Fostering best practice methods and translation in animal research

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Supervisors' Statement

As supervisors of Heidi Louise Morahan's doctoral work, we certify that we consider her thesis entitled "Fostering best practice methods and translation in animal research" to be suitable for examination.

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Statement of Originality

I, **Heidi Louise Morahan**, declare that the content in this thesis is my own original work and it has not been submitted to any other university or institution as part of or a whole requirement for any higher degree.

I declare that I am the principal researcher of all work included in this thesis, including work published with multiple authors. I certify that the intellectual content of this thesis is the product of my own work and that all assistance received in preparing this thesis and sources have been acknowledged.

The ethics approval (2019/1502) from the University of Sydney Animal Ethics Committee was granted for the third study presented in this thesis. The ethics approval (2022/472) from the University of Sydney Human Ethics Committee was granted for the fourth study presented in this thesis. Participants in this study were required to read a participant information statement, and informed consent was gained before data collection.

I understand if my candidature is successful, my thesis will be lodged with the Director of University Libraries and made available for immediate use.

Signed:

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Table of Contents

Publications and Conference Proceedings

The following peer reviewed publications arose directly from research conducted as part of my PhD candidature:

Published papers

Morahan, H. L., Leenaars, C. H. C., Boakes, R. A., & Rooney, K. B. (2020). Metabolic and behavioural effects of prenatal exposure to non-nutritive sweeteners: A systematic review and meta-analysis of rodent models. *Physiology & Behavior*, 213, 112696.

Morahan, H. L., Leenaars, C. H. C., Boakes, R. A., & Rooney, K. B. (2021). Metabolic and behavioural effects in offspring exposed to maternal sucrose consumption: a systematic review and meta-analysis of data from rodent models. *Journal of Developmental Origins of Health and Disease*, 12(4), 603–618.

Morahan, H., & Rooney, K. (2022). An Ecological Validity Model for the Prevention of Obesity: Non-Nutritive Sweetener Consumption in Rats and the Effects of Switching from Sugar-Sweetened to Diet Beverages. *Nutrients*, 14(13), 2758.

Submitted papers under review

Morahan, H., Hancock, N., Bero, L., & Rooney, K. (2023). "It seems really hard to justify killing animals": A qualitative study of early career researchers conducting animal experiments. *[currently under review with Laboratory Animals]*

Morahan, H., Cohen, S., Bero, L., & Rooney, K. (2023). The culture of care to enhance laboratory animal personnel well-being and the quality of animal research: A scoping review. *[currently under review with Laboratory Animals]*

The following additional publications were completed during the PhD candidature:

Additional publications

Lee, C., Yee, J., Morahan, H., Ross, L., & Edwards, K. (2022). The effect of aerobic exercise on tumour delivery: A systematic review and meta-analysis. *Supportive Care in Cancer*, 30(11), 1-17.

Lee, C., Yee, J., Morahan, H., & Edwards, K. (2022). Letter to the Editor on: "The effects of physical exercise on tumour vasculature: Systematic review and meta-analysis", *International Journal of Sports Medicine*, 43(03), 286-287.

The following presentations arose directly from research conducted as part of the PhD candidature:

Conference Presentations

Poster Presentations (in person)

Morahan, H., Boakes, R., & Rooney, K. (2019). A translational model for consumption of commercially available non-nutritive sweetened beverages in female rats. *Australian and New Zealand Obesity Society – Australasian Society for Lifestyle Medicine – International Chair for Cardiometabolic Risk 2019 Annual Scientific Meeting, Sydney, Australia*

Morahan, H. L., Leenaars, C. H. C., Boakes, R. A., & Rooney, K. B. (2019). Metabolic effects in offspring exposed to maternal non-nutritive sweetener consumption during pregnancy: A

systematic review and meta-analysis in animal models. *Australian and New Zealand Obesity Society – Australasian Society for Lifestyle Medicine – International Chair for Cardiometabolic Risk 2019 Annual Scientific Meeting, Sydney, Australia*

Oral Presentations (on-line; COVID-19 impacted)

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Morahan, H. L., Leenaars, C. H. C., Boakes, R. A., & Rooney, K. B. (2020). Metabolic and behavioural effects in offspring exposed to maternal sucrose consumption: A systematic review and meta-analysis of data from rodent models. *Australian and New Zealand Obesity Society - The Austral-Asia Obesity Research Update 2020, Virtual Conference, Australia*

Abstract

This thesis began with clearly defined research aims and a plan to explore the impact maternal nutrition has on the metabolic development of the child. Specifically, to investigate the metabolic effects from prenatal exposure to highly sweetened diets, such as those that contain excess sugar or non-nutritive sweeteners. In this research field, animal experiments play a crucial role to help understand mechanistic links between genetic and environmental influences during early life, and most studies have been conducted in rodent models. Therefore, the starting point for this thesis was to synthesise the totality of published scientific evidence by performing two preclinical systematic reviews; the first on maternal non-nutritive sweetener consumption and the second on maternal sucrose consumption. Unfortunately, findings from both studies highlighted many of the included animal experiments were poorly designed, poorly reported, and often lacked real consideration for animal to human translation.

I became concerned about the clinical relevance and impact these historical animal studies had on advancing our understanding of maternal nutrition and its subsequent influence on a child's metabolic health in human contexts. As a result, the seemingly obvious path was to attempt to perform and report an animal study of high scientific quality and rigour and importantly, develop an ecologically valid model of sweetener consumption in the hope of using this model to enhance translational capacity in a future maternal rodent study. I was fully committed and believed this experiment had the potential to enhance clinical relevance. Following several months of testing, and at the completion of the study, a deep uneasiness developed, and questions emerged whether it was worth conducting further animal studies. It was at this point in my PhD journey where, despite best efforts, I became uncertain about the relevance of my own animal study. How could I justify using another animals' life when I was openly concerned about the impact my future research might have on human health? Moreover, it felt

ethically wrong to continue animal experiments just to complete this PhD candidature. It was at this critical juncture that I decided my research path would be more impactful by advocating for improvement to the scientific quality of animal studies. It was hoped this improvement might reveal greater enhancement of clinical animal to human translation, although currently there is limited or no evidence to support this. From this point in the candidature, a completely different path emerged and the chapters in this thesis follow that journey.

As such, this thesis uses a mixed methodological approach to investigate research aims which are broadly separated into two parts, but intrinsically linked by the overarching theme of fostering animal best practice methods and translation. The first part of this thesis in Chapters 1 to 3 investigates the metabolic effects of a highly sweetened maternal diet and enhancing translational capacity for NNS consumption. And the second part in Chapters 4 and 5 explores the factors that influence early career researchers that use animals, on how they make important decisions during preclinical research and identifies effective strategies to support the researchers.

This thesis consists of six chapters. Two preclinical systematic reviews on existing literature researching the effects of maternal NNS and sucrose consumption, and one rodent study to determine an ecologically valid model of NNS consumption constitute the first part of my PhD journey. A human qualitative study of early career researchers, a scoping review on the potential for a culture of care to enhance study quality, and a final chapter exploring the overall conclusions and future directions of this research constitute part two of my PhD journey.

Chapters 1 and 2: Systematic Reviews – Metabolic effects of prenatal exposure to non-nutritive sweeteners and sucrose

Chapter 1 was peer-reviewed and published in Physiology & Behavior Journal in September 2019. Morahan, H. L., Leenaars, C. H. C., Boakes, R. A., & Rooney, K. B. (2020). Metabolic and behavioural effects of prenatal exposure to non-nutritive sweeteners: A systematic review and meta-analysis of rodent models. Physiology & Behavior, 213, 112696.

Chapter 2 was peer-reviewed and published in the Journal of Developmental Origins of Health and Disease in July 2020. Morahan, H. L., Leenaars, C. H. C., Boakes, R. A., & Rooney, K. B. (2021). Metabolic and behavioural effects in offspring exposed to maternal sucrose consumption: A systematic review and meta-analysis of data from rodent models. Journal of Developmental Origins of Health and Disease, 12(4), 603–618.

These chapters review all the published data on maternal consumption of highly sweetened diets during pre-pregnancy, pregnancy and/or weaning and the relationship between the offspring's body weight, weight gain, glycaemic control and feeding behaviours.

In Chapter 1 we conducted a preclinical systematic review and meta-analysis specifically assessing maternal NNS consumption. Following a thorough database search, 24 papers that met the specified criteria were included. It reported on a variety of different NNSs investigated, however aspartame and saccharin dominated the studies. Study quality was assessed using two methods; the SYRCLE Risk of Bias Tool for internal validity and adherence to the ARRIVE guidelines checklist for reporting quality.

Although meta-analysis results showed maternal consumption of NNSs did not increase offspring body weight, it could not elucidate if high dosages of sweetener reduced the palatability of food or fluid, and this may have confounded results. Moreover, most papers were predisposed to bias and showed low levels of adherence to reporting guidelines. Analyses also highlighted several study design weaknesses that had the potential to impact results and

translational capacity. This included inappropriate concentrations, timing of intervention administration, animal model and sweetener choice.

In Chapter 2, we present a second preclinical systematic review and meta-analysis assessing maternal sucrose consumption, but limited concentrations to 8-20% *w/v*, comparable to those seen in commercially available sugar-sweetened beverages consumed by humans. Using similar methodology as seen in chapter one, 15 studies were identified for qualitative synthesis, of which 11 studies were included in the meta-analysis. Results showed maternal sucrose exposure was associated with an increased risk of obesity and poor glycaemic outcomes in adult offspring; however, there was bias towards inclusion of male rats. The risk of bias and reporting adherence mirrored that of the first systematic review, clouding our interpretation of results.

In both reviews, we learnt that previous literature in this field did not adequately model the way humans consume NNS beverages and were often poorly designed and reported. In chapter three we sought to address this gap by aiming to find an ecologically valid model of NNS consumption to enhance translational capacity. We defined ecological validity, in the context of animal experiments, as research findings where laboratory tests represent real-world conditions. In addition, systematic review data contributed to an evidence-based choice of rodent strain to use for the experiment.

Chapter 3: An ecological validity model in rodent models for the prevention of obesity

Chapter three was published in Nutrients Journal in July 2022. Morahan, H., & Rooney, K. (2022). An Ecological Validity Model for the Prevention of Obesity: Non-Nutritive Sweetener Consumption in Rats and the Effects of Switching from Sugar-Sweetened to Diet Beverages. Nutrients, 14(13), 2758. https://doi.org/10.3390/nu14132758

Chapter 3 presents the results from a series of experiments where female and male Sprague Dawley rats were offered commercially available NNS drinks: Diet Coke™, Sprite™, Cottee's sugar free cordial™ and Remedy Kombucha™. The primary behavioural outcome was consumption of NNS drinks during acceptance and preference testing, initially performed in naïve animals then retested following a four-week period of chronic sucrose exposure. The rats then switched to drinking their assigned NNS for another 4-week period, mimicking the human behaviour of replacing sugar-sweetened beverages for diet beverages. The primary metabolic outcome was fat pad mass to assess if there was any metabolic recovery from damage caused by excessive sugar intake.

As predicted, Sprite™, which contained a reduced sucrose concentration mixed with the NNS stevia, was consumed in high volumes (50.9 +/-1.6ml/day) and further metabolic damage was evident following the switch. Rats accepted Diet Coke™, although lower intakes were recorded compared to that of Sprite™ volumes (17.5+/-1.5ml/day; $p<0.05$). Remedy Kombucha™ and Cottee's sugar free cordial™ were accepted, however intakes were minimal (9.6+/-0.4ml/day and 6.8+/-0.6ml/day respectively) suggesting these beverages were not reliable models. Limited metabolic recovery was seen in rats that switched from sucrose to NNS beverages.

From the outset, we anticipated results from this experiment would guide the future study design of a maternal rodent study. However, deep introspective reflection uncovered significant concerns about the clinical relevance and lack of impact that further animal experiments may have on human outcomes. The bigger question was: is it ethically right to design another project simply for PhD achievement and is this responsible science? With the desire to do something more impactful, it was decided the broader aim of fostering best practices and translation would be better satisfied by exploring how less experienced researchers, those who contribute to vast numbers of animal experiments, make important decisions on research practices. This change of focus is presented as a qualitative study in Chapter 4.

Chapter 4: "It seems really hard to justify killing animals": A qualitative study of early career researchers conducting animal experiments.

This chapter has been submitted for peer-review in Laboratory Animals Journal by Sage Publications on the 14th of August 2023. It is a qualitative study on early career researchers (ECRs) exploring what shaped their decisions on research and reporting practices and understanding the barriers they encountered, which could be targeted for future resource development. Using semi-structured interviews, we collected and analysed data from 13 ECRs using constant comparison techniques, reflexivity, and an iterative approach.

While the primary line of questioning consistently focussed on influences that shaped an ECRs research practice, a secondary theme organically emerged. As such, the results are presented across two major themes that were drawn from coding of data: 1) Learning from self and others, which encompassed various learning pathways and research practices of ECRs; and 2) Emotional and ethical dilemmas on self, which encompassed the emotionality ECRs experienced when working with research animals.

We heard that ECRs learning pathways were heavily influenced by supervisory relationships, laboratory practices and literature more than institutional training. We also revealed there were low levels of awareness of best practice recommendations and that ECRs showed a desire to engage in dedicated workshops on designing and reporting animal research.

The unexpected finding of the emotional and ethical turmoil many ECRs experienced highlighted the need to provide more emotional support for researchers, and although some sub-themes were not able to be linked to poor research practices, this behaviour could not be ruled out. In chapter five, we further explore this line of enquiry.

Chapter 5: The culture of care to enhance best practice in animal research: A scoping review

This chapter presents a scoping review providing a comprehensive overview of the concept of Culture of Care (CoC) in animal research and its relationship with scientific quality. Results from 48 included peer reviewed publications identified and mapped data that focussed on the prevalence of work-related stressors in laboratory animal professionals, their possible effects on study quality and key strategies to create a positive CoC to support researcher well-being. We identified compassion fatigue as the most investigated stress with widespread prevalence across North America and Europe. While many strategies had been suggested, there was limited data supporting success and no direct data was available to link work-related stress with research quality despite many publications describing this association.

Chapter 6: Conclusions and Future Directions

Chapter six concludes this thesis by commenting on the main findings of the research studies and providing recommendations for future directions for supporting animal researchers to foster best practice research methods and deliver more responsible science.

Chapter 1: Metabolic and behavioural effects of prenatal exposure to non-nutritive sweeteners: A systematic review and meta-analysis of rodent model

Chapter 1 has been published as:

Morahan, H. L., Leenaars, C. H. C., Boakes, R. A., & Rooney, K. B. (2020). Metabolic and behavioural effects of prenatal exposure to non-nutritive sweeteners: A systematic review and meta-analysis of rodent models. *Physiology & Behavior*, *213*, 112696.

Statement from co-authors confirming authorship contribution of the PhD candidate

The co-authors of the paper: *Metabolic and behavioural effects of prenatal exposure to non-nutritive sweeteners: A systematic review and meta-analysis of rodent models. Physiology & Behavior, 213, 112696* confirm that Heidi Louise Morahan has made the primary contribution to this study in each of the following areas:

- Conception and design of the research
- Collection and analysis of data, interpretation of the findings
- Writing of the manuscript and critical appraisal of content

In addition to the statements above, in cases where I am not the corresponding author of a published item, permission to include the published material has been granted by the corresponding author.

Heidi Louise Morahan Date: 26 September 2023

As supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

Associate Professor Kieron Rooney Date: 26 September 2023

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Metabolic and behavioural effects of prenatal exposure to non-nutritive sweeteners: A systematic review and meta-analysis of rodent models

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ABSTRACT

Little is known about possible effects of maternal non-nutritive sweetener (NNS) consumption on the metabolic health of a child. Animal models of maternal NNS consumption during pregnancy or weaning have yielded widely varying results, and there appears to be no clear consensus on the consequences for offspring body weight, glycaemic control or sweet preference choices. Moreover, heterogeneity in study design has hampered a clear focus for future research relevant to human health. In an effort to bring clarity, we have conducted a systematic review and meta-analysis (protocol no: CRD42018109509) in animal models (rat or mouse) of maternal NNS feeding (compared to water or basal diet) during pre-gestation, pregnancy or lactation. Four databases were searched from inception to 15th September 2018: PubMed, EMBASE, SCOPUS and Web of Science. We present maternal and offspring data from 24 included studies, which have been quantitatively analysed after study quality assessment, to identify relationships between maternal diet and offspring body weight (BW), feeding behaviour and glycaemic control. In 11 data sets, exposure to NNS reduced maternal BW during pregnancy, with no effect on litter outcomes. Meta-analyses on offspring BW during weaning (1123 offspring) and adulthood (646 offspring) identified small decreases in BW for both sexes. Subgroup analyses revealed reductions in BW of rat, but not mouse models. High dosage appears to be a potential factor for reduced palatability that could influence BW results; however, a lack of reported data limited our ability to confirm. Despite this, and the fact many papers were predisposed to bias, the balance of evidence suggests a maternal NNS diet during pregnancy or lactation did not increase the body weight in offspring.

1. Introduction

Consumption of non-nutritive sweeteners (NNSs) has dramatically increased over the last decade [1]. Widespread recommendations to reduce added sugar intake, heightened consumer awareness and implementation of sugar taxes have been catalysts for the greater consumption of NNS-supplemented food and beverages [2,3]. The use of NNSs as a partial or full sugar replacement conveniently sweeten foods whilst reducing energy content and are commonly marketed to consumers wanting to manage body weight. Recent systematic reviews report reductions in energy intake, body weight [4] and BMI [5] when NNS sweeteners were consumed in place of sugar. Nevertheless, links have been claimed between the consumption of NNSs and obesity, alterations to glycaemic control, poor appetite control and changes to the microbiome in humans and animals [6–9].

The prevalence of NNS consumption during pregnancy has risen by nearly 50% over the last 15 years, corresponding with trends in the general population [1]. Approximately one quarter of pregnant women reported drinking or eating NNS products in recent years. From a public health perspective, it is of significant interest to explore if possible adverse effects may be provoked during prenatal exposure through developmental programming. It is well known that maternal nutrition is an important factor for the long-term health of the child and dietary insults can interfere with interactions between genetic and environmental influences during prenatal life [10]. Some recent data identified NNS beverage consumption during pregnancy was associated with an increased risk of infant obesity and elevated BMI [11,12]. However, another study found no link to mid-childhood adiposity [13]. The reported observations could be either a direct effect of NNS on metabolism or a behavioural effect on eating; but of course, correlation studies

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are limited in their ability to determine causal relationships [14].

