is now recognised that sex differences in ischaemic heart disease result in disparities in treatment outcome as well as increased morbidity and mortality in women post-myocardial infarction (Aggarwal et al., 2018). Another large cohort study, the European Prospective Investigation into Cancer and Nutrition (EPIC), has reported sex differences in the prevalence of certain diet-related cancers (Zamora-Ros et al., 2018); these could be attributed to differences in the activity of metabolic enzymes in men and women (e.g., Dellinger, Garcia, & Meyskens, 2014). There are numerous reports of sex differences in the responses to inflammatory stimuli in experimental animals, a finding confirmed in two studies of healthy volunteers (Rathod et al., 2017), and a recent report indicates genetically dependent sex differences in the incidence of irritable bowel syndrome (Bonfiglio et al., 2018). These few examples of the differences in healthy volunteers and patients provide a strong rationale for the consideration of sex when investigating pathways of disease, identifying novel targets and teasing out the pharmacology of novel therapeutics.

There is now tangible evidence for a growing appreciation within the research community that sex/gender research is important. For instance, a PubMed search of the term "sex difference" in 2018 identifies 41 reviews. In particular, the growth of sex/gender research in pharmacology has stimulated the commissioning of a *BJP* Themed Issue on this topic in 2019. In this themed issue alone, there are reviews and original articles demonstrating that sex is an important determinant of physiology, disease phenotype, and drug response from diverse systems of the body spanning across schizophrenia (Gogos, Ney, Seymour, Van Rheenen, & Felmingham, 2019), fear sensation (Clark et al., 2019) to metabolism (Henstridge, Abildgaard, Lindegaard, & Febbraio, 2019) and obesity (Taylor, Ramirez, Musail, & Sullivan, 2019). A focus upon sex driving diversity in drug response is also evident from the literature: Recent examples include that airway smooth muscle, isolated from obese females, manifests greater agonist-induced excitation–contraction coupling than those derived from lean males, females, or from obese males (Orfanos et al., 2018) and that sex differences in the thermoregulatory and behavioural responses to the CNS stimulant, cathinone (Alsufyani & Docherty, 2017), have been identified. However, a notable complication is that the response of interest can depend on an interaction between sex and genotype, as was the case for the vulnerability to obesity in a strain of genetically altered mice (Pillidge, Heal, & Stanford, 2016).

Unlike the *BJP* of 1946, sex is mentioned in the *BJP* of today. Of 27 consecutive original articles (published in Vol 176 issue 3–7), 22 were animal studies of which 19 reported the sex of the animals used, which is a substantial improvement. However, of these 19, 12 used only males and five used only females. There were also six studies using human tissues (tissue, blood, or cell culture) of which two specified a female source. Cell culture and cell lines were used in 16 studies, of which two cell cultures were described as being derived from females. None of the studies compared sexes. We contend that whilst the reporting of sex in *BJP* is encouraging, it is not enough to state the sex of the animals used, and so, to improve our practices further, we encourage researchers to study both sexes.

3 | REASONS FOR STUDYING BOTH MALES AND FEMALES

Arguably, the most important reason to study both sexes is to facilitate successful translation of science with goals that improve human well-being, regardless of sex. It should be obvious that translation of encouraging preclinical findings with new molecular entities, or even repurposing of drugs, risks failure if the supporting evidence is based on research using males only.

Furthermore, substantial evidence shows that the stage of oestrous/menstrual cycle, not just sex, can affect the response to drugs (e.g., in cardiac electrophysiology; see Salama & Bett, 2014). Such findings underpin the conventional view that responses of females to experimental challenges are more variable than males. It should be acknowledged that this concept has been challenged recently following a meta-analysis of a wide range of studies of both mice (Prendergast, Onishi, & Zucker, 2014) and rats (Becker, Prendergast, & Liang, 2016), which led to the conclusion that "intrinsic variability … is at least as great in males as variability associated with the females estrous cycle." Nevertheless, the potential problems that could arise from studying only males, when the intention is to extrapolate the findings to the whole population, are obvious.