In the absence of prospective human trials, we analysed animal studies. A number of reviews [15,16] have explored maternal NNS consumption and metabolic implications in offspring. Some of the experimental studies in rat and mouse models focussed on offspring weight, behaviour and glycaemic outcomes [17–23]. Differing results have only generated further controversy in this intensely debated field. One such example was the observation that aspartame promoted increased offspring bodyweight following maternal consumption during pregnancy [19], where others identified no change [24–26]. In part, differences may be due to variability between study methodologies, animal species and strains. The choice of sweetener may introduce variations in results as individual NNSs may elicit different metabolic or sensory responses according to their distinct biological fate [27]. Moreover, the timing of NNS exposure complicates inter-study comparisons, as dams can be fed prior to mating, during pregnancy and/or lactation.

As such, we have conducted a systematic review to clearly collate and summarise the total body of evidence for metabolic and behavioural effects in offspring exposed prenatally to NNS diets. Based on all available evidence, we aim to identify areas for future research that may be relevant for human health, assist in the development of appropriate study designs and reduce unnecessary repetition of already performed animal studies.

2. Methods

We report our review in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA 2009) Guidelines (S1. PRISMA Checklist). The protocol was developed with the SYstematic Review Centre for Laboratory animal Experimentation's (SYRCLE) Protocol template, Version 2.0 [28], and registered with PROSPERO (Protocol number : 42017109509; Available from http://www.crd.york.ac.uk/PROSPERO/display_record.php?ID=CRD42018109509) on November 8th, 2018 and the Systematic Review Facility for preclinical studies (http://syrf.org.uk/protocols/) on November 1st, 2018.

The key research question is: what are the metabolic and behavioural effects in offspring exposed prenatally to non-nutritive sweeteners (NNS)?

2.1. Search strategy and study selection

The following databases were searched: PubMed (all years), EMBASE via OvidSP (1947 to present), Web of Science (all years) and SCOPUS (all years) on the 15th September 2018 and updated iteratively (final search 4th February 2019). An extensive search strategy was constructed using keywords, related synonyms and medical subject headings (MeSH). The final search strategy for each database can be found in S2. Table. Search Strategy. No date restrictions were applied. References extracted from each database were combined and duplicates manually removed in EndNote reference management software (EndNote™ X8) Screening for inclusion was performed by two independent reviewers. Inclusion followed our predefined criteria seen in Table 1. Only studies describing rodents (rat or mouse) consuming NNS as part of their *ad libitum* diet (i.e. NNS available in addition to or mixed with chow or drinking water) were included, to model human consumption. The NNS had to be fed to dams prenatally and/or during gestation and/or during lactation. No limitation was placed on dosage. We defined NNS as any artificially synthesised or natural sweetener that contributes a negligible energy content. They are classified as a broad range of sweetening compounds with differing molecular structures and vary in the way each is absorbed, metabolised and excreted. Included and excluded sweeteners and biological fates of common NNSs can be found in the Supplementary File 3.

Table 1
Eligibility criteria.

Inclusion criteria
• Controlled interventional studies
• Rodent studies (rat or mouse)
• Maternal NNS diet during pre-gestation and/or gestation and/or lactation
• Free feeding models
exclusion criteria
• Non experimental studies
• Non English studies
• No offspring outcomes assessed
• No appropriate non-NNS control group for comparison; including nicotine or ethanol comparators for models of prenatal alcohol exposure
• Maternal dietary interventions with high fat total energy intake > 10%; including westernised, junk, cafeteria, obesogenic or high-fat high-sugar diets
• Intragastric or intraperitoneal feeding models

Inclusion and exclusion criteria developed *in a priori* and used to assess papers during study selection.

2.2. Grouping of papers

For analyses, included papers were divided over four categories according to the primary outcome measure (category descriptors found in S4. Table). The first category comprised studies in which metabolic and behavioural measures of the offspring were the primary outcomes. The second category comprised studies in which the primary measures were offspring's sweet taste preference. The third category comprised studies in which the potential toxicological effects of compulsory consumption of a sweetener were of primary concern. The fourth category comprised studies in which neoplastic incidence in offspring was the primary concern.

2.3. Data extraction

Bibliographical details, experimental conditions, sample size, unit of measurement, animal characteristics (species, strain, source, maternal age and weight), maternal dietary intervention (NNS type, dosage, administration during pre-gestation and/or gestation and/or lactation periods, liquid or solid, free access or timed), additional offspring interventions, maternal and offspring outcomes and funding/sponsorship were extracted using a customised data extraction form (S5. Table). Maternal and offspring data for body weight, body composition, litter outcomes, food and fluid intake and glycaemic control were collected as mean and standard error of the mean (SEM) or standard deviation (SD) and entered into an excel spreadsheet. If required, a digital ruler (Pixel Ruler version 3.1) was used for extraction from graphs. If data were not reported or further information was required, attempts to contact the corresponding author were made. Where relevant, separate groups of animals receiving different treatments were treated as separate data sets. Experimental results were summarised as a percentage change of NNS-exposed animals compared to the control group.

2.4. Meta-analysis

Following completion of data extraction, it became evident that sufficient information was available to perform meta-analyses. Meta-analyses were then planned for maternal and offspring body weight, and for litter size. A separate protocol was registered with SyRF on the 28th November 2018 (http://syrf.org.uk/protocols/). Relevant data were extracted as mean and SD (SEM was converted to SD where necessary). Following recent meta-analyses of animal data [29–31], different groups of animals from the same paper were included as separate data sets, and thus effectively treated as independent experiments. We performed random-effects meta-analyses using R Version 3.5.1 (2018-07-02) "Feather Spray" for standardised mean differences (SMD) and

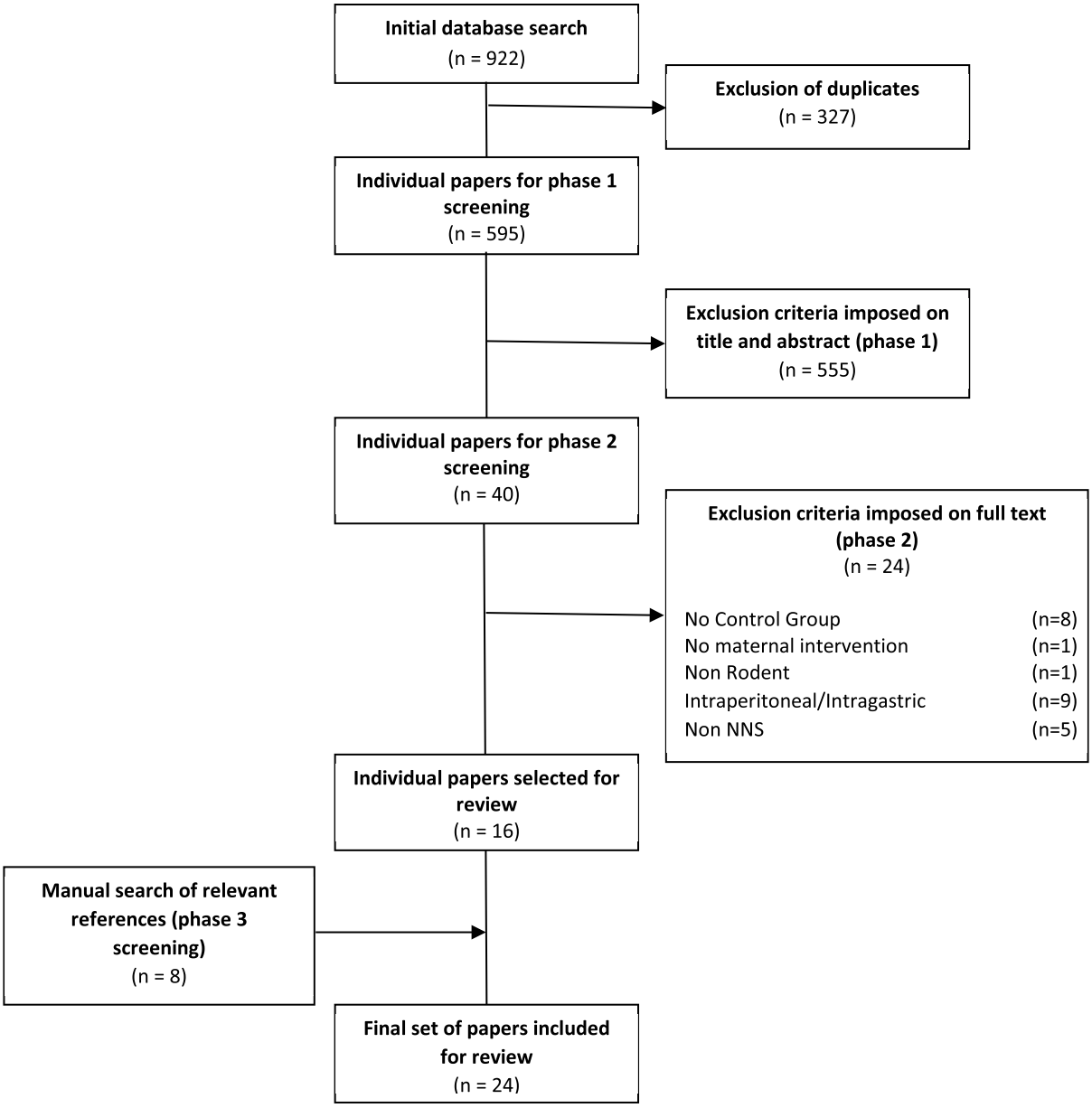

Fig. 1. Flow diagram of the paper selection.

corresponding 95% confidence intervals. The *metacont* function from the *meta* package was used for standard analyses; the rma function from the *metafor* package was used for the meta-regression. As several papers included data from multiple experimental groups compared to the same control group, a correction was made using the following equation: $N_{\text{corrected control}} = N_{\text{control}}$/no. of experimental groups. All data are presented as forest plots drawn using the forest function from the *metafor* package.

Maternal body weight was extracted for gestation day 21 (GD21). If such data were not available at this exact time-point, the next closest time-point within the gestational period was selected. Many studies included multiple experimental groups, therefore each group was considered as a separate data set in analyses. To assess the effect of maternal NNS exposure on litter size, the number of pups per litter was analysed for six papers, which yielded 15 separate experimental groups. Offspring body weight (BW) was extracted for weaning at PND21 and adulthood, at PND 30–140. Sub-group analyses on offspring and adult BW were performed for sex and species to explore heterogeneity. A meta-regression analysis was performed to analyse the effect of PND on offspring BW in adulthood. Additionally, we explored the possibility of bias to favourable results in publications funded by the artificial sweetener industry through sub-group analysis.

2.5. Study quality assessment

2.5.1. Risk of bias

The included studies were assessed for internal validity using SYRCLE's Risk of Bias (RoB) Tool [32]. This 10-item validated tool has been adapted from the Cochrane RoB Tool [33] for use in systematic reviews on animal models and is recommended by SYRCLE, CAMARADES and NC3R. Rationale for each risk of bias category has been previously described [32]. Items relating to six types of bias: selection, performance, detection, attrition, reporting and other were judged as 'yes' (low risk of bias), 'no' (high risk of bias) or 'unclear' (unable to clearly assign risk of bias) using the reported signalling questions [32]. As industry-funding has previously been shown to be a potential bias toward positive reporting in artificial sweetener studies [34], an in-dustry funding source signalling question was added (item 10). Equally,

we could not assume that independently funded research carried no bias (e.g. white hat bias) [35], however assessment was outside the scope of this review.

2.5.2. ARRIVE guidelines checklist

In addition to RoB, we evaluated reporting quality by assessing adherence to the Animal Research: Reporting of in Vivo Experiments (ARRIVE) Guidelines Checklist [36]. This checklist was developed in 2010 as a comprehensive tool to improve reporting in animal studies. Historically, poor reporting in animal studies has contributed to a lack of reproducibility, animal waste and failure in translation to human trials [37]. Assessment of reporting allows greater transparency for missing information and thus aids in evaluating the reliability of each study. We have modified and developed the ARRIVE Guidelines into evaluation descriptors and a reporting system (S6. Table. Arrive Descriptors) similar to evaluation tools previously described [38,39]. Briefly, the 20-item checklist has been extended to include 40 associated sub-items relevant to this review. Items are described as 'fully reported', partially reported or non-reported. For items with associated sub-items fully reported means that all sub-items were reported, partially reported that not all sub-items are fully reported, and not reported that none of the sub-items was reported.

3. Results

3.1. Study selection

Initial database searches yielded 922 papers, of which 595 remained after removal of duplicates. Screening based on exclusion criteria was applied to title and abstract (phase 1; 555 papers excluded) and to full text (phase 2; 24 papers excluded) which resulted in 16 included papers. During full-text screening, eight papers were excluded due to the absence of a non-NNS control group, one paper was excluded as no maternal intervention was applied, one paper was excluded as it was conducted in humans, nine papers were excluded because they were non free-feeding models (such as intragastric or intraperitoneal administration) and five papers were excluded as the intervention was not considered to involve a non-nutritive sweetener (e.g. D-tagatose and sugar alcohols). A full list of excluded papers and exclusion reasons, along with type of NNS is presented in the Supplementary files (S7. Table Excluded Papers). Searching reference lists yielded a further eight papers for inclusion. In total, 24 papers were identified for assessment in the systematic review (Fig. 1).

3.2. Study characteristics

Study characteristics varied for (rodent) species and strains, maternal age and weight, type and timing of NNS administered and dosage (See S8. Table. Study Characteristics). Mice (C57BL/6J and ICR) were used for the majority of metabolic and behavioural and sweet taste preference papers [17–23,40,41], only one study used Wistar rats [42]. Toxicology and neoplastic papers [24,25,43–52] commonly used rat models (Sprague Dawley, Hans Wistar and F344), although two studies (both by the same author) used Swiss mice to investigate potential carcinogenic effects of aspartame [26,53]. A variety of sweeteners (acesulfame potassium - 2 papers; advantame - 1 paper; arruva -1 paper; aspartame - 9 papers; rebaudioside A - 1 paper; saccharin - 7 papers; sucralose - 3 papers; sucralose mixed with acesulfame potassium - 1 paper) were administered in the animal's drinking water or mixed with food (See Table 2). In one study, the sweetener sucralose was provided in a restricted amount of drinking water to mimic human daily consumption [21]. Not always reported, the use of a variety of chow suppliers contributed to differences in composition (S9. Chow composition).

Considerable variation in maternal dosage was present, ranging from equivalent to human acceptable daily intake (ADI) [21] to several thousand fold greater than ADI [24]. It should be noted that toxicological studies commonly administered high doses as the experimental objectives included evaluation of a sweetener's safety for general use in foods. For several studies, [20,22,47,49,50,52,53], we could not calculate the dosage in mg/kg/day due to insufficient data. Timing and duration of maternal exposure also varied greatly, from 70 days pre-gestation to gestation and/or weaning. A summary of maternal dietary interventions and study design is shown in Table 2.

A complete list of all maternal and offspring outcomes for individual studies was tabulated prior to defining relevant metabolic and behavioural outcomes (S10. Table – Maternal and Offspring Outcomes).

3.3. Maternal outcomes

An overview of maternal results is shown in Table 3. From the 24 included papers, several dam groups were exposed to varying concentrations of NNS or the same concentration at differing time-points; consequently, 59 separate data sets were included for review. Maternal body weight, food and fluid intakes and litter effects were seldom reported for studies in the behavioural and metabolic, sweet taste preference and neoplastic categories. Additionally, several papers reported outcomes without providing data (as indicated in Table 3. with asterisk symbol *). No paper investigated maternal glycaemic outcomes, such as glucose or insulin levels or glucose tolerance.

3.3.1. Maternal bodyweight and bodyweight gain

Nine papers gave rise to 29 data sets reporting on maternal body weight during gestation (14/29), lactation (3/29) or both (12/29) as seen in Table 3. Nine data sets reported a significant reduction in body weight (BW) compared to control fed dams (6-14%) when measured around gestation day 21 (GD21). One of these sets reported a decrease at both gestation and lactation and one showed no observable change during gestation but a significant increase during lactation. A meta-analysis performed on extractable data from four papers and eleven data sets identified a decrease in maternal BW measured at approximately GD21 (SMD = −0.34, 95% CI = −=0.59, −0.08, I^2 = =12%) with a low heterogeneity, as shown in Fig. 2. In over half of these groups (7/11), dams were exposed to NNS 14–70 days pre-pregnancy through to parturition [45,50], whereas the remainder were exposed during pregnancy only from gestational day 6–21 [23,43]. Of particular note, the NNS dosage for all groups exceeded human ADI by several hundred fold and the majority were classified as toxicology studies.

BW gain during pregnancy was measured in 15 experimental sets with six reporting significant reductions (8–19%) relative to non-NNS fed control dams. BW measured during the lactation period, in 15 experimental sets, generally detected no effect. The one paper reporting an increase [51] did not provide data.

3.3.2. Maternal food and fluid intake

Food intake measured during the time of conception, gestation or lactation was reported in eight studies. There was variation in the duration of time that food consumption was measured, ranging from 3 to 7 days. Thus, we averaged to a mean intake of grams/animal/day for each individually housed dam. The majority of studies reported no change in food intake, although two high dosage papers [50,51] observed decreased chow consumption and increased water intake across the same time-period, suggestive of reduced NNS palatability. High concentrations of many NNSs present a bitter aftertaste or have other post-ingestive factors that may lead animals to display a pattern of avoidance [54,55]. One paper investigating rebaudioside A [45] demonstrated a nine per cent increase in food intake during the last week of lactation. Only five studies measured maternal fluid intake as an outcome; two observed no change in intake [40,47] and three reported increased water consumption [49–51].

Table 2
Study design and maternal dietary intervention summary. The comparator (control) was plain water where NNS was added to drinking water or standard chow where NNS was mixed with chow.

Study ID/Year	Animal Species	Animal Strain	NNS type and dose	Equivalent dose mg/kg BW/day	Administration of NNS	Duration of maternal exposure PG	G	L	Additional offspring exposure
Sweet taste preference studies									
Choo 2018	mouse	C57BL/6	Sucralose 2ml of 6.7mM	5.331	oral via liquid ration daily	28	Y	PND1-14	—
Li 2013	mouse	ICR	Ace K 5, 12.5, 25 & 50mM	NR[b]	added to drinking water *ad libitum*	—	—	PND 4-21	—
Zhang 2011	mouse	ICR	Ace K 5g/kg preg only & lact only	400-600	mixed with standard chow *ad libitum*	—	GD6-21 preg group	PND 1-21 lact group	—
Metabolic and behavioural studies									
Zhang 2018	mouse	C57BL/6	Saccharin 2% (w/v)	NR	added to drinking water *ad libitum*	21	Y	Y	—
Olivier-VS 2019	mouse	C57BL6	Sucralose & Ace K (combined) ADIx1 & ADIx2	ADIx1: 5 & 15 ADIx2: 10 & 30 (sucralose & Ace K respectively)	pipetted onto pellet daily	—	Y	Y	—
Collison 2016	mouse	C57BL/6J	Aspartame 0.25g/L	55.14	added to drinking water *ad libitum*	21	Y	PND0-28	weaning to 20 weeks age
Parlee 2014	mouse	C57BL/6J	Saccharin 3% (w/v)	NR[b]	added to drinking water *ad libitum*	—	—	Y	—
Collison 2012a	mouse	C57BL/6J	Aspartame 0.25g/L	55.14	added to drinking water *ad libitum*	21	Y	Y	weaning to 20 weeks age
Collison 2012b	mouse	C57BL/6J	Aspartame 0.25g/L	55.14	added to drinking water *ad libitum*	21	Y	Y	weaning to 20 weeks age
von Poser Toigo 2015	rat	Wistar	a) Aspartame 2g/L b) Saccharin 1.35g/L	a) 300 – 400[a] b) 200 – 270[a]	added to drinking water *ad libitum*	30	Y	—	—
Toxicology studies									
Brathwaite 2013	rat	SD	Arruva (Monatin) 15000, 30000 & 50000ppm	1259, 2564 & 4185	mixed with standard chow *ad libitum*	—	GD6-21	—	—
Otabe 2011	rat	SD (CD)	Advantame 2000, 10000 & 50000ppm	164, 833 & 4410	mixed with standard chow *ad libitum*	70	Y	Y	weaning to 2nd generation
Curry 2008	rat	Hans Wistar	Rebaudioside A 7500, 12500 & 25000ppm	669, 1115 & 2273	mixed with standard chow *ad libitum*	70	Y	Y	weaning to 2nd generation
Kille 2000	rat	SD (CD)	Sucralose 0.3, 1.0 & 3.0% of diet	500, 1825 & 5750	mixed with standard chow *ad libitum*	14	Y	Y	weaning to 2nd generation
Reilly 1990	rat	SD	Aspartame 500mg/kg	NR[b]	added to drinking water *ad libitum*	—	Y	Y	—
Holder 1987	rat	SD	Aspartame 0.007, 0.036, 0.18 & 0.9% w/v	14, 68, 347 & 1614	added to drinking water *ad libitum*	12	Y	Y	weaning to PND38
Brunner 1979	rat	SD	Aspartame 2, 4 and 6% of diet	1580, 3220 & 4970	mixed with standard chow *ad libitum*	>14	Y	Y	—
Lapointe 1979	rat	SD	Sodium Saccharin 0.4mg/ml	NR[b]	mixed with standard chow *ad libitum*	14	Y	Y	—
Neoplastic studies									
Soffritti 2016	mouse	swiss	Sucralose 500, 2000, 8000 & 16000ppm	NR[b]	mixed with standard chow *ad libitum*	—	GD12-21	Y	weaning to natural death
Soffritti 2010	mouse	swiss	Aspartame 2000, 8000, 16000 & 32000ppm	247, 987, 1919 & 3909	mixed with standard chow *ad libitum*	—	GD12-21	Y	weaning to natural death
Soffritti 2007	rat	SD	Aspartame 400 & 2000ppm	20 & 200	mixed with standard chow *ad libitum*	—	GD12-21	Y	weaning to natural death
Cohen 1995	rat	SD & F344	Sodium Saccharin 0.4mg/ml	NR**	mixed with standard chow *ad libitum*	14	Y	Y	weaning to PND91
Garland 1991	rat	SD	Sodium Saccharin 7.5% of diet	1283[a]	mixed with standard chow *ad libitum*	28-42	Y	Y	weaning to PND30
Taylor 1980	rat	SD (CD)	Sodium Saccharin 0.01, 0.1, 1.0, 5.0 & 7.5% of diet	NR[b]	mixed with standard chow *ad libitum*	49	Y	Y	weaning to 23 months age

Abbreviations: PG – pre-gestation (days), G – gestation (days), L – lactation (days), Y – yes, GD – gestational days, PND – post natal days, NR – not reported, SD – Sprague Dawley, Ace K – Acesulfame Potassium

[a] Dosage (m/kg BW/day) not reported by authors; estimated dosage calculated based on average food/fluid consumption and body weight;

[b] Unable to calculate dosage (mg/kg BW/day) due to insufficient data

Table 3
Maternal results for body weight at conception, gestation and lactation, body weight gain, litter outcomes and food and fluid intakes at conception, gestation and lactation.

Study ID/Year	Experimental Group	Species/ Strain	*n*	Conception	Gestation and Lactation						
				Age	BW	BW	BW Gain	Pups/ Litter	Litter size	Sex ratio	Food intake	Fluid intake
Sweet taste preference studies												
Choo 2018	Sucralose 5.3mg/kg BW/day	m/ C57BL/6	—	PND 84	—	—	—	—	—	—	—	—
Li 2013	Ace K 5mM	m/ ICR	7	—	—	—	—	—	—	—	—	—
	Ace K 12.5mM	m/ ICR	7	—	—	—	—	—	—	—	—	—
	Ace K 25mM	m/ ICR	7	—	—	—	—	—	—	—	—	—
	Ace K 50mM	m/ ICR	7	—	—	—	—	—	—	—	—	—
Zhang 2011	Ace K 5g/kg (*preg*)	m/ ICR	6	PND 56	—	NS (G,L)	—	—	NS	NS	NS (G)	—
	Ace K 5g/kg (*lact*)	m/ ICR	6	PND 56	—	NS (G,L)	—	—	NS	NS	NS (L)	—
Metabolic and behavioural studies												
Zhang 2018	Saccharin 2% (w/v)	m/ C57BL/6	—	PND 77-105	—	—	—	—	NS	NS	—	NS
Olivier-VS 2019	Sucralose/Ace K ADIx1	m/ C57BL/6	10	PND 70	—	—	—	—	NS*	NS*	—	—
	Sucralose/Ace K ADIx2	m/ C57BL/6	10	PND 70	—	—	—	—	NS	NS	—	—
Collison 2016	Aspartame 0.25g/L	m/ C57BL/ 6J	7-12	PND 63	—	—	—	—	—	—	—	—
Parlee 2014	Saccharin 3% (w/v)	m/ C57BL/ 6J	5	PND 70	—	—	—	—	—	NS*	—	—
Collison 2012a	Aspartame 0.25g/L	m/ C57BL/ 6J	—	PND 63	—	—	—	—	—	—	—	—
Collison 2012b	Aspartame 0.25g/L	m/ C57BL/ 6J	7-10	PND 63	—	—	—	—	—	—	—	—
von Poser Toigo 2015	Aspartame 2g/L	r/ WS	4	PND 120	—	—	—	—	—	—	—	—
	Saccharin 1.35g/L	r/ WS	4	PND 120	—	—	—	—	—	—	—	—
Toxicology studies												
Brathwaite 2013	Arruva 15,000 ppm	r/ SD	25	PND 83	NS	NS (G)	NS(G)	NS	NS	NS	NS (L)	—
	Arruva 30,000 ppm	r/ SD	25	PND 83	NS	NS (G)	↓11 (G)	NS	NS	NS	NS (L)	—
	Arruva 50,000 ppm	r/ SD	25	PND 83	NS	↓7(G)	↓19 (G)	NS	NS	NS	NS (L)	—
Otabe 2011	Advantame 2000 ppm	r/ SD (CD)	30	PND 112	NS*	NS (G,L) *	NS (G,L) *	NS	NS	NS	NS (C,G,L)	—
	Advantame 10,000 ppm	r/ SD (CD)	30	PND 112	NS*	NS (G,L) *	NS (G,L) *	NS	NS	NS	NS (C,G,L)	—
	Advantame 50,000 ppm	r/ SD (CD)	30	PND 112	NS*	NS (G,L) *	NS (G,L) *	NS	NS	NS	NS (C,G,L)	—
Curry 2008	Reb A 7500 ppm	r/ HWS	30	PND 112	—	NS (L)†	—	NS	NS	—	NS (C,G,L)	—
	Reb A 12,500 ppm	r/ HWS	30	PND 112	—	NS (L)†	—	NS	NS	—	NS (C,G);↑9 (L)	—
	Reb A 25,000 ppm	r/ HWS	30	PND 112	—	NS (L)†	—	NS	NS	—	NS (C,G);↑9 (L)	—
Kille 2000	Sucralose 0.3% Fa	r/ SD (CD)	30	—	↓6	↓6 (G)	↓8 (G)	NS	NS	—	—	—
	Sucralose 1.0% Fa	r/ SD (CD)	30	—	↓9	↓8 (G)	NS (G)	NS	NS	—	—	—
	Sucralose 3.0% Fa	r/ SD (CD)	30	—	↓14	↓14(G)	NS (G)	NS	NS	—	—	—
	Sucralose 0.3% Fb	r/ SD (CD)	30	—	↓6	↓6 (G)	↓11 (G)	NS	NS	—	—	—
	Sucralose 1.0% Fb	r/ SD (CD)	30	—	↓7	↓6 (G)	↓12 (G)	NS	NS	—	—	—
	Sucralose 3.0% Fb	r/ SD (CD)	30	—	↓8	↓13 (G)	↓9 (G)	NS	NS	—	—	—
Reilly 1990	Aspartame 500mg/kg	r/ SD	—	—	NS	NS (G,L) *	NS (G,L)	NS*	NS*	—	—	NS
Holder 1987	Aspartame 0.007% (w/ v)	r/ SD	10	—	NS	NS (G,L)	—	—	NS*	—	—	—
	Aspartame 0.036% (w/ v)	r/ SD	10	—	NS	NS (G,L)	—	—	NS*	—	—	—
	Aspartame 0.18% (w/v)	r/ SD	10	—	NS	NS (G,L)	—	—	NS*	—	—	—
	Aspartame 0.9% (w/v)	r/ SD	10	—	NS	NS (G,L)	—	—	NS*	—	—	—
Brunner 1979	Aspartame 2% diet	r/ SD	—	—	NS*	NS (G)	—	—	NS*	—	NS*	—
	Aspartame 4% diet	r/ SD	—	—	NS*	NS (G)	—	—	NS*	—	NS*	—
	Aspartame 6% diet	r/ SD	—	—	NS*	NS (G)	—	—	NS*	—	NS*	—
Lapointe 1979	Na Saccharin 0.4mg/ml	r/ SD	5	—	—	—	NS (G,L)	—	NS	—	NS*	↑*
Neoplastic studies												
Soffritti 2016	Sucralose 500 ppm	m /swiss	20-30	PND 91	—	—	—	NS	—	—	—	—
	Sucralose 2000 ppm	m /swiss	20-30	PND 91	—	—	—	NS	—	—	—	—
	Sucralose 8000 ppm	m /swiss	20-30	PND 91	—	—	—	NS	—	—	—	—
	Sucralose 16,000 ppm	m /swiss	20-30	PND 91	—	—	—	NS	—	—	—	—
Soffritti 2010	Aspartame 2000 ppm	m /swiss	20-30	PND 91	—	—	—	NS	—	—	—	—
	Aspartame 8000 ppm	m /swiss	20-30	PND 91	—	—	—	NS	—	—	—	—
	Aspartame 16,000 ppm	m /swiss	20-30	PND 91	—	—	—	NS	—	—	—	—
	Aspartame 32,000 ppm	m /swiss	20-30	PND 91	—	—	—	NS	—	—	—	—
Soffritti 2007	Aspartame 400 ppm	r /SD	12-16	—	—	—	—	—	—	—	—	—
	Aspartame 2000 ppm	r /SD	12-16	—	—	—	—	—	—	—	—	—
Cohen 1995	Na Saccharin 0.4 mg/ml *Exp1*	r/ SD	17	PND 70	NS	↓8 (G)	—	NS	NS	NS	↓*	↑
	Na Saccharin 0.4 mg/ml *Exp1*	r/ F344	5	PND 70	NS	NS (G)	—	NS	NS	NS	↓*	↑
	Na Saccharin 0.4 mg/ml *Exp2*	r/ SD	40	PND 70	NS	NS(G);↑(L)	—	NS	NS	NS	↓*	↑

(*continued on next page*)

Table 3 (*continued*)

Study ID/Year	Experimental Group	Species/ Strain	*n*	Conception Age	Gestation and Lactation BW	BW Gain	Pups/ Litter	Litter size	Sex ratio	Food intake	Fluid intake
Garland 1991	Na Saccharin 7.5% diet	r /SD	30	PND 70-84	↓10*	↓ (G,L) *	NS(G);↑(L) *	NS	NS	NS	↓(C); NS (G,L)	↑
Taylor 1980	Na Saccharin 0.01% diet	r /SD (CD)	48	PND 70	—	—	—	—	—	—	—	—
	Na Saccharin 0.1% diet	r /SD (CD)	48	PND 70	—	—	—	—	—	—	—	—
	Na Saccharin 1.0% diet	r /SD (CD)	48	PND 70	—	—	—	—	—	—	—	—
	Na Saccharin 5% diet	r /SD (CD)	48	PND 70	—	—	—	—	—	—	—	—
	Na Saccharin 7.5% diet	r /SD (CD)	48	PND 70	—	—	—	—	—	—	—	—

Abbreviations: BW – body weight; n – sample size; m – mouse; r – rat; ICR – Institute of Cancer Research; WS – Wistar; HWS – Hans Wistar; SD – Sprague Dawley; SD (CD) – Sprague Dawley (caesarean derived strain); NS – not significant; *preg* – pregnancy group; *lact* – lactation group; C – conception; G – gestation day 21; L – lactation day 21; Ace K – acesulfame potassium; Reb A – rebaudioside A; Na saccharin – sodium saccharin; mg – milligram; g – gram; kg – kilogram; mM – millimolar; ml – millilitre; L – litre; ADI – acceptable daily intake; w/v – weight per volume; ppm – parts per million; Fa – parent generation a group; Fb – parent generation b group; *Exp1* – experimental group 1; *Exp2* – experimental group 2; (↓x) or (↑x) denotes reported percent change from the control group; (↓) or (↑) denotes reported change without magnitude; (—) not recorded; (L)†- taken on PND28; * - Data not provided.

3.3.3. Litter outcomes

Data relating to litter outcomes were extracted from 16 studies. Here, we describe the following litter outcomes associated with maternal NNS exposure: the number of live pups per litter, litter size and sex ratio. 15 experimental groups were included in the meta-analysis with no effect identified (SMD = −0.15, 95% CI = −0.35, 0.05, I^2 = =0%; Figure shown in S11. Litter Meta-analysis).

3.4. Offspring results

Detailed study characteristics, BW at birth, weaning and adulthood, BW gain and body composition for offspring are shown in Table 4. Multiple maternal interventional groups resulted in 59 offspring data sets being included. However, data were not always reported for each outcome. Only a small number of studies (5/24) investigated glycaemic control outcomes. These results are presented separately (Table 5).

3.4.1. Offspring birthweight

For the purpose of this study, we defined birthweight as an offspring's weight measured at either GD0, GD1 or during foetal morphological assessment. Data was extracted from 30 groups. However, there was insufficient information available on sample size, SD or SEM for meta-analyses. Only one experimental group reported a reduction in birthweight for males and females [43]. This was the highest concentration group in a toxicity study on arruva, a monatin salt isomer sweetener. All other groups reported no significant effect on birthweight.

3.4.2. Offspring bodyweight at weaning

Over half the studies (13/24) reported offspring's BW at weaning (PND21). Further data sets were obtained when separating groups by sex. Overall, 18 experimental groups gave rise to 1123 offspring included in the meta-analysis (Figure 3A). No significant effect in an offspring's BW at weaning was observed; however, high heterogeneity was noted (SMD = −0.31; 95% CI = − =0.67, 0.05; I^2 = =86%). Further exploration by subgroup analysis for sex (Fig. 3B) found a significant decrease in BW for both males (SMD = − =0.70; 95% CI = − =1.19, −0.21; I^2 = =72%;) and females (SMD = − =0.84; 95% CI = − =1.36, −0.32; I^2 = =68%;) when measured at PND21 (Fig. 3B). Studies with only combined-sex groups identified no effect (SMD = 0.28; 95% CI = − =0.09, 0.65; I^2 = =68%). When sub grouped by species (Fig. 3C), only rats displayed a small yet significant reduction in BW at weaning (SMD = − =0.54; 95% CI = − =1.00, −0.07; I^2 = =84%) whereas mice displayed no effect (SMD = 0.07; 95% CI = 0.13, 0.27; I^2 = =0%). Of note, mice data were extracted from behavioural preference studies of lower dose acesulfame potassium exposure during gestation and lactation, whereas the majority of rat data were extracted from toxicological studies of various sweeteners at significantly higher dosages.

3.4.3. Offspring bodyweight at adulthood

Data was available for the majority of papers (18/24) and the general meta-analysis included 646 offspring. Overall, we identified a significant reduction in offspring BW measured during adulthood following maternal exposure to NNS during pre-pregnancy, pregnancy and/or lactation (SMD = − =0.95; 95% CI = − =1.64, −026; I^2 = =92%) as seen in Fig. 4A. Sub-group analyses determined that females (SMD = − =2.44; 95% CI = − =4.52, −0.36; I^2 = =94%) maintained the BW reduction into adulthood, whereas the males (SMD = − =0.47; 95% CI = − =1.39, 0.44; I^2 = =86%) and combined-sex groups displayed no effect (SMD = − =0.92; 95% CI = − =2.14, 0.30; I^2 = =95%) (Fig. 4B.). Although the results may be skewed by data from one study reporting significant BW loss in females [20]. Sub-grouped by species, mice identified no significant

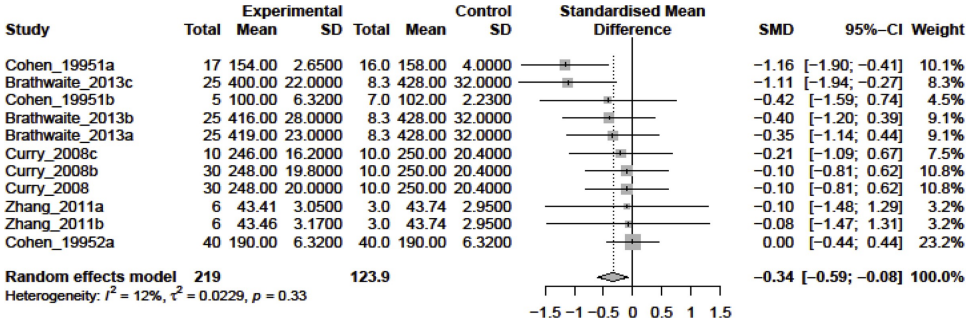

Fig. 2. Meta-analysis of maternal outcomes for body weight. Maternal BW measured at approximately GD21. Overall, standardised mean difference (SMD) and 95% confidence interval (CI; upper; lower limits) were estimated by random effects meta-analysis.