Perhaps the new experimental environment will stimulate much-needed, systematic studies of the effects of the oestrous cycle on drug actions. In the meantime, it is widely agreed that the problem of sex differences in response to an experimental challenge can no longer be ignored, to the extent that some national funding agencies such as the National Institutes of Health (NIH) (USA) and the Swedish Research Council are now requiring consideration of sex and/or gender in all applications. Importantly, this mandate includes cells maintained in vitro, not just whole animals (https://orwh.od.nih.gov/sex-gender/nih-policy-sex-biological-variable). This aspect is critical since, for instance, electrophysiological studies of isolated cardiac muscle cells (Lu et al., 2006) can be used to assess the propensity of drugs to cause the ventricular arrhythmia of Torsade de Pointes (Cubeddu, 2003), which is more common in females. There is also evidence for sex differences even at the level of individual cardiomyocytes (Mason & MacLeod, 2009; Salama & Bett, 2014). Importantly, it is not common practice for authors to state the sex of the individual (human or animal) from which primary cells have been derived (particularly if these cells have been purchased) and rare for

any mention of the sex of the original cell for cell lines, with the obvious exceptions of cell lines such as the Chinese hamster ovary (CHO).

Another important justification for studying both sexes is to address concerns regarding the ethical burden of surplus breeding. In the UK alone, 1.81 million non-genetically altered animals (91% of which were mice or rats) were bred for scientific procedures in 2017 but were killed, or died, without being used in any regulated procedure (Gov.uk, 2018). Some of these animals would have been used for collecting tissue for studies post mortem, but many would have been surplus stock and much of this surplus would comprise female animals. This attrition should be taken into account when considering compliance with the 3Rs (Replacement, Reduction, Refinement), notably in respect of Reduction (Russell & Burch, 1959).

4 | *BJP* POLICY

The *British Journal of Pharmacology* has decided to rectify this neglect of sex as a research variable, and we recommend that all future studies published in this journal should acknowledge consideration of the issue of sex. In the ideal scenario for in vivo studies, both sexes will be included in the experimental design. However, if the researcher's view is that sex or gender is not relevant to the experimental question, then a statement providing a rationale for this view will be required. Obvious examples of this would include studies of reproduction, genitalia, the actions of sex hormones, or sex-specific diseases and the use of immortalised cell lines. An example of an article in a recent issue of this journal where details of the numbers of each sex of the animals that were used for each stage of the study were given is Clark et al. (2019).

We acknowledge that the economics of investigating the influence of sex on experimental outcomes will be difficult until research grant-funding agencies insist that researchers adapt their experimental designs, in order to accommodate sex as an experimental variable and provide the necessary resources. In the meantime, manuscripts based on studies that have used only one sex or gender will continue to be published in *BJP*. However, we will require authors to include a statement to justify a decision to study only one sex or gender. In such cases, the conclusions should be expressed cautiously; if only males were used in the experiment, there should be an explicit statement to the effect that findings from the work might not be replicated in females.

Even once funding agencies have amended their approach, articles based on fundamental studies of pharmacological targets in vitro (such as receptors and enzymes), in single cell lines, will still be considered for publication in *BJP*, but we would need experimental confirmation that any findings from primary cells that underpin the main conclusion have been replicated in both sexes, unless there is an explicit justification for not doing so.

We shall review and revise this policy continually, to ensure it remains consistent with that of research funders as they move towards the mandatory study of both sexes.

5 | IMPLICATIONS FOR THE DESIGN AND STATISTICAL ANALYSIS OF STUDIES USING BOTH SEXES

To support researchers, we offer some considerations, below, that might help when designing future experiments.

5.1 | Implications for parametric analysis

In the light of evidence, albeit now controversial, that sex can influence the variability of the response of interest, authors should ensure that the data from studies using both sexes comply with the homogeneity of variance assumption that must be met for valid use of parametric statistics. The need to comply with this assumption, even for simple experimental designs, is explained in Curtis et al. (2018). A suitable transformation of the data (e.g., logarithm or square root) should be applied to the responses, if necessary, before carrying out any parametric analysis.