Table 4
Offspring outcomes for body weight at birth, weaning and adulthood, body weight gain and body composition.

Study ID/Year	Experimental Group	Species/Strain	*n*	Body weight / Growth						Body Composition
				Bwt (~PND 0)	Weaning (~PND 21)	Adult	Age adult	BW gain	Age BW gain	
Sweet taste preference studies										
Choo 2018	Sucralose 5.3 mg/kg BW/day	m/ C57BL/6	21	—	—	↓7(M); NS (F)	PND 56	—	—	—
Li 2013	Ace K 5 mM	m/ ICR	56	—	NS	NS	PND 49	—	—	—
	Ace K 12.5 mM	m/ ICR	56	—	NS	NS	PND 49	—	—	—
	Ace K 25 mM	m/ ICR	56	—	NS	↓5	PND 49	—	—	—
	Ace K 50 mM	m/ ICR	56	—	NS	↓10	PND 49	—	—	—
Zhang 2011	Ace K 5 g/kg (*preg*)	m/ ICR	71	NS	NS	NS (M)	PND 56	—	—	—
	Ace K 5 g/kg (*lact*)	m/ ICR	69	NS	NS	NS (M)	PND 56	—	—	—
Metabolic and behavioural studies										
Zhang 2018	Saccharin 2% (w/v)	m/ C57BL/6	15	NS*	NS*	NS*	PND 90	—	—	—
Olivier-VS 2019	Sucralose/Ace K ADIx1	m/ C57BL/6	30	NS	NS	—	—	—	—	—
	Sucralose/Ace K ADIx2	m/ C57BL/6	30	NS	↓10	—	—	—	—	—
Collison 2016	Aspartame 0.25g/L (M only)	m/ C57BL/6J	18	—	—	↑9 (M)	PND 140	—	—	↑32(M)∞
Parlee 2014	Saccharin 3% (w/v)	m/ C57BL/6J	25	—	NS (M);↓11(F)	NS (M);↓10(F)	PND 98	—	—	↓(M);NS(F)¤
Collison 2012a	Aspartame 0.25g/L	m/ C57BL/6J	12	—	—	NS	PND 119	↑36 (M); NS(F)	PND 42-119	↑167(M)∞
Collison 2012b	Aspartame 0.25g/L	m/ C57BL/6J	18	—	—	NS	PND 119	↑11 (M); NS(F)	PND 42-119	NS∞
von Poser Toigo 2015	Aspartame 2g/L	r/ WS	16	—	—	—	—	↑190 (M); NS(F)‡	PND 80-110‡	NS‡^
	Saccharin 1.35g/L	r/ WS	16	—	—	—	—	NS (M;F) ‡	PND 80-110‡	NS‡^
Toxicology studies										
Brathwaite 2013	Arruva 15,000 ppm	r/ SD	370	NS (M;F)	—	—	—	—	—	—
	Arruva 30,000 ppm	r/ SD	368	NS (M;F)	—	—	—	—	—	—
	Arruva 50,000 ppm	r/ SD	363	↓9(M); ↓8(F)	—	—	—	—	—	—
Otabe 2011	Advantame 2000 ppm	r/ SD (CD)	395	NS*	NS*	NS*	—	NS*	—	—
	Advantame 10,000 ppm	r/ SD (CD)	386	NS*	NS*	NS*	—	NS*	—	—
	Advantame 50,000p pm	r/ SD (CD)	354	NS*	NS*	NS*	—	NS*	—	—
Curry 2008	Reb A 7500 ppm	r/ HWS	25-30	NS (M;F)	NS (M;F)	—	—	—	—	—
	Reb A 12,500 ppm	r/ HWS	25-30	NS (M;F)	NS (M;F)	—	—	—	—	—
	Reb A 25,000 ppm	r/ HWS	25-30	NS (M;F)	↓6(M); ↓8(F)	—	—	—	—	—
Kille 2000	Sucralose 0.3% Fa	r/ SD (CD)	339	NS	NS†	NS (M); ↓8(F)	PND 70	↓7(M); ↓9(F)	PND 0-70	—
	Sucralose 1.0% Fa	r/ SD (CD)	370	NS	NS†	NS (M); ↓9(F)	PND 70	NS (M); ↓11(F)	PND 0-70	—
	Sucralose 3.0% Fa	r/ SD (CD)	364	NS	NS†	NS (M); ↓11(F)	PND 70	↓7(M); ↓12(F)	PND 0-70	—
	Sucralose 0.3% Fb	r/ SD (CD)	364	NS	NS†	—	—	—	—	—
	Sucralose 1.0% Fb	r/ SD (CD)	370	NS	NS†	—	—	—	—	—
	Sucralose 3.0% Fb	r/ SD (CD)	435	NS	NS†	—	—	—	—	—
Reilly 1990	Aspartame 500 mg/kg	r/ SD	—	—	NS	—	—	—	—	—
Holder 1987	Aspartame 0.007% (w/v)	r/ SD	—	—	NS††	NS	PND 38	—	—	—
	Aspartame 0.036% (w/v)	r/ SD	—	—	NS††	NS	PND 38	—	—	—
	Aspartame 0.18% (w/v)	r/ SD	—	—	NS††	NS	PND 38	—	—	—
	Aspartame 0.9% (w/v)	r/ SD	—	—	NS††	NS	PND 38	—	—	—
Brunner 1979	Aspartame 2% diet	r/ SD	—	—	—	—	—	—	—	—
	Aspartame 4% diet	r/ SD	—	—	—	—	—	—	—	—
	Aspartame 6% diet	r/ SD	—	—	—	↓30(M); ↓14(F)	PND 30(M); 44(F)	—	—	—
Lapointe 1979	Na Saccharin 0.4 mg/ml	r/ SD	111	NS (M;F)	—	—	—	NS*	—	—
Neoplastic studies										
Soffritti 2016	Sucralose 500 ppm	m /swiss	219	—	—	NS	PND 56	—	—	—
	Sucralose 2000 ppm	m /swiss	140	—	—	NS	PND 56	—	—	—
	Sucralose 8000 ppm	m /swiss	134	—	—	NS	PND 56	—	—	—
	Sucralose 16,000 ppm	m /swiss	131	—	—	NS	PND 56	—	—	—
Soffritti 2010	Aspartame 2000 ppm	m /swiss		—	—	NS	PND 56	—	—	—
	Aspartame 8000 ppm	m /swiss	—	—	—	NS	PND 56	—	—	—
	Aspartame 16,000 ppm	m /swiss	—	—	—	NS	PND 56	—	—	—
	Aspartame 32,000 ppm	m /swiss	—	—	—	NS	PND 56	—	—	—

(*continued on next page*)

Table 4 (*continued*)

Study ID/Year	Experimental Group	Species/Strain	*n*	Body weight / Growth		Adult	Age adult	BW gain	Age BW gain	Body Composition
				Bwt (~PND 0)	Weaning (~PND 21)					
Soffritti 2007	Aspartame 400 ppm	r /SD	—	—	—	NS	PND 112	—	—	—
	Aspartame 2000 ppm	r /SD	—	—	—	NS	PND 112	—	—	—
Cohen 1995	Na Saccharin 0.4 mg/ml *Exp1*	r/ SD	29	NS (M;F)	NS (M;F)	↓30(M)	PND 91	—	—	—
	Na Saccharin 0.4 mg/ml *Exp1*	r/ F344	23	NS (M;F)	—	NS	PND 91	—	—	—
	Na Saccharin 0.4 mg/ml *Exp2*	r/ SD	40	NS (M;F)	↓19(M); ↓18(F)	—	—	—	—	—
Garland 1991	Na Saccharin 7.5% diet	r /SD	287	NS (M;F)	↓40(M); ↓36(F)	↓36(M); ↓39(F)	PND 30	—	—	—
Taylor 1980	Na Saccharin 0.01% diet	r /SD (CD)	20	NS (M;F)*	NS (M;F)*	NS (M;F)*	PND 182	NS	PND 0-210	—
	Na Saccharin 0.1% diet	r /SD (CD)	20	NS (M;F)*	NS (M;F)*	NS (M;F)*	PND 182	NS	PND 0-210	—
	Na Saccharin 1.0% diet	r /SD (CD)	20	NS (M;F)*	NS (M;F)*	NS (M;F)*	PND 182	NS	PND 0-210	—
	Na Saccharin 5% diet	r /SD (CD)	20	DNC	DNC	DNC	PND 182	NS	PND 0-210	—
	Na Saccharin 7.5% diet	r /SD (CD)	20	DNC	DNC	DNC	PND 182	↓7(M); NS (F)	PND 0-210	—

Abbreviations: Ace K – acesulfame potassium; ADI – acceptable daily intake; Age adult – age at time of adult BW measurement; age BW gain – number of days BW gain measured; BW – body weight; Bwt – birthweight; DNC – data not clear; *Exp1* – experimental group 1; *Exp2* – experimental group; Fa – parent generation a group; Fb – parent generation b group; g – gram; HWS – Hans Wistar; ICR – Institute of Cancer Research; kg – kilogram; L – litre; m – mouse; mg – milligram; mM – millimolar; ml – millilitre; n – sample size; Na saccharin – sodium saccharin; NS – not significant; ppm – parts per million; PND – post natal day; r – rat; Reb A – rebaudioside A; SD – Sprague Dawley; SD (CD) – Sprague Dawley (caesarean derived strain); w/v – weight per volume; WS – Wistar; (↓x) or (↑x) denotes reported percent change from the control group; (↓) or (↑) denotes reported change without magnitude; (—) not recorded; † -weight taken on PND25; †† - weight taken on PND26; ‡ -weight gain reported after 30 days of chocolate consumption *ad libitum*; ^ - abdominal fat pad weights; ∞ - white adipose tissue; ¤ - nuclear magnetic resonance (NMR) analysis

change (SMD = − −=0.50; 95% CI = − −=1.21, 0.21; $I^{2\ =}$ =92%) and rats showed a considerable decrease (SMD = − −=3.05; 95% CI = − −=5.10, −1.00; $I^{2\ =}$ =85%), although significant heterogeneity still existed (Fig. 4C). Variability in the timing of measuring adulthood BW ranged from 30 to 140 days of age and meta-regression modelling found this had no detectable effect on BW as an outcome, $p = 0.0727$. Sub-group analysis for industry funding as a source of potential bias showed that papers with industry funding generally found no effect on body weight (SMD = − −=0.50; 95% CI = − −=1.21, 0.21; $I^{2\ =}$ =92%) and papers with no industry funding generally found reduced body weights (SMD = − −=3.05; 95% CI = − −=5.10, −1.00; $I^{2\ =}$ =85%). See Fig. 4D.

3.4.4. Offspring bodyweight gain and body composition

Bodyweight gain was determined by measuring changes in body weight over a specified time-period. Four papers (17 datasets) reported on BW gain with three datasets reporting a reduction in males and females [46]. In direct contrast, two papers [17,18] reported males having substantial increases in offspring BW gain compared to control (11–36%) and a third reported a dramatic increase of 190% following consumption of novel food (chocolate) over a 30-day period [42]. Body composition was assessed either directly by nuclear magnetic resonance analysis [20] or indirectly by visceral fat mass following resection of abdominal fat pads [17–19,42]. Parlee et al. [20] reported elevated lean mass in male offspring at 8 and 13 weeks of age with concurrent fat mass reduction. No change was evident for females even though there was a reported decrease in female BW beginning postnatal week three. A substantial elevation in visceral fat was observed in male offspring at 20 weeks of age by the Collison group [17,19], corresponding with increases in BW and BW gain.

3.4.5. Offspring glycaemic control outcomes

Limited evidence from four papers provided data on fasting or random-fed glucose levels measured in blood or plasma in offspring. Collison et al. [17–19] reported substantial increases in glucose levels in both male (49–2%) and female (25–60%) mice exposed to aspartame from 21 days pre-gestation to the end of weaning. Here, the offspring were exposed to NNS after they were weaned from dams until approximately 20 weeks of age. Similarly, von Poser Toigo et al. [42] found an increase in fasting blood glucose levels (FBGL) in offspring of mothers fed aspartame (males 26%; females 29%); although in this case, exposure lasted from 30 days pre-gestation to the end of pregnancy and was investigated in rats. They also reported no significant changes in FBGL for a saccharin-exposed group. Differing from these results, Olivier-Van Stichelen et al. [41] observed a significant decrease in glucose levels in mice where dams were exposed to a combination of sucralose and acesulfame potassium during gestation and lactation, however FBGL was measured at a considerably earlier age (PND20) compared to the previous studies (PND112-133). Two papers [17, 19] performed random-fed insulin tolerance tests (ITT) on mice aged 19 weeks. Measured as total area under the curve, results displayed a sexually dimorphic response with males displaying increased insulin levels (20–26%) relative to the control group. (See Table 5).

3.4.6. Offspring food and fluid intake

Large variations were observed in measurement methodologies in the eleven papers reporting offspring food intake and nine papers offspring fluid intakes. Several provided data for weekly consumption from birth to cull [20,25,26,52,53], reported as either weekly or cumulative mean intake, whereas others measured at single [19,21] or dual time-points [17,18]. The majority of studies calculated the intake by dividing the overall cage consumption by the number of animals per cage [17,18,25,26,42,53], and commonly, the unit of measurement was not clear [17,20,21,52]. A full summary of food and fluid intakes is provided in Supplementary files (S12. and S13. Table. Offspring food and fluid intakes). With regard to food consumption, no significant

Table 5
Offspring outcomes for glycaemic control.

Study ID	Experimental group	Species/strain	*n*	Age	FGL	RF GL	FPI	RF ITT AUCtotal
Olivier-VS 2019	Sucralose/Ace K ADIx1	m/ C57BL/6	30	PND 20	NS	—	—	—
	Sucralose/Ace K ADIx2	m/ C57BL/6	30	PND 20	↓9	—	—	—
Collison 2016	Aspartame 0.25 g/L (males)	m/ C57BL/6J	18	PND 133	—	↑10 (M)	NS(M)	↑26 (M)
Collison 2012a	Aspartame 0.25 g/L	m/ C57BL/6J	18	PND 133	↑49 (M); ↑25 (F)	↑20 (M); NS(F)	NS(M;F)	↑21 (M); NS(F)
Collison 2012b	Aspartame 0.25 g/L	m/ C57BL/6J	18	PND 133	↑62 (M); ↑60 (F)	*	*	*
Vp Toigo 2015	Aspartame 2 g/L	r/ WS	16	PND 112	↑26 (M); ↑29 (F)	—	—	—
	Saccharin 1.35 g/L	r/ WS	16	PND 112	NS (M;F)	—	—	—

Abbreviations: Ace K – acesulfame potassium; ADI – acceptable daily intake; Age – age of glycaemic control measurement; AUCtotal – area under the curve total; F – female; FGL – fasting glucose levels; FPI – fasting plasma insulin; g – grams; ITT – insulin tolerance test; L – litre; M – male; m – mouse; n – sample size; NS – not significant; PND – post natal days; r – rat; FR – random-fed; WS – Wistar; (↓x) or (↑x) denotes reported percent change from the control group; (↓) or (↑) denotes reported change without magnitude; (—) not recorded; *Data reported for same group of animals as Collison (2012a).

difference was reported in nine of the eleven studies, one observed a small decrease in the first three days post weaning that recovered by PND30 [51] and another reported males reduced their food intake across the first 24 weeks of life [52]. Interestingly, both studies exposed offspring to high concentrations of sodium saccharin (7.5% of diet) for up to 49 days pre-gestation and continued the intervention post-weaning through to adulthood. Similarly, fluid consumption data was not reported in 15 papers, and in the studies that did report it, the majority (8/9 papers) observed no effect relative to control.

3.4.7. Sweet taste preference outcomes

Only three papers [21–23] reported outcomes relating to sweet taste acceptance and preference in offspring treated with NNS prenatally. As previously described by Myers and Sclafani [56], preference is considered the choice of one flavour over another and can be measured by brief behavioural assays, whereas acceptance is the absolute amount of a tastant consumed, which is commonly measured over longer periods such as 24 or 48 hours (h). For the purpose of this review, we will refer to 48-h two-bottle testing as 'acceptance' testing. The first two papers, authored by the same group, examined the developmental modulation in adult offspring's sweet taste responses following maternal exposure to acesulfame potassium when mixed in drinking water during either pregnancy or lactation [22] or lactation alone [23]. Their initial study reported a reduction in the preference threshold for acesulfame potassium in both pregnancy and lactation groups relative to control during a 48-h acceptance test, suggesting lower concentrations elicit a sweet taste [22]. Preference ratios were observed to be higher than control. Their subsequent study investigated multiple concentrations administered in chow and fed to dams during lactation [23]. In direct contrast, they reported an increase in the preference threshold, suggesting higher concentrations are required to elicit a sweet taste. Observed preference ratios were reduced, particularly in the low to moderate concentrations. Notably, acesulfame K dosage provided to dams significantly exceeded human ADI guidelines. The final paper investigating sweet taste preference [21] had two key differences from the other described work. First, the sweetener sucralose was fed to dams during both pregnancy and lactation and second, the dosage was more relevant to human ADI. This paper also included a second comparator group, being sucrose. The authors reported no difference in response to sweet taste preference during a brief access lickometer test and no change in total intake or preference ratio during a two-bottle 48-h acceptance test to either sucralose or sucrose.

3.5. Study quality assessment

3.5.1. Risk of bias

The results of bias assessment are presented in Fig. 5. Results are reported as the percentage of papers per item categorised as low risk of bias (yes), high risk of bias (no) or unclear risk of bias.

Three items (1 to 3) related to selection bias. Only two (8%) of the included papers reported randomisation of animal allocation to create comparable groups using adequate methods, such as computer-generated randomisation or number tables. 11 papers reported using randomisation yet failed to describe the method, therefore were judged 'unclear'. The remaining 11 papers did not mention randomisation at all. Eight (33%) of the papers described similar baseline characteristics for maternal age and weight or weight matching of groups. None of the 24 included papers reported concealing allocation to treatment groups from the investigators. Performance bias relates to systematic differences in the management of care provided to experimental groups [32]. In animal studies, this involves husbandry and blinding of caregivers to an animals' interventional group. The potential for bias was judged high as none of the papers described randomly housing animals and only one paper (4%) described blinding investigators during the study (question 4 and 5).

Detection bias (question 6 and 7) may be introduced by differences in the way outcomes are assessed. Only three (13%) of included papers described randomly selecting animals for outcome assessment, and only one (4% of papers) described blinding the assessor.

Concerning attrition bias (question 8), over two thirds of studies indicated a reason for the observed attrition or exclusion of data, yet 25% were unclear. The majority of studies were assessed as free of selective outcome reporting by comparing methods and results within the paper (question 9). However, it must be noted that no paper made the study protocol available, limiting the reliability of this assessment. Sixteen (67%) papers were assessed as free from potential risk of bias due to industry funding sources (question 10) and one (4%) was unclear. Individual study results for risk of bias can be found in the Supplementary files (S14. Table RoB Results).

3.5.2. Reporting adherence to ARRIVE guidelines

Generally, low to moderate adherence to the guidelines was observed in the majority of studies, with only one item (appropriate title) judged as fully reported. Of concern, two items were assessed as 100% partially reported: item 6 (study design) and item 18 (interpretation and scientific implications). This was predominantly due to lack of reporting of blinding measures, unit of analysis, animal model limitations and study implications for the 3R's in animal research.

Several important items were less than 60% fully reported, including experimental procedures (54% FR; 46% PR), experimental animals (21% FR; 79% PR); mostly due to lack of reported dam details, housing and husbandry (92% PR; 8% NR), sample size (4% FR; 83% PR; 13%NR), allocation of animals to experimental groups (4% FR; 58% PR; 38% NR), statistical methods (50%; 46% PR; 4% NR), baseline data (38% FR; 21% PR; 42% NR) and generalisability/translation (54% FR, 25% PR, 21% NR). Overall adherence of the ARRIVE guidelines per item and individual study assessment can be found in the supplementary files (S15 and S16. Table ARRIVE Adherence Results).

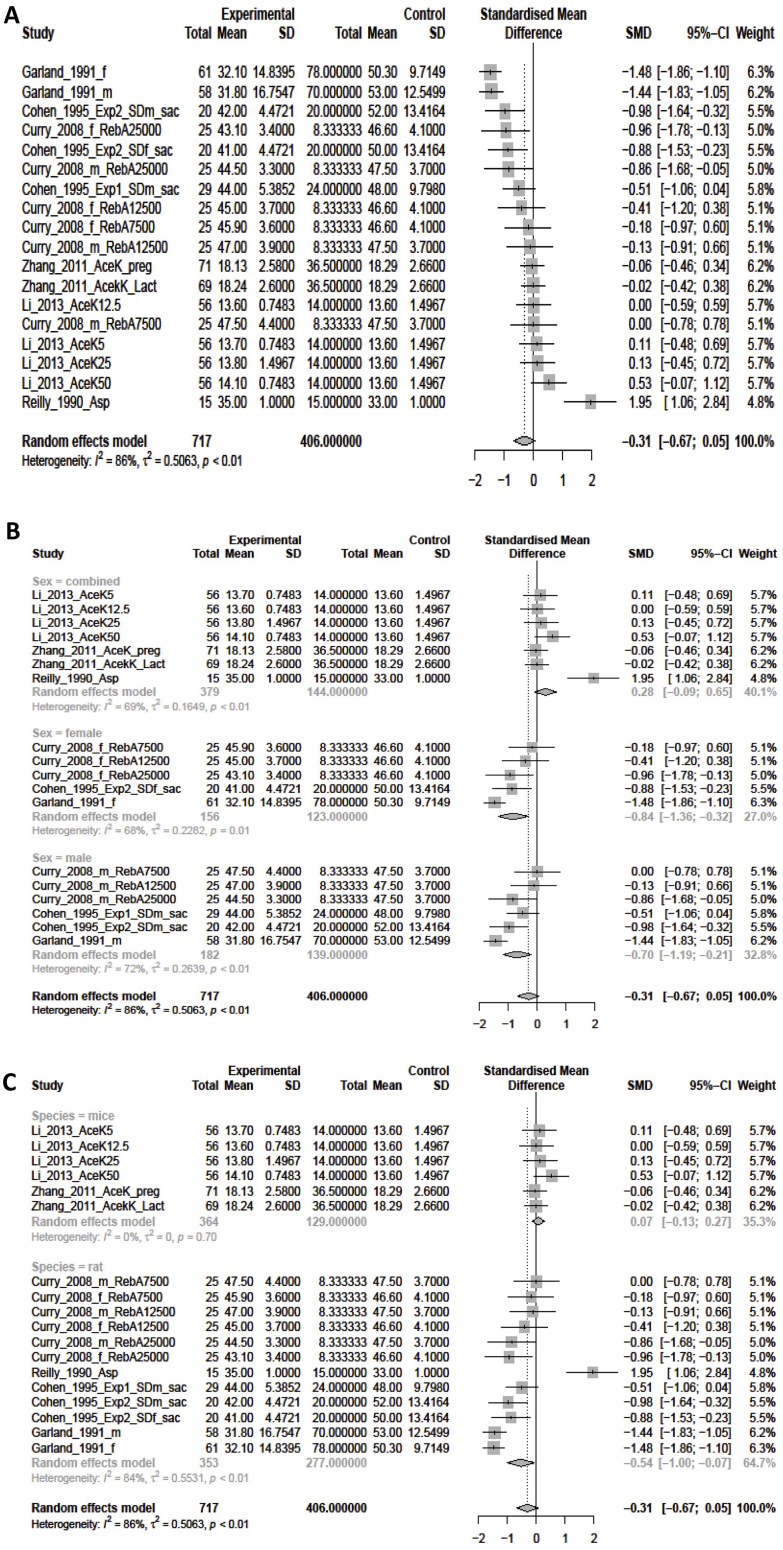

Fig. 3. Meta-analysis of wean bodyweight in 1123 included offspring exposed to maternal NNS diets. **A**. combined sex BW, **B**. offspring BW subgroup analysis for sex; separated by males, females and combined sex groups, **C**. offspring subgroup analysis for species; separated by rat and mouse. Estimated standardised mean differences (SMD) and 95% confidence interval (CI; upper; lower limits) were estimated by random effects analysis with heterogeneity expressed as I^2 and tau^2.

4. Discussion

This comprehensive systematic review and meta-analysis was conducted to identify metabolic and behavioural effects in offspring exposed to non-nutritive sweetened maternal diets in rodent models. Included were papers where NNS was administered *ad libitum* during pre-gestation and pregnancy, pregnancy alone or during lactation. We accepted papers that provided offspring with additional NNS exposure after weaning ceased, to capture relevant data.

Whilst meta-analyses show evidence for minor reductions to BW in dams and adult offspring, it was impossible to disassociate if reduced weight gain was a direct physiological action from NNSs or if

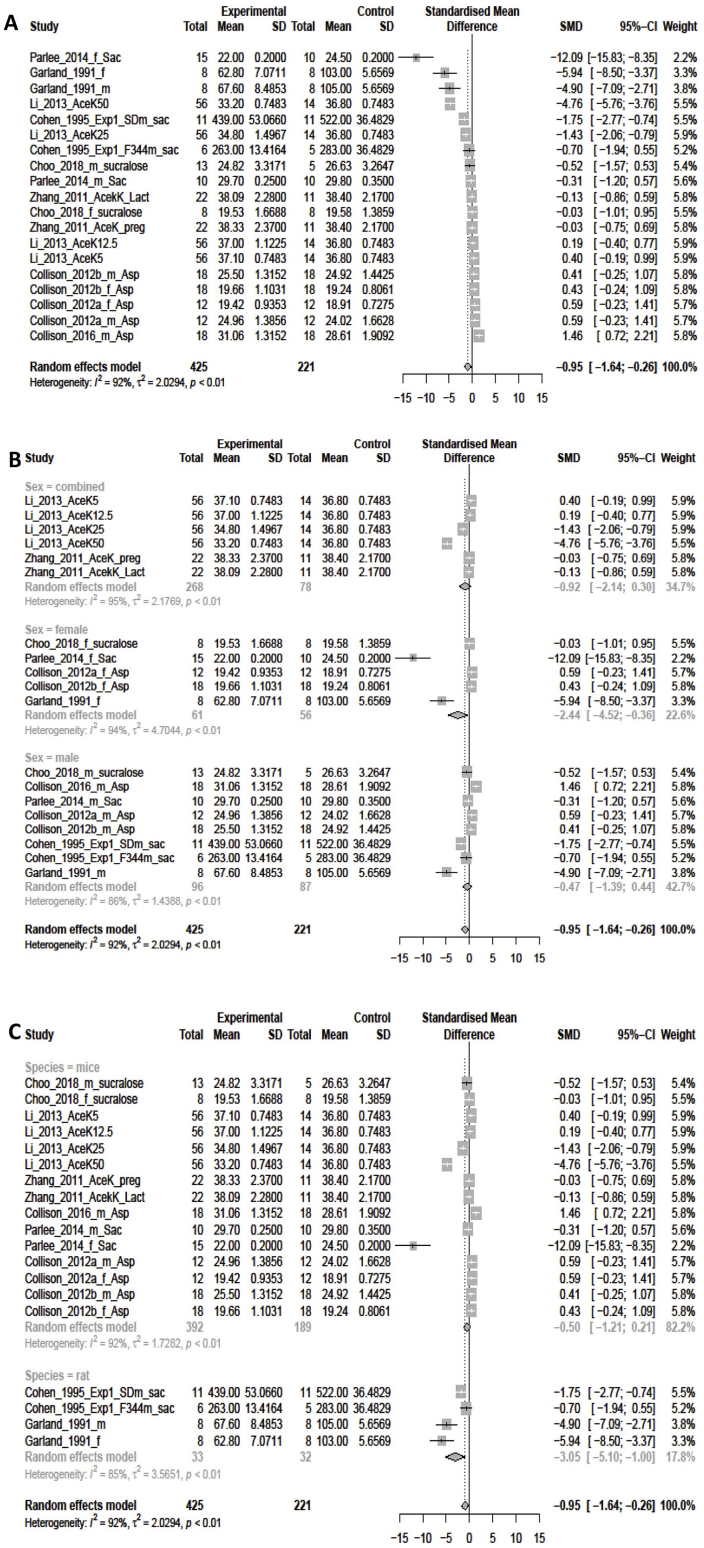

Fig. 4. Meta-analysis of adult bodyweight in 646 included offspring exposed to maternal NNS diets. **A**. Combined sex adult offspring BW, **B**. Subgroup analysis for sex; separated by males, females and combined sex groups, **C**. offspring subgroup analysis for species; separated by rat and mouse. **D.** Subgroup analysis for potential funding risk; separated by industry funded and not industry funded papers. Estimated standardised mean differences (SMD) and 95% confidence interval (CI; upper; lower limits) were estimated by random effects analysis with heterogeneity expressed as I^2 and tau^2.

contributions from unpalatable concentration was a factor. Nevertheless, the bulk of evidence suggest offspring weight did not increase. This outcome is important as it is well known that maternal and childhood obesity are linked to poor metabolic health [57–59]. For other metabolic and behavioural outcomes such as body composition, glycaemic control and sweet taste preference, no overall conclusions could be drawn due to a paucity of data. Moreover, there was significant risk of internal bias across the majority of studies, mainly due to a lack of reporting of randomisation and blinding.

The current analysis was extensive, yet challenged by significant

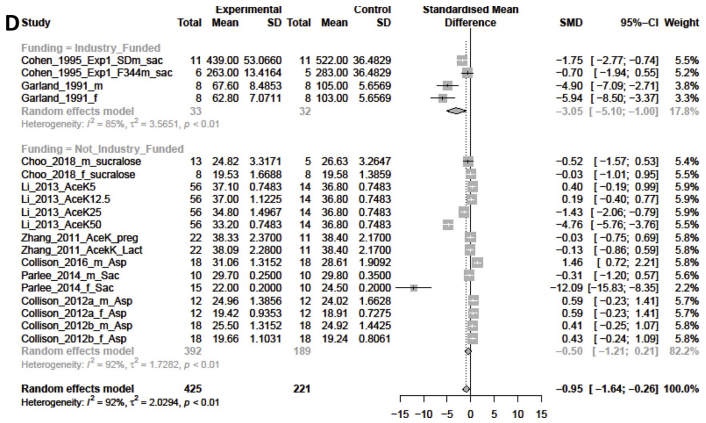

Fig. 4. (*continued*)

variability in study design. Given these limitations and the inconclusive nature of some results, the focus turned to present a broader summary of evidence, including gaps in the current literature and recommendations for the future. Several weaknesses were uncovered in the included studies, which have real potential to impact results. This included inappropriate choice of concentration, NNS type, animal model, timing of dietary administration and study design. Taken together, relevance for human metabolic health was difficult to interpret. Accordingly, guidelines have been proposed for key methodological considerations and priorities in determining prenatal effects of NNS in rodent models.

Whilst we recognise certain papers were specifically designed to inform health bodies on toxicological, reproductive and neoplastic effects during pregnancy by administering extreme concentrations, many papers focusing on metabolic and behavioural effects also used supra-physiological doses [20,23,40,42]. Despite strain differences, rodents in general tend to avoid higher concentrations of NNSs due to their bitter aftertaste [54,55], leading to decreased food or fluid consumption. As previously mentioned, the inclusion of high dosage data sets may have skewed our reported results towards bodyweight reduction. A lack of reported data on either BW, food/fluid intake or concentration meant we were unable to calculate dosage in mg/kg/day to determine if this may be the case. To eliminate this weakness in study design, future researchers should carefully select and report concentrations more relevant to levels used in the human food supply.

Nevertheless, a small number of studies reported an increase in the adiposity of male offspring following maternal consumption of aspartame at levels approximating human ADI in C57BL/6J mice [17–19] and high doses in Wistar rats [42]. Given these particular mouse and rat strains do not perceive aspartame as sweet [60,61], one would assume the observed adiposity was not related to behavioural responses, such as stimulated intake or altered preference for sweet foods. There may be other biological mechanisms to explain the reported findings. Some animal studies suggest alterations to gut hormone secretions [62], appetite regulation [8,63] or the microbiota [9] can have implications on

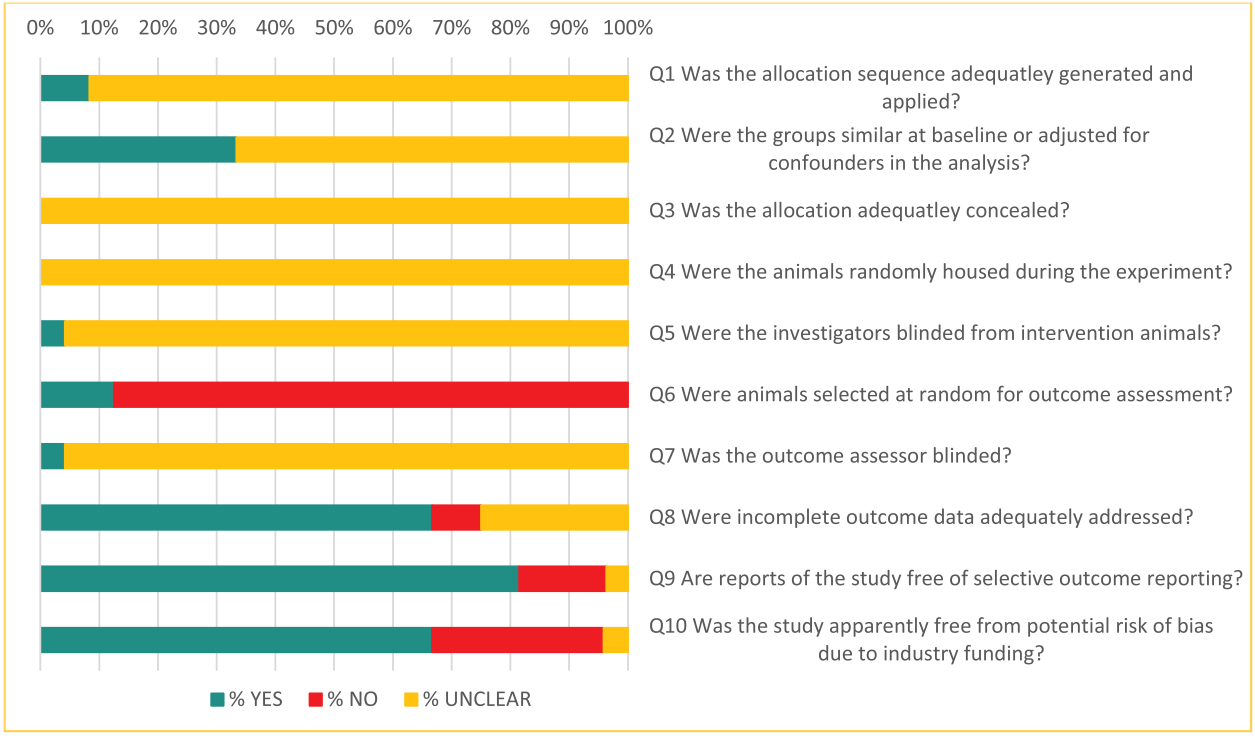

Fig. 5. Risk of Bias reported per item according to SYRCLE's tool [32]. Rationale and further detail for the above items was also reported in this tool. Percentages (0–100%) refer to the percentage of included studies with a particular risk of bias score. Two independent reviewers assessed each study for these items and classified as follows: 'YES' = low risk of bias; 'NO' = high risk of bias; 'UNCLEAR' = unable to estimate / assess risk of bias.

body weight and glycaemic control, although most of this work was conducted using saccharin. Further investigation must take into consideration that individual sweeteners have differing biological pathways, which may in turn display a variety of metabolic responses [64]. For example, some NNSs pass through the gastrointestinal tract unchanged, whilst others are rapidly metabolised or absorbed in the small intestines. (See S9.) This implies that perinatal impacts may also be a function of gastrointestinal permeability to the NNS under investigation and the animal model it presents.

Of the included studies, aspartame and saccharin dominated the investigations, yet these make up only a portion of NNSs in the human food supply and are rarely added to products as the sole sweetener. Furthermore, the majority of papers do not mimic the way humans consume NNS, specifically in sweetener combination or feeding patterns. For example, diet beverages often blend two or more NNSs to enhance the sweet taste profile. Yet effects of blended sweeteners are rarely studied in animal studies, let alone maternal investigations. Surprisingly we identified one maternal paper investigating consumption of blended sucralose and Acesulfame K pipetted onto pellets at physiological concentrations [41]. Of interest was that both sweeteners were detected in the mothers' breastmilk and pups' blood and/or faeces at similar, albeit low concentrations. This suggested NNS transmission occurred in perinatal life. Although the presence of NNS in breastmilk may not be sufficient evidence to predict a physiological effect, the authors observed extensive metabolic and gut microbial changes in offspring, suggesting increased potential risk for future metabolic disease. It would be of significant interest if these findings relate to other combinations of NNSs in beverages and foods; including stevia, which has been rapidly introduced into the food chain in recent years [1]. Moving forward, a valid animal model for consumption of commercially available NNS products, for translational relevance to humans, would be of value to assess in both maternal and non-maternal feeding paradigms.