5.2 | Randomisation

Overall sex differences and, more importantly, interactions between experimental interventions and sex (i.e., the effect of the intervention differs in the two sexes) cannot be inferred if males and females are studied in separate time frames. It is not correct for animals from both sexes to be fully randomised within the study. If animals are randomised across treatment interventions, then this could lead to an unequal replication of males versus females across all the treatment groups. If there are overall differences between males and females, then this imbalance will bias the treatment comparisons. The technique of "blocking" or stratification must be used so that each treatment group is replicated equally often in both sexes, that is, randomise what looks like a mini-study in each sex.

Another example is when the study is to be carried out, at intervals, over a long period of time, with each subject being tested only once. In such cases, it would be more appropriate to treat both "day" and "sex" as blocking factors, that is, for equal numbers of male and female subjects to be tested, in parallel, on each day. The distribution of the different levels of the experimental factor (e.g., drug treatment) should be the same for both sexes, within each block, but randomised or counterbalanced (as in a Williams Latin Square) over the whole series of test days.

In conscious animal experiments, a potential confounder is that the response of interest might be affected by the close proximity of an animal of the opposite sex. We have no specific recommendation on how to deal with this, and it should be borne in mind that this situation will replicate their "real world." We ask authors merely to consider whether or not males and females should be physically separated, to ensure that sight and smell are not an issue that could confound the results, and to report on how this was addressed when carrying out the study. Obviously, it would not be advisable to house males and females in different rooms because that would undermine the need

for the animals to be exposed to the same environmental factors in a properly controlled experiment.

5.3 | Blinding

When carrying out studies in vivo, it can be difficult for experimenters to be blind to the sex of the animal. Nevertheless, as is currently the policy for this journal, blinding for this factor should be in place whenever possible. For example, samples can be coded and the order of processing them can be randomised after taking them from the animals. There should also be blinding during the statistical analysis of the data, as is the case for all other factors. However, blinding at this late stage will not resolve problems arising from any subjective bias that has been introduced during earlier stages of the experiment.

5.4 | Approaches to experimental design to enable consideration of sex

An inevitable consequence of studying both sexes is that each study will need more animals than would be necessary if only a single sex is used. However, by adopting a factorial design, with "Sex" and "Treatment" (e.g., a range of different test drugs or doses) as the experimental factors, a relatively small number of animals of each sex can provide sufficient information on the overall effect of the experimental factors.

If the objectives of the experiment rest on comparing specific pairs of treatment group means, from experiments with a factorial design, using a post hoc test (e.g., male vs. female at a specific test drug dose, or a specific test drug vs. vehicle for a single sex) then, to comply with this journal's rubric, sample sizes must be $N \geq 5$ for each combination of Sex and Treatment. It follows that an experiment investigating the effects of three drug doses (or three different drugs) plus vehicle, in both sexes (with each animal receiving only one treatment), would need a total of at least 40 animals (see Table 1).

However, using a factorial experimental design and two-way ANOVA to analyse the data implies that information from the raw data will be shared across all the factor levels. As a consequence, the 40-animal design risks wasting animals if the impact of Sex upon the measured variable was not a primary outcome measure of the study, which would compromise Reduction (as in Russell & Burch, 1959). This is especially likely to arise when the objective of the experiment is solely to test whether "Treatment" (across a range of test groups of drugs or doses) has an overall effect on the variable of interest. However, in this article, we are arguing that, for preclinical research to be more representative of the human setting, both sexes should be incorporated into the experimental design. In such cases, where the impact of sex on a specific treatment is not one of the experimental outcomes being interrogated, then we suggest that using only three animals for each combination of factors can sometimes be adequate. This is because equal numbers of males and females for each combination of Sex and Treatment yields a total of six animals for each level of Treatment. Providing that there is no evidence for an interaction between the two factors, which would be evident from the ANOVA analysis, then it is valid to combine the data from the two sexes and proceed with an analysis of any main effect of Treatment.