Timing of dietary manipulation is an equally important aspect of study design in maternal feeding paradigms. This review identified half of the studies extended NNS exposure from prenatal to postnatal periods by administering additional NNS diets to offspring following weaning, the majority being toxicological and neoplastic papers [24–26,44–46,50–53]. However three experiments, focussing on metabolism, fed the offspring aspartame into adulthood (20 weeks of age) [17–19]. Confounding of offspring data due to direct and chronic exposure cannot be excluded. Moreover, this limited the ability to assess a temporal impact of NNS exposure during pre-gestation, gestation and/or lactation. These are critical periods of neonatal development when the rapid rate of DNA synthesis is vulnerable to nutritional influences, and timing may be as important as the insult [10,65]. Although our review focussed specifically on maternal NNS consumption, models of over-nutrition suggest the lactation period to be a crucial period for metabolic developmental programming, and effects may be further heightened when feeding occurs during both gestation and lactation [66,67]. The recommendation to cease exposure at parturition and/or weaning or to utilise a cross-fostering approach may sufficiently achieve the delineation of temporality if hypotheses include developmental programming.

Arguably, the most disappointing aspect of this review was identifying a lack of reporting of maternal results. An early focus was to identify a relationship between maternal diet and offspring effects. Yet our interpretation of the maternal metabolic state and offspring development was bounded by the fact extracted data did not always include a dam and her corresponding offspring. None of the included papers focussing on metabolism and/or behaviour, reported relevant maternal outcomes, such as body weight, body composition, feeding behaviour or glycaemic control [17–20,40,41]. This appears to be a consistent pattern in animal research, with previous systematic reviews of maternal feeding citing a paucity of maternal results with a singular focus on offspring outcomes [66,68,69]. Given the mounting human evidence observing that altered maternal metabolic state is associated with increased risk for childhood adiposity and poor glucose tolerance [11,57–59], it seems not only prudent, but overdue that future animal studies investigating offspring prenatally exposed to NNS also report relevant maternal outcomes.

Due to rising interest in the use of NNS as a sugar replacement, glycaemic control was considered a relevant outcome for this review. With only two high risk studies from the same group performing dynamic assessment for whole body insulin disposal [17,18] and a further three reporting fasting blood glucose levels [19,41,42], we identified a clear gap in the research. Results suggest male mice exposed to aspartame from pre-gestation to 20 weeks of age displayed reduced insulin sensitivity, along with elevated fasting glucose levels during random-fed insulin tolerance tests (ITT). However, we cannot ascertain each animal was in a similar 'fed' state at the commencement of the ITT ensuring consistent baseline values, as reporting on feeding methodology was unclear [17,18]. It is apparent further high quality studies with appropriately reported dynamic assessments are needed to link offspring glucose homeostasis and maternal NNS consumption.

An overarching concern in this review is the high risk of bias demonstrated for the majority of studies following critical appraisal. Additionally, there is potential bias in NNS research due to funding sources [34], which can promote over-estimations of interventional effects [70,71]. As such, future animal studies must ensure appropriate randomisation and blinding methods and adhere to reporting guidelines to improve methodological quality and reduce replication of unnecessary experiments.

This review has several strengths, including rigorous study quality

Table 6
Considerations for future experiments to improve quality and translation.

NNS type and dosage	• Translational consideration is recommended in the selection of NNSs, i.e. widely consumed and ecologically relevant. • The biological pathways of the chosen NNS/s and/or metabolites may determine physiological interactions and perinatal impact. • Concentrations at human physiological levels are recommended for metabolic and behavioural studies.
Animal model	• Species and strain differences exist in perception of sweetness for individual NNSs. This may differ to human perception and post-ingestive responses.
Timing of intervention	• If primary hypotheses involve developmental origins of disease, consideration of timing is critical e.g., exposure pre-gestation, during pregnancy and/or lactation suggested for metabolic programming. Direct exposure in offspring after weaning may confound effects.
Study design	• Randomisation by computerised RNG or similar during allocation and outcome assessment is recommended. • Blinding of allocation to groups and outcomes assessor is recommended, where possible. • Power analysis for appropriate sample size. • Relevant baseline characteristics for dams, e.g. weight, age, glycaemic values, sweet preference ratios etc. • Litter (not individual offspring) used as the unit of analysis.
Potential outcomes to be measured	• Relevant maternal and offspring outcomes to identify potential relationships, e.g. dynamic assessment of glycaemic control, fat mass assessment as measure of adiposity, blood chemistry, microbiota.
Reporting	• Accurate reporting adhering to ARRIVE guidelines for improvement of transparency. • Accurate reporting of randomisation and blinding. • Selection of animal model and its relevance to other biological systems including humans.

assessment, inclusive data capture across extensive primary outcomes, transparency on reporting methodology and inclusion of meta-analyses with subgroup analyses. To our knowledge, this is the first fully comprehensive systematic review summarising all available evidence on this highly relevant topic.

Future recommendations

The current landscape appears to be shifting towards a greater understanding that each NNS has the potential to elucidate its own metabolic or sensory effect. We recommend future systematic reviews investigate maternal consumption of individual sweeteners in animal models, when sufficient evidence becomes available. In addition, we offer important considerations in design, quality and reporting of animal experiments concerning the impact of NNS during pregnancy that were identified in this systematic review (Table 6).

Conclusions

While meta-analyses results suggest maternal NNS consumption during gestation and/or lactation reduced body weight in pregnant dams and their adult offspring, we could not determine if a physiological effect or if unpalatable concentrations contributed to this reduced weight gain. Regardless, the bulk of evidence suggests when aggregated together, NNSs do not increase offspring BW in rodents exposed during prenatal life, which is contrary to human observational findings [11,12]. Limited evidence was available for glycaemic outcomes and sweet taste preferences in offspring. This review advances our understanding on this complex topic by identifying prior study weaknesses and outlining key considerations for future investigations. We hope the information presented assists in guiding future translational research.

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CRediT authorship contribution statement

H.L. Morahan: Conceptualization, Data curation, Formal analysis, Writing - original draft. **C.H.C. Leenaars:** Data curation, Formal analysis, Validation, Writing - review & editing. **R.A. Boakes:** Supervision, Writing - review & editing. **K.B. Rooney:** Conceptualization, Formal analysis, Supervision, Validation, Writing - review & editing.

Declaration of Competing Interest

None.

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Supplementary materials

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Supplementary 1. PRISMA 2009 Checklist

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	3, 4
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	4, 5
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	2,5,7
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	5,6, S.3
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	6, 7
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	S.2
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	6, 7, 8
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	7, 8, S.4, S.5
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	6, 7, 8
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	8, 9, 10, S.6
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	7, 8
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	8

Supplementary 1. PRISMA 2009 Checklist

Section/topic	#	Checklist item	Reported on page #
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	8, 9
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	8
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	10,11, S.7
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	11, 12, S.8, S.9, S.10
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	32, 33, 34, S.14, S.15, S.16
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	15-32
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	16, 24-28
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	32-34
Additional analysis	23	Give result1s of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	S11, 23-26,
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	34, 40
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	34-40
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	34-40
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	41

Supplementary 1. PRISMA 2009 Checklist

From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi:10.1371/journal.pmed1000097

For more information, visit: **www.prisma-statement.org**.

Supplementary 2. Final Search Sets

PubMed Search Set

PubMed was searched using the following combination of Medical Subject Headings (MeSH) terms and relevant free terms in title and/or abstract [Tiab]. In addition, an adaptation from the SYRCLE PubMed animal filter (Hoojiman et. al 2010) designed to maximise retrieval of all animal experiments in rodent models was used.

(Epigenesis, Genetic[Mesh:noexp] OR Embryonic and Fetal Development[Mesh:noexp]) OR Epigenomics[Mesh] OR Embryonic Development[Mesh:noexp] OR Fetal Development[Mesh:noexp] OR Fetal Weight[Mesh] OR Fetal Nutrition Disorders[Mesh] OR Gene-Environment Interaction[Mesh] OR Phenotype[Mesh:noexp] OR "Foetal development"[Title/Abstract] OR "Fetal development"[Title/Abstract] OR "Foetal programming"[Title/Abstract] OR "Fetal programming"[Title/Abstract] OR Epigenetic[Title/Abstract] OR Offspring[Title/Abstract] OR "Phenotyp* programming"[Title/Abstract] OR "Phenotyp* development"[Title/Abstract] OR "Developmental programming"[Title/Abstract] OR "Epigenetic inheritance"[Title/Abstract]) AND (Non-Nutritive Sweeteners[Mesh] OR Sweetening Agents[Mesh] OR Stevia[Mesh] OR stevioside [Supplementary Concept] OR rebaudioside A [Supplementary Concept] OR Saccharin[Mesh] OR Aspartame[Mesh] OR Sugar Alcohols[Mesh] OR Sorbitol[Mesh:NoExp] OR Mannitol[Mesh:NoExp] OR Erythritol[Mesh:NoExp] OR Xylitol[Mesh] OR lactitol [Supplementary Concept] OR Dithioerythritol[Mesh] OR Galactitol[Mesh] OR Inositol[Mesh] OR Triose Sugar Alcohols[Mesh] OR Ribitol[Mesh] OR "non-nutritive sweeten*"[Title/Abstract] OR "artificial* sweet*"[Title/Abstract] OR Stevia[Title/Abstract] OR "Steviol glycoside*"[Title/Abstract] OR Saccharin[Title/Abstract] OR Sucralose[Title/Abstract] OR Aspartame[Title/Abstract] OR "Acesulfame K"[Title/Abstract] OR Neotame[Title/Abstract] OR Advantame[Title/Abstract] OR "Sugar alcohol*"[Title/Abstract] OR Sorbitol[Title/Abstract] OR Mannitol[Title/Abstract] OR Maltilol[Title/Abstract] OR Erythritol[Title/Abstract] OR "Hydrogenated starch hydrolysates"[Title/Abstract] OR "HSH"[Title/Abstract] OR Xylitol[Title/Abstract] OR Isomalt[Title/Abstract] OR Lactitol[Title/Abstract] OR "NNS"[Title/Abstract] OR "Non-caloric sweet*"[Title/Abstract] OR "Low calorie sweet*"[Title/Abstract] OR "Natural sweet*"[Title/Abstract] OR "Lo calorie sweet*"[Title/Abstract] OR Thaumatin[Title/Abstract])]) AND ("Mothers"[Mesh] OR "Maternal Exposure"[Mesh] OR Maternal Nutritional Physiological Phenomena[Mesh] OR "Prenatal Exposure Delayed Effects"[Mesh] OR "Pregnancy"[Mesh:noexp] OR "Prenatal Nutritional Physiological Phenomena"[Mesh] OR Lactation[Mesh:noexp] OR Weaning[Mesh] OR Maternal[Title/Abstract] OR Gestat*[Title/Abstract] OR "in-utero"[Title/Abstract] OR "inutero"[Title/Abstract] OR pregnan*[Title/Abstract] OR offspring*[Title/Abstract] OR prenatal[Title/Abstract] OR perinatal[Title/Abstract] OR lactat*[Title/Abstract] OR Mother*[Title/Abstract] OR embryo*[Title/Abstract] OR intrauterine[Title/Abstract] OR "pre-gestation"[Title/Abstract] OR "early exposure"[Title/Abstract] OR "early life exposure"[Title/Abstract] OR wean*[Title/Abstract]) AND ("animal experimentation"[MeSH Terms] OR "models, animal"[MeSH Terms] OR "invertebrates"[MeSH Terms] OR "Animals"[Mesh:noexp] OR "animal population groups"[MeSH Terms] OR animals[tiab] OR animal[tiab] OR mice[Tiab] OR mus[Tiab] OR mouse[Tiab] OR murine[Tiab] OR woodmouse[tiab] OR rats[Tiab] OR rat[Tiab] OR murinae[Tiab] OR muridae[Tiab])

Supplementary 2. Final Search Sets

Embase Search Set

Embase was searched using the following combination of Subject Headings and relevant keywords in addition to an adapted SYRCLE Embase animal filter (Hoojiman et. al 2010).

(exp maternal nutrition/ OR maternal exposure/ OR exp prenatal exposure/ OR pregnancy/ OR progeny/ OR exp perinatal period/ OR lactation/ OR mother/ OR gestation period/ OR Maternal.tw. OR Gestat*.tw. OR In utero.tw. OR Pregnan*.tw. OR Offspring.tw. OR Prenatal.tw. OR Perinatal.tw. OR Lactat*.tw. OR Mother*.tw. OR Embryo*.tw. OR Intrauterine.tw. OR Pre-gestat*.tw. OR (Early adj 2 exposure).tw. OR Wean*.tw.) AND (sweetening agent/ OR nonnutritive sweetener/ OR Stevia rebaudiana/ OR steviol/ OR Stevia/ OR stevioside/ OR saccharin/ OR sucralose/ or aspartame/ OR acesulfame/ OR neotame/ OR sugar alcohol/ OR sorbitol/ OR mannitol plus sorbitol/ OR mannitol/ OR maltitol/ OR erythritol/ OR starch hydrolysate/ OR xylitol/ OR isomalt/ OR lactitol/ OR thaumatin/ OR non-nutritive sweeten*.tw. OR artificial* sweet*.tw. OR Stevia.tw. OR Steviol glycoside*.tw. OR Saccharin.tw. OR Sucralose.tw. OR Aspartame.tw. OR Acesulfame K.tw. OR Neotame.tw. OR Advantame.tw. OR Sugar alcohol*.tw. OR Sorbitol.tw. OR Mannitol.tw. OR Maltitol.tw OR Erythritol.tw. OR Hydrogenated starch hydrolysates.tw. OR HSH.tw. OR Xylitol.tw. OR Isomalt.tw. OR NNS.tw. OR Non-caloric sweet*.tw. OR Low calorie sweet*.tw. OR Lo calorie sweet*.tw. OR Natural sweet*.tw. OR Thaumatin.tw.) AND (Exp fetus development/ OR epigenetics/ OR fetus growth/ OR exp prenatal growth/ OR ecp ecological genetics/ OR exp perinatal development/ OR exp prenatal development/ OR development/ OR phenotypic plasticity/ OR F$etal development.tw. OR F$etal programming.tw. OR Epigenetic.tw. OR Offspring.tw. OR Phenotyp* programming.tw. OR Phenotyp* development.tw. OR Developmental programming.tw. OR Epigenetic inheritance.tw.) AND (exp animal experiment/ or exp animal model/ or exp experimental animal/ or exp transgenic animal/ or exp male animal/ or exp female animal/ or exp juvenile animal/ OR animal/ OR rodentia.mp. OR rodent.mp. OR rodents.mp. OR murinae.mp. OR mouse.mp. OR mice.mp. OR mus.mp. OR musculus.mp. OR murine.mp. OR woodmouse.mp. OR apodemus.mp. OR rat.mp. OR rats.mp. OR rattus.mp. OR norvegicus.mp.)

Scopus and Web of Science Search Set

Scopus and Web of Science will be searched using relevant keywords as seen below:

(maternal OR gestat* OR "In utero" OR inutero OR pregan* OR offspring OR prenatal OR perinatal OR lactat* OR mother* OR embryo* OR intrauterine OR "Pre-gestat*" OR "early exposure" OR "early life exposure" OR wean*) AND ("non-nutritive sweeten*" OR "artificial sweet*" OR Stevia OR "Steviol glycoside*" OR Saccharin OR Sucralose OR Aspartame OR "Acesulfame K" OR Neotame OR Advantame OR "Sugar alcohol*" OR Sorbitol OR Mannitol OR Maltitol OR Erythritol OR "Hydrogenated starch hydrolysates" OR "HSH" OR Xylitol OR Isomalt OR Lactitol OR "NNS" OR "non-caloric sweet*" OR "Low calorie sweet*" OR Lo calorie sweet*" OR "Natural sweet*" OR Thaumatin) AND "Fetal development" OR "Foetal programming" OR epigenetic OR offspring OR "Phenotyp* programming" OR "Phenotyp* development" OR "Developmental programming") AND (rodentia OR rodent OR rodents OR murinae OR mouse OR

mice OR mus OR musculus OR murine OR woodhouse OR apodemus OR rat OR rats OR rattus OR norvegicus OR "animal experiment" OR "animal model" OR "experimental animal" OR "transgenic animal" OR "male animal" OR "female animal" OR "juvenile animal" OR animal)

Supplementary 3. Included and Excluded Sweeteners & Biological Fate

Definition: **Non-nutritive sweeteners** (NNS) are products added to food and beverages to provide sweetness with no or negligible energy content.

Included NNS (artificial and natural) containing negligible energy content

Non-nutritive sweetener (artificially synthesised)*
Acesulfame Potassium (commonly known as Ace K)
Advantame
Alitame
Aspartame
Aspartame-acesulfame salt
Cyclamate
Neotame
Saccharin
Sucralose
Non-nutritive sweetener (naturally derived)
Monatin R-R isomer
Monk Fruit Extract (commonly known as luo han guo)
Steviol glycosides (commonly known as stevia, steviol, stevioside, rebaudioside A)

*as defined by Foods Standards Australia and New Zealand (FSANZ) Food Standards Code

Sweeteners that have less energy content (kilojoules per gram) than sugars, yet still contribute to overall energy intake, will be **excluded** (also known as low calorie sweeteners, reduced calorie sweeteners, modified carbohydrate sweeteners).

Excluded NNS contributing to energy intake

Sweeteners with reduced energy content*
Sugar Alcohols (erythritol, hydrogenated starch hydrosolates, isomalt, lactitol, mannitol, maltitol, sorbitol, xylitol)
Tagatose
Thaumatin

* compared to sugars

Supplementary 3. Included and Excluded Sweeteners & Biological Fate

Biological Fate of Common NNSs	
Acesulfame K	Rapidly absorbed in the small intestines, through the systemic circulation and excreted in the urine unchanged
Advantame	Majority is rapidly hydrolysed in the small intestines into metabolites (advantame acids) which are excreted in faeces. Small amounts of metabolites are excreted in the urine.
Aspartame	Rapidly metabolised in the small intestines prior to entering the systemic circulation. The three sub-components: aspartic acid, phenylalanine and methanol are metabolised the same way as natural dietary sources.
Cyclamate	Rapidly metabolised and excreted in the urine and faeces unchanged. At certain levels and different species, cyclamate can be fermented by gut bacteria into its metabolite cyclohexylamine.
Saccharin	Majority of saccharin rapidly absorbed in the small intestines, through the systemic circulation and excreted in the urine unchanged. Small portion excreted in faeces unchanged.
Steviol Glycoside	Remains unchanged in the small intestines. Gut bacterial in large intestine hydrolyse by removing the glycoside chain (fermented in large intestine) and steviol is absorbed into the systemic circulation. Steviol is primarily metabolised in the liver to steviol glucuronide which is excreted in urine.
Sucralose	Little to no absorption or no metabolism by gut bacteria. Excreted in the faeces unchanged.

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Supplementary 4. Descriptors for Categories

Papers have been grouped according to the primary aim / objective of the study.

Descriptors for four distinct categories – toxicology, neoplastic, metabolic and/or behavioural and sweet preference testing

Sweet Taste Preference Studies: To be included in this category, a study must report
• on the investigation of sweet taste preferences in offspring from maternal exposure to a sweetener an primary aim, objective or statement in the abstract or introduction • results of preference or acceptance testing in offspring from maternal exposure to a sweetener
Metabolic and/or behavioural: To be included in this category, a study must report
• on metabolic or behavioural outcomes in offspring exposed during pre-gestation and/or gestation and/or lactation sweetener. Reported as a primary aim, objective or statement in the abstract or introduction. • primary metabolic outcomes to be included: weight or weight gain, body composition, glycaemic control. Primary behavioural outcomes to be included: feeding/fluid intakes, consumption of novel foods
Toxicology: To be included in this category, a study must report either
• on the toxicity or safety of a sweetener in offspring exposed during pre-gestation and/or gestation and/or lactation, to assist in predicting possible adverse events in humans. Reported as a primary aim, objective or statement in the abstract or introduction. • on the reproductive toxicological investigation of a sweetener to assist in predicting possible adverse events in humans. Reported as a primary aim, objective or statement in the abstract or introduction. • on the investigation of neuro-toxicological effects in offspring exposed during pre-gestation and/or gestation and/or lactation, to assist in predicting possible adverse events in humans. Reported as a primary aim, objective or statement in the abstract or introduction. • “toxicity study” as part of the title or abstract
Neoplastic: To be included in this category, a study must report
• on the investigation of neoplastic incidence in offspring from maternal exposure to a sweetener Reported as a primary aim, objective or statement in the abstract or introduction.

Supplementary 5. Table. Customised data extraction form

Data Extraction Template: Maternal Non-Nutritive Sweetener Systematic Review		
Coder Initials:		
Date Started:		
Date Completed:		
Study Identification		
Study ID:		
Author/s:		
Year of Publication:		
Title of Publication:		
Journal:		
Volume/Pages:		
Language:		
Location (Country):		
Study Design		
Primary aim of study:		
Study design:		
Experimental conditions:		
Control group:		
Number of animals in control group:	*n* (dams)	*n* (offspring)
Unit of measurement:	Individual Offspring	Litter
Animal Model Characteristics		
Rodent species:		
Rodent strain (as stated by author):		
Maternal age at intervention commencement (weeks +/-SD):		
Maternal weight at intervention commencement (grams +/- SD):		
Dietary Intervention Characteristics		
Type of non-nutritive sweetener/s:		
Non-nutritive sweetener dosage: How did the study define the dosage? What concentration/s were used?		
Timing and duration of maternal dietary intervention: • Pre-gestation: • Gestation: • Lactation :		
Administration of maternal dietary intervention:		
Additional maternal intervention/s other than non-nutritive sweetener:		
Offspring dietary intervention/s:		
Timing and duration of offspring dietary intervention:		
Additional offspring intervention/s other than non-nutritive sweeteners:		

Supplementary 5. Table. Customised data extraction form

Outcomes				
List of all measurable outcomes in study:				
Metabolic Outcome: Offspring Insulin Sensitivity				
As measured by: 1. **Fasting Glucose Level (blood or plasma)** 2. **Fasting Insulin Level** 3. **Glucose Tolerance Test** 4. **Insulin Tolerance Test**				
Validated outcome tool used				
Unit/s reported				
Unit of measurement (litter or individual)				
Offspring age at testing (PND)				
Number of offspring per litter (standardised)				
	Control Group		Intervention Group	
	Female offspring	Male offspring	Female offspring	Male offspring
Mean +/- SD				
Level of significance (p- value or CI)				
% change from control				
Direction of result	Favours Control		Favours Intervention	
Behavioural Outcome: Food / Fluid Intake				
Measured by: 1. **Chow or Novel Food Consumption (home cage)** 2. **Fluid Intake (home cage)** 3. **Acceptance or Preference Test (home cage)**				
Validated outcome tool used				
Unit/s reported				
Unit of measurement (litter or individual)				
Offspring age at testing				
Number of offspring per litter (standardised)				
	Control Group		Intervention Group	
	Female offspring	Male offspring	Female offspring	Male offspring
Mean +/- SD or % or Preference Ratio (if different units)				
Level of significance (p- value or CI)				

Supplementary 5. Table. Customised data extraction form

% change from control				
Direction of result	Favours Control		Favours Intervention	
Outcome: Pregnancy Related Measures				
Measured by: **1. Birthweight** **2. Litter Size** **3. Litter ratio**				
Validated outcome tool used				
Unit/s reported				
Unit of measurement (litter or individual)				
	Control Group		Intervention Group	
	Female offspring	Male offspring	Female offspring	Male offspring
Birth weight (grams) Mean +/- SD				
Level of significance (p- value or CI)				
% change from control				
Direction of result	Favours Control		Favours Intervention	
Litter Size (mean +/- SD)				
Level of significance (p- value or CI)				
% change from control				
Direction of result	Favours Control		Favours Intervention	
Outcome: Body Composition				
Measured by: **1. Body Weight / Body Weight Changes** **2. Abdominal Fat Dissection** **3. NMR**				
Validated outcome tool used				
Unit/s reported				
Unit of measurement (litter or individual)				
Offspring age when measurement taken (i.e. neonate, weaning, adolescent, adult, cull)				
Number of offspring per litter (standardised)				
	Control Group		Intervention Group	
	Female offspring	Male offspring	Female offspring	Male offspring
Body weight (grams) Mean +/- SD				
Level of significance (p- value or CI)				
% change from control				
Direction of result	Favours Control		Favours Intervention	
Total abdominal fat mass (grams) Mean +/- SD				
Level of significance (p- value or CI)				

Supplementary 5. Table. Customised data extraction form

% change from control				
Direction of result	Favours Control		Favours Intervention	
Funding sponsorship reported	Yes		No	
Industry funding	Yes		No	

Supplementary 6. ARRIVE Descriptors

Study Quality Assessment: ARRIVE Guidelines Reporting Evaluation Descriptors

ARRIVE Checklist	Item/ sub-items	Evaluation	Descriptor for Evaluation
Title	1	FR / PR / NR	Accurate and concise description of article content
Abstract	2	FR / PR / NR	Accurate summary of background, research objectives including details of species/strain, key methods, principal findings and conclusions
			Introduction
Background	3a.	R / NR	Sufficient scientific background (relevant references) for motivation/context of study and explain experimental approach and rationale
	3b.	R / NR	Why rodent model was chosen and study's relevance to human biology
Objectives	4	FR / PR / NR	Objectives and hypotheses accurately described
			Methods
Ethical statement	5	FR / PR/ NR	Ethical review permissions/licences and relevant guidelines for care and use of animals in research
Study design	6a.	R / NR	Number of experimental and control groups
	6b.	R / NR	Randomisation measures applied during study
	6c.	R / NR	Blinding measures applied during study
	6d.	R / NR	Experimental unit reported i.e. individual animal or litter
Experimental procedures	7a.	R / NR	Description of how the experiment was performed (including procedures and equipment/suppliers used)
	7b.	R / NR	Details of timing of experimental procedures performed (i.e. time of day or timing of light/dark cycle or time according to study flow chart)
	7c.	R / NR	Details on where each experimental procedure/s was carried out (e.g. drinking cage for sweetener preference testing, Water Maze testing, home cage)
	7d.	R / NR	Rationale for why procedures were performed
Experimental animals	8a.	R / NR	Detail of rodent species
	8b.	R / NR	Details of strain
	8c.	R / NR	Details of source of rodent
	8d.	R / NR	Detail of age of dam
	8e.	R / NR	Detail of weight of dam
	8f.	R / NR	Details of health or test status (i.e. experimentally naïve, virgin)
Housing and husbandry	9a.	R / NR	Details of cage type and dimensions for each stage of experimental study
	9b.	R / NR	Details of bedding material/environmental enrichment

Supplementary 6. ARRIVE Descriptors

	9c.	R / NR	Details of housing during each stage of experimental study (i.e. group or individually housed)
	9d.	R / NR / NA	Details of number of cage companions if group housed
	9e.	R / NR	Details of facility (i.e specific pathogen free)
	9f.	R / NR	Details of light/dark cycle timing
	9g.	R / NR	Details of temperature/humidity
	9h.	R / NR	Details of food and water (composition, source, access or delivery mode)
	9i.	R / NR	Details of welfare-related assessments and interventions prior, during or after experiment; not related to adverse event
Sample size	10a.	R / NR	Details of total number of rodents used (both breeding programme and offspring) for each experiment and experimental group
	10b.	R / NR	Explanation for how the number of rodents was arrived at (i.e. sample size calculation, power analysis)
	10c.	R / NA	Number of independent replications performed (Not applicable if not reported)
Allocating animals to experimental groups	11a.	R / NR	Details of how rodents were allocated to experimental groups (randomisation or weight/sex matching)
	11b.	R / NR	Details of the order in which rodents in each experimental group were treated and assessed
Experimental outcomes	12	FR / PR / NR	Clearly defined the primary and secondary experimental outcomes assessed
Statistical methods	13a.	R / NR	Details of statistical methods used for each analysis
	13b.	R / NR	Clearly defined unit of measurement for each data set i.e. individual animal or litter
	13c.	R / NR	Testing for normality was performed
Results			
Baseline data	14	FR / PR / NR	Details of baseline characteristics and health status of rodents in each experimental group
Numbers analysed	15a.	R / NR	Reported the number of rodents in each group included in each analysis
	15b.	R / NR / NA	Reported and explained why rodents excluded from analysis, if any (Not applicable if no exclusions)
Outcomes and estimations	16	FR / PR / NR	Results reported for each analysis carried out with a measure of precision (i.e. standard error or confidence interval)
Adverse events	17a.	R / NR / NA	Details of all adverse events in each experimental group reported (Not applicable if no adverse events expected e.g. embryology/toxicology studies)
	17b.	R / NR	Reported any modification to experimental protocols made to reduce adverse events
Discussion			
Interpretation/scientific implications	18a.	R / NR	Results interpreted, taking into account the study's objectives and hypotheses, current theory and other relevant studies
	18b.	R / NR	Discuss any limitation of the study, including potential source of bias, animal model limitation and imprecision associated with results
	18c.	R / NR	Described implications of methods/ findings of the study for the 3R's of the use of animals in research
Generalisability/translation	19	FR / PR / NR	Commented on whether the findings are likely to translate to other species or systems, including relevance to human biology

Supplementary 6. ARRIVE Descriptors

Funding	20	FR / PR / NR	Listed all funding sources and the role of the funder/s in the study

Abbreviations: FR Fully Reported; R Reported; PR Partially Reported; NR Not Reported; NA Not applicable

Supplementary 7. Excluded papers

Excluded papers from full-text screening (phase 2) with reason and type of NNS

Reason for Exclusion	*Number of studies (n)*	*Excluded Studies*	*Type of NNS*
No appropriate non NNS control group for comparison: - including ethanol or nicotine interventional studies using saccharin as a comparator for prenatal exposure models to alcohol or nicotine	*n* = 8	Brady, M. L., et al. (2011). "NMDA receptor subunit levels are altered in purified synaptic membranes isolated from the dentate gyrus of a mouse model of prenatal alcohol exposure." Alcoholism: Clinical and Experimental Research, 36; 3 pp 457-466	Saccharin
		Caldwell, K. K., et al. (2014). "Prenatal alcohol exposure is associated with altered subcellular distribution of glucocorticoid and mineralocorticoid receptors in the adolescent mouse hippocampal formation." Alcoholism: Clinical and Experimental Research, 38; 2 pp 392-400	Saccharin
		Caldwell, K. K., et al. (2015). "The impact of prenatal alcohol exposure on hippocampal-dependent outcome measures is influenced by prenatal and early-life rearing conditions." Alcoholism: Clinical and Experimental Research, 39; 4 pp 631 – 639	Saccharin
		Fox, K. A., et al. (2012). "Sex-specific effects of nicotine exposure on developmental programming of blood pressure and vascular reactivity in the C57Bl/6J mouse." American Journal of Obstetrics and Gynaecology, 207; 3 pp 208.e1-9	Saccharin
		O'Neill, H.C., et al. (2018). "The interaction of the Chrna5 D398N variant with developmental nicotine exposure." Genes, Brain and Behaviour, 17; 7 pp e12474	Saccharin
		Rodriguez, C.I., et al. (2016). "Moderate Prenatal Alcohol Exposure Alters Functional Connectivity in the Adult Rat Brain." Alcoholism: Clinical and Experimental Research, 40; 10 pp 2134-2146	Saccharin

Supplementary 7. Excluded papers

		Rodriguez, C.I., et al., (2016). "Effects of sex and housing on social, spatial, and motor behavior in adult rats exposed to moderate levels of alcohol during prenatal development." Behavioural Brain Research, 313 pp 233-243 Sanchez, J.J., et al., (2017). "Prenatal alcohol exposure is a risk factor for adult neuropathic pain via aberrant neuroimmune function." Journal of Neuroinflammation, 14; 1 pp 254	Saccharin Saccharin
No maternal intervention exposure during pre-gestation and/or gestation and/or lactation	*n* = 1	Schoenig, G. P., Anderson, R. L. (1985). "The effects of high dietary levels of sodium saccharin on mineral and water balance and related parameters in rats." Food and Chemical Toxicology, 23; 4-5 pp 465-474	Saccharin
Non rodent study	*n* = 1	Stone, D., Matalka, E., Pulaski, B. (1971). "Do artificial sweeteners ingested in pregnancy affect the offspring?" Nature, 231; 5297 pp 53	Cyclamate
Non free feeding models: - intragastric gavage (*n* = 8) - intraperitoneal administration (*n* = 1)	*n* = 9	Abd Elfatah, A. A., Ghaly, I. S., Hanafy, S. M. (2012). "Cytotoxic effect of aspartame (diet sweet) on the histological and genetic structures of female albino rats and their offspring." Pakistan Journal of Biological Sciences, 15; 19 pp 904-18 Damasceno, D. C., (2003). "Effects of a saccharin and cyclamate mixture on rat embryos." Veterinary and Human Toxicology, 45; 3 pp 157-159 Fritz, H., Hess, R., (1968). "Prenatal development in the rat following administration of cyclamate, saccharin and sucrose." Experientia, 24; 11 pp 1140-1141 Kille, J. W., et al., (2000). "Sucralose: assessment of teratogenic potential in the rat and the rabbit." Food and Chemical Toxicology, 38 Suppl 2 pp S43-52	Aspartame Saccharin Cyclamate and saccharin Sucralose

Supplementary 7. Excluded papers

		Martins, A. T., et al. (2010). "Effect of Sodium Cyclamate on the Rat Fetal Exocrine Pancreas: a Karyometric and Stereological Study." International Journal of Morphology, 28; 3 pp 899-904	Cyclamate
		McAnulty, P. A., et al. (1989) "Absence of developmental effects in CF-1 mice exposed to aspartame in utero." Fundamentals of Applied Toxicology, 13; 2 pp 296-302	Aspartame
		Otabe, A. et al., (2011). "Evaluation of the teratogenic potential of N-[N-[3-(3-hydroxy-4-methoxyphenyl) propyl]-alpha-aspartyl]-L-phenylalanine 1-methyl ester, monohydrate (advantame) in the rat and rabbit." Food and Chemical Toxicology, 49 Suppl 1 pp S60-69	Advantame
		Schmahl, D., Habs, M. (1980). "Absence of carcinogenic response to cyclamate and saccharin in Sprague-Dawley rats after transplacental application." Arzneimittelforschung, 30; 11 pp 1905-1906	Cyclamate and saccharin
		Tanaka, R. (1964). "The toxicity of synthetic sweetening agents to mouse embryos." Japanese journal of public health, 11; 14 pp 909-915	Cyclamate and saccharin
Not included as intervention not a defined non-nutritive sweetener (S3. Table Included and excluded NNS): - sugar alcohol (sorbitol, mannitol, maltitol, erythritol, hydrogenated starch hydrolysates, xylitol, isomalt and lactitol)	*n* = 5	Canimoglu, S., Rencuzogullari, E. (2013). "The genotoxic and teratogenic effects of maltitol in rats." Toxicology and Industrial Health, 29; 10 pp 935-943	Maltitol
		Cardoso, F. S., et al. (2016). "Exposure to sorbitol during lactation causes metabolic alterations and genotoxic effects in rat offspring." Toxicology Letters, 260 pp 36-45	Sorbitol
		Kruger, C. L., et al. (1999). "Developmental toxicity study of D-tagatose in rats." Regulatory Toxicology and Pharmacology, 29; 2 Pt 2 pp S29-35	D-tagatose

Supplementary 7. Excluded papers

		Waalkens-Berendsen, D. H. et al., (1989). "Developmental toxicity of isomalt in rats." Food and Chemical Toxicology, 27; 10 pp 631-637 Waalkens-Berendsen, D. H., et al., (1990). "Embryotoxicity/teratogenicity of isomalt in rats and rabbits." Food and Chemical Toxicology, 28; 1 pp 1-9	Isomalt Isomalt