This scenario illustrates the importance of prospective definition of the experimental objective(s) and careful planning of the experimental design, which should include a sample size estimation that is documented in the manuscript.

For more advice and information on this topic, see: Bate and Clark (2014); Percie du Sert et al. (2017); the NC3Rs Experimental Design Assistant (https://www.nc3rs.org.uk/experimental-designassistant-eda); Festing et al. (2016).

TABLE 1 Showing the total number of animals for each of the experimental factors, Sex and Treatment, when five different animals (i.e., $N = 5$) are assigned to each combination of these two factors

Factor 2: Treatment (e.g., different compounds or doses)	Factor 1: Sex		
	Male	Female	Total
Vehicle	5	5	**10**
Drug A or low dose	5	5	**10**
Drug B or intermediate dose	5	5	**10**
Drug C or high dose	5	5	**10**
Total	**20**	**20**	**40**

5.5 | The oestrous cycle

Unless the objective of the study is specifically to investigate drug-induced responses at specific stages of the oestrous cycle, we shall not require authors to record or report this information in this journal. This is not least because procedures to determine oestrous status are moderately stressful and an interaction between the stress response and stage of the oestrous cycle could affect the experimental outcome. However, authors should be aware that the stage of the oestrous cycle may affect response to drugs particularly in behavioural studies, as reported for actions of cocaine in rats and mice (Calipari et al., 2017; Nicolas et al., 2019).

6 | SUMMARY

- *BJP* now requires sex to be considered as an experimental variable for all experimental reporting. This will affect the details of the experimental design that are documented or, in the absence of a design incorporating both sexes, a full justification for that approach.
- We recommend that all experiments (in vitro, in vivo, and ex vivo) should include both sexes, unless there is a specific justification or exemption, such as when using immortalised cell lines or tissue derived from a sex organ.
- Multifactorial designs should be used to study the overall effects of Sex, Treatment (i.e., the experimental intervention), and their interaction.

- Authors should consider the implications for the study findings when testing males and females in close proximity, particularly in behavioural studies, and describe these implications in the discussion of the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

James R. Docherty[1*]
S. Clare Stanford[2*]
Reynold A. Panattieri[3]
Steve P.H. Alexander[4]
Giuseppe Cirino[5]
Christopher H. George[6]
Daniel Hoyer[7,8,9]
Angelo A. Izzo[5]
Yong Ji[10]
Elliot Lilley[11]
Christopher G. Sobey[12]
Phil Stanley[13]
Barbara Stefanska[14]
Gary Stephens[15]
Mauro Teixeira[16]
Amrita Ahluwalia[17]

[1]*Royal College of Surgeons in Ireland, Dublin, Ireland*
[2]*University College London, UK*
[3]*Rutgers University, USA*
[4]*University of Nottingham, UK*
[5]*University of Naples Federico II, Italy*
[6]*Swansea University, UK*
[7]*The University of Melbourne, Australia*
[8]*Florey Institute of Neuroscience and Mental Health, Australia*
[9]*The Scripps Research Institute, La Jolla, USA*
[10]*Nanjing Medical University, Nanjing, China*
[11]*Research Animals Department, Science Group, RSPCA, West Sussex, UK*
[12]*La Trobe University, Bundoora, VIC, Australia*
[13]*Preclinical Research Statistics, UCB Pharma, Slough, UK*
[14]*The University of British Columbia, Canada*
[15]*University of Reading, UK*
[16]*Federal University of Minas Gerais, Belo Horizonte, Brazil*
[17]*William Harvey Research Institute, Queen Mary University of London, London, UK*

*Authors made equal contributions to this article.

Correspondence
Amrita Ahluwalia, British Journal of Pharmacology, The Schild Plot, 16 Angel Gate, City Road, London EC1V 2PT, UK.
Email: info@bps.ac.uk

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