Supplementary File 9. Chow composition and administration

Study ID/Year	Chow Composition	NNS type and dose	Administration of NNS
	Sweet Taste Preference Studies		
Choo 2018	Not reported	Sucralose	oral via liquid ration daily
Li 2013	Not reported	Ace K	added to drinking water *ad libitum*
Zhang 2011	Purina Lab Chow 24.5% protein, 50.3% carbohydrate, and 4.4% fat; 3.93 kcal/g gross energy; 0.31% sodium, 0.99% potassium, and 1.46% calcium	Ace K	mixed with standard chow *ad libitum*
	Metabolic and Behavioural Studies		
Zhang 2018	Not reported	Saccharin	added to drinking water *ad libitum*
Olivier-VS 2017	regular NIH-07 chow diet 22.5 % protein, 4.5 % fat, 4.5 % fiber	Sucralose & Ace K combined	pipetted onto pellet daily
Collison 2016	Not reported	Aspartame	added to drinking water *ad libitum*
Parlee 2014	Purina Lab Diet Catalog number 5008 23.5% protein, 6.5% fat, 3.8% fiber; 4.15 kcal/g gross energy	Saccharin	added to drinking water *ad libitum*
Collison 2012a	Not reported	Aspartame	added to drinking water *ad libitum*
Collison 2012b	Not reported	Aspartame 0	added to drinking water *ad libitum*
von Poser Toigo 2015	Not reported	a) Aspartame b) Saccharin	added to drinking water *ad libitum*
	Toxicology Studies		
Brathwaite 2013	Rodent LabDiet 5002 24.1% protein, 13.2% fat, 62.7 carbohydrates 4.3% fibre; 4.09 kcal/g gross energy	Arruva (Monatin)	mixed with standard chow *ad libitum*
Otabe 2011	UAR VFR1 Certified (Usine d'Alimentation Rationnelle in France, Charles River UK, Margate, Kent, England) 19.11% protein, 4.75% fat, 3.85% fibre, 4.6% sucrose, 35.41% starch, 3.4 kcal/g Atwater fuel energy	Advantame	mixed with standard chow *ad libitum*
Curry 2008	SDS VRF1 powdered certified rodent chow (Special Diet Services, Witham, Essex) 19.11% protein, 4.75% fat, 3.85% fibre, 4.6% sucrose, 35.41% starch, 3.4 kcal/g Atwater fuel energy	Rebaudioside A	mixed with standard chow *ad libitum*

Supplementary File 9. Chow composition and administration

Kille 2000	Labsure Laboratory Animal Diet No. 2, K & K (Greeff Chemicals Ltd) Composition not reported	Sucralose	mixed with standard chow *ad libitum*
Reilly 1990	Formulab Chow 5008 (Ralston Purina Co.) Composition not reported	Aspartame	added to drinking water *ad libitum*
Holder 1987	Purina Rat Chow Composition not reported	Aspartame	added to drinking water *ad libitum*
Brunner 1979	Purina Rat Chow Composition not reported	Aspartame	mixed with powered chow *ad libitum*
Lapointe 1979	Not reported	Sodium Saccharin	mixed with standard chow *ad libitum*
	Neoplastic Studies		
Soffritti 2016	Corticella diet Laboratorio Dottori Piccioni (Milan, Italy) 12% water; 24% raw protein; 3.5% raw fat; 5.5% raw fibers; 10.5% ashes; and 56.5% non-nitrogen extracts	Sucralose	mixed with standard chow *ad libitum*
Soffritti 2010	Corticella diet Laboratorio Dottori Piccioni (Milan, Italy) 12% water; 24% raw protein; 3.5% raw fat; 5.5% raw fibers; 10.5% ashes; and 56.5% non-nitrogen extracts	Aspartame	mixed with standard chow *ad libitum*
Soffritti 2007	Corticella diet Laboratorio Dottori Piccioni (Milan, Italy) 12% water; 24% raw protein; 3.5% raw fat; 5.5% raw fibers; 10.5% ashes; and 56.5% non-nitrogen extracts	Aspartame	mixed with standard chow *ad libitum*
Cohen 1995	Prolab 3200 rat chow (Agway, Inc., St. Marys, OH) Composition not reported	Sodium Saccharin	mixed with standard chow *ad libitum*
Garland 1991	Purina Rodent Chow No. 5002 24.1% protein, 13.2% fat, 62.7% carbohydrate 4.3% fiber; 4.09 kcal/g gross energy	Sodium Saccharin	mixed with standard chow *ad libitum*
Taylor 1980	Ground Purina Lab Chow plus 1.5% sodium carbonate	Sodium Saccharin	mixed with standard chow *ad libitum*

Supplementary 10. Maternal and Offspring Outcomes

Complete list of maternal and offspring outcomes

Study ID	Species/Strain	NNS Type	Maternal Outcomes	Offspring Outcomes
Sweet Taste Preference Studies				
Choo 2018	C57BL/6 mice	Sucralose		Sweet taste behaviour assay - lickometer, sucrose and sucralose preference testing, fungiform papilla density, taste bud isolation and RNA extraction, Total RNA extraction, reverse transcription and gene expression of sweet receptors
Li 2013	ICR mice	Ace K		body weight, sweetener preference testing, sweet taste receptor T1R2, T1R3, transduction protein Gα-gustducin, TRPM5, and the receptors of regulated protein Ob-Rb and CB1
Zhang 2011	ICR mice	Ace K	litter size, sex ratio, body weight, chow consumption	body weight, sweetener preference testing
Metabolic and Behavioural Studies				
Zhang 2018	C57BL/6 mice	Saccharin	Gestational length, litter size, sex ratio, fluid intake	Body weight, Behavioural testing: locomotor activity, spatial working memory, elevated plus maze, object based attention, novel object recognition, cliff avoidance
Olivier-VS 2019	C57BL/6 mice	Ace K mixed with Sucralose	NNS bioavailability in blood, faeces and breast milk, litter size and sex ratio	NNS detection in blood, faeces and urine, fasting glucose levels, body weight, hepatic weight, fat content and glycogen storage, metabolites, gene expression for liver detoxification metabolism, gut microbiome
Collison 2016	C57BL/6J mice	Aspartame		Body weight, food/fluid intake; Behavioural testing: open field activity, light/dark transition test, novel object recognition, Morris water maze; Insulin Tolerance Test, white adipose tissue, interscapular fat and pancreatic mass, glucose, plasma insulin and corticosterone levels

Supplementary 10. Maternal and Offspring Outcomes

Parlee 2014	C57BL/6J mice	Saccharin	Litter size	Body weight, body composition (at 3,8 and 13 weeks), Adipocyte size and number, serum insulin, IGF-1, testosterone, blood glucose, glucose and insulin tolerance tests (GTT and ITT), Bone density and bone marrow adipocytes, ex vivo insulin secretion and adiponectin expression
Collison 2012a	C57BL/6J mice	Aspartame		Food/fluid intake (data not shown), body weight change, abdominal fat dissections, fasting serum glucose, insulin and lipids, insulin tolerance test, Morris Water Maze Test for spatial learning, non-cognitive behaviour, spatial memory
Collison 2012b	C57BL/6J mice	Aspartame		Food/fluid intake, body weight change, abdominal fat dissections, fasting serum glucose, insulin and lipids, insulin tolerance test
von Poser Toigo 2015	Wistar rat	Aspartame & Saccharin		Behavioural Procedures; open field testing, elevated plus maze; Feeding behaviour; novel food consumption (fruit loops), chocolate and chow consumption; Plasma lipids and glucose levels, weight gain
Toxicology Studies				
Brathwaite 2013	Sprague Dawley rat	Arruva (Monatin)	maternal observations, body weight, gravid uterine weight, feed consumption, feed efficiency and test article intake, Laparohysterectomy, litter size	Morphological examination
Otabe 2011	Sprague Dawley rat (CD)	Advantame	clinical observations, mortality, body weight, food/fluid intake, oestrous cycles, mating performance and fertility, gestation length, litter size, sex ratio	offspring survival, physical and reflex maturation development, morphology, sperm analysis and morphology, body weight, organ weight, sexual maturation, food/fluid intake
Curry 2008	Hans Wistar rat	Rebaudside A	clinical observations, body weight, food/fluid intake, oestrous cycles, mating performance and fertility, gestation length, litter size	offspring survival, sperm analysis and morphology, body weight, organ weight, sexual maturation

Supplementary 10. Maternal and Offspring Outcomes

Kille 2000	Sprague Dawley rat (CD)	Sucralose	Body weight, food/fluid intake , clinical observations, mating performance and fertility, gestation length and index, litter size and sex ratio	fetal birthweight, offspring body weight, organ weight, food/fluid intake
Reilly 1990	Sprague Dawley rat	Aspartame	Body weight and fluid consumption, litter size	Body weight, amine neurotransmitter binding kinetics and brain metabolites, plasma and brain amino acid levels
Holder 1987	Sprague Dawley rat	Aspartame	Fluid/food intake, maternal retrieval, litter size	Morphological and reflex development, body weight, food/fluid intake, radial arm maze test, milk maze
Brunner 1979	Sprague Dawley rat	Aspartame	Body weight, body weight, food consumption, litter size and sex ratio	Fetal Outcomes: birthweight, mortality Pre-weaning observations: physical and reflex maturation development, swimming development, open field activity Post-weaning observations: open field activity, running wheel activity, rotorod balancing testing, active and passive avoidance testing
Lapointe 1979	Sprague Dawley rat	Sodium Saccharin	Body weight, food/fluid intake, gestation length, litter size	fetal birthweight, food/fluid intake, morphological examination, gross behavioural criteria
Neoplastic Studies				
Soffritti 2016	Swiss mice	Sucralose		food consumption, body weight, survival rate, neoplastic incidence
Soffritti 2010	Swiss mice	Aspartame		food consumption, body weight, survival rate, neoplastic incidence
Soffritti 2007	Sprague Dawley rat	Aspartame		food consumption, body weight, survival rate, neoplastic incidence
Cohen 1995	Sprague Dawley rat	Sodium Saccharin	Food/fluid intake, Litter size, sex ratio, body weight	Birthweight, body weight, bladder urothelial examination
Garland 1991	Sprague Dawley rat (CD)	Sodium Saccharin	Haematology, litter size and sex ratio	Survival rates, fetal birthweight, body weight, food/fluid intake, urine, bladder and liver chemistry, haematology, serum vitamin and lipid concentrations

Supplementary 10. Maternal and Offspring Outcomes

Taylor 1980	Sprague Dawley rat (CD)	Sodium Saccharin	Body weight, food/fluid intake, haematological tests	Body weight, Morphological examination, histopathological tissue examination, organ weights, urinary bladder examination

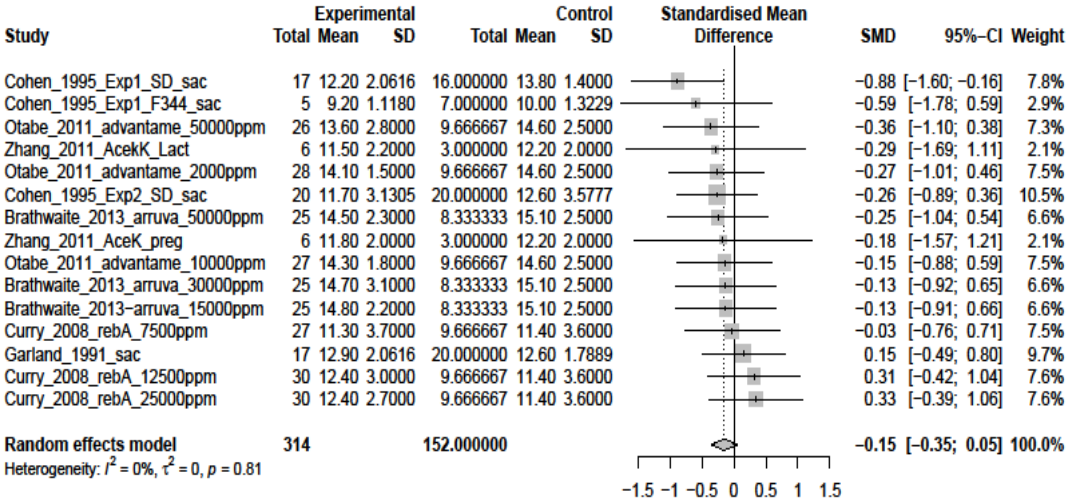

Figure 1. Meta-analysis of maternal outcomes for litter outcomes. Overall, standardised mean difference (SMD) and 95% confidence interval (CI; upper; lower limits) were estimated by random effects meta-analysis.

Supplementary 12. Offspring Food Intakes

Study ID	*Unit of Measurement*	*Methodology*	*Summary of offspring food intake results*	*Category*
Brathwaite 2013	N/A	N/A	N/A; Foetal findings only	Toxicology
Brunner 1979	NR	NR	NR	Toxicology
Choo 2018	unclear	Taken at 8 weeks; reported as g/48hrs	No difference between control and sucralose groups	Sweet Taste Preference
Cohen 1995	NR	NR	NR	Neoplastic
Collison 2012a	unclear	Taken at 7 & 15 weeks; measured over 4-day period by weighing food pellets	Food consumption no effect; Data not shown	Metabolic and Behavioural
Collison 2012b	overall cage intake divided 3 animals to give animal consumption	Taken at 7 & 15 weeks; measured by weighing food pellets and reported as grams	No difference between control and aspartame groups in males and females	Metabolic and Behavioural
Collison 2016	overall cage intake divided 3 animals to give animal consumption	Taken at 7 weeks; measured by weighing food pellets and reported as grams	No difference between control and aspartame group in males (females not included in this study)	Metabolic and Behavioural
Curry 2008	NR	NR	NR	Toxicology
Garland 1991	individual animal	measured over 3 day interval immediately after weaning (PND21) followed by daily measurements to PND30; measured in g/rat/day	First three days post weaning, significant decrease in food consumption; Progressive increase in food consumption for next 6 days; PND27-30 no difference between control and NaSac groups in males and females when adjusted for body weight	Neoplastic
Holder 1987	NR	NR	NR	Toxicology
Kille 2000	NR	NR	NR	Toxicology
Lapointe 1979	NR	NR	NR	Toxicology

Supplementary 12. Offspring Food Intakes

Li 2013	NR	NR	NR	Sweet Taste Preference
Olivier-Van Stichelen 2019	NR	NR	NR	Metabolic and Behavioural
Otabe 2011	NR	NR	NR	Toxicology
Parlee 2014	unclear	measured weekly during postnatal weeks 8-12*; reported as 4-week cumulative food intake in grams (*weaned onto westernised diet containing 41% fat until 14 weeks of age)	No significant difference in food intake between control and saccharin groups in males and females	Metabolic and Behavioural
Reilly 1990	NR	NR	NR	Toxicology
Soffritti 2007	Meaured per cage and divided by number of animals/cage	measured once per week from 6 weeks age for first 13 weeks then every 2 weeks until 110 weeks of age	No significant difference in food intake between control and aspartame groups in males and females	Neoplastic
Soffritti 2010	Meaured per cage and divided by number of animals/cage	measured once per week from 6 weeks age for first 13 weeks then every 2 weeks until 110 weeks of age	No significant difference in food intake between control and sucralose groups in males and females	Neoplastic
Soffritti 2016	Meaured per cage and divided by number of animals/cage	measured once per week from 6 weeks age for first 13 weeks then every 2 weeks until 110 weeks of age	No significant difference in food intake between control and aspartame groups in males and females	Neoplastic

Supplementary 12. Offspring Food Intakes

Taylor 1980	unclear	measured weekly from 0-24 weeks of age; reported as cumulative food intake 0-12 weeks and 0-24 weeks in grams for males and 0-8 weeks and 0-30 weeks for females	Males on 7.5% NaSac group showed decreased food intake and weight gain for 0 -12 weeks; decreased food intake and feed efficiency over 24 weeks. Females showed no effect for 0-8 weeks however over the first 30 weeks there was a decrease in feed efficiency in 7.5% NaSac group	Neoplastic
von Poser Toigo 2015	Measured per cage and divided by 3-4 animals/cage	chow consumption compared to chocolate consumption was measured for 30 days from PND80-110	no significant difference between chow and chocolate consumption	Metabolic and Behavioural
Zhang 2011	NR	NR	NR	Sweet Taste Preference
Zhang 2018	NR	NR	NR	Metabolic and Behavioural

Supplementary 13. Offspring fluid intakes

Study ID	*Unit of Measurement*	*Methodology*	*Summary of offspring fluid intake results*	*Category*
Brathwaite 2013	N/A	N/A	N/A; Foetal findings only	Toxicology
Brunner 1979	NR	NR	NR	Toxicology
Choo 2018	unclear	Taken at 8 weeks; reported as ml/48hrs	No difference between control and sucralose groups	Sweet Taste Preference
Cohen 1995	NR	NR	NR	Neoplastic
Collison 2012a	unclear	Taken at 7 & 15 weeks; measured over 4-day period by weighing water bottles	Fluid consumption no effect; Data not shown	Metabolic and Behavioural
Collison 2012b	overall cage intake divided 3 animals to give animal consumption	Taken at 7 & 15 weeks; measured by weighing water bottles	7 week old males had reduced fluid intake compared to control; no difference in 7 week females; By 15 weeks, no effect on fluid consumption in males or females	Metabolic and Behavioural
Collison 2016	overall cage intake divided 3 animals to give animal consumption	Taken at 7 weeks; measured by weighing water bottles	No difference between control and sucralose groups (females not included in this study)	Metabolic and Behavioural
Curry 2008	NR	NR	NR	Toxicology
Garland 1991	individual animal	measured over 3 day interval immediately after weaning (PND21) followed by daily measurements to	Between PND25-30 water consumption increased in NaSac group compared to control	Neoplastic

Supplementary 13. Offspring fluid intakes

		PND30; measured in g/rat/day		
Holder 1987	NR	NR	NR	Toxicology
Kille 2000	NR	NR	NR	Toxicology
Lapointe 1979	NR	NR	NR	Toxicology
Li 2013	NR	NR	NR	Sweet Taste Preference
Olivier-Van Stichelen 2019	NR	NR	NR	Metabolic and Behavioural
Otabe 2011	NR	NR	NR	Toxicology
Parlee 2014	NR	NR	NR	Metabolic and Behavioural
Reilly 1990	NR	NR	NR	Toxicology
Soffritti 2007	Meaured per cage and divided by number of animals/cage	measured once per week from 6 weeks age for first 13 weeks then every 2 weeks until 110 weeks of age	No significant difference in fluid intake between control and aspartame groups in males and females	Neoplastic
Soffritti 2010	Meaured per cage and divided by number of animals/cage	measured once per week from 6 weeks age for first 13 weeks then	No significant difference in fluid intake between control and aspartame groups in males and females	Neoplastic

Supplementary 13. Offspring fluid intakes

		every 2 weeks until 110 weeks of age		
Soffritti 2016	Measured per cage and divided by number of animals/cage	measured once per week from 6 weeks age for first 13 weeks then every 2 weeks until 110 weeks of age	No significant difference in fluid intake between control and sucralose groups in males and females	Neoplastic
Taylor 1980	NR	NR	NR	Neoplastic
von Poser Toigo 2015	Measured per cage and divided by 3-4 animals/cage	evaluated during treatments	each animal found to consume approximately 30mL fluid/day; Data not shown	Metabolic and Behavioural
Zhang 2011	NR	NR	NR	Sweet Taste Preference
Zhang 2018	NR	NR	NR	Metabolic and Behavioural

Supplementary File 15. Adherence of ARRIVE guidelines per item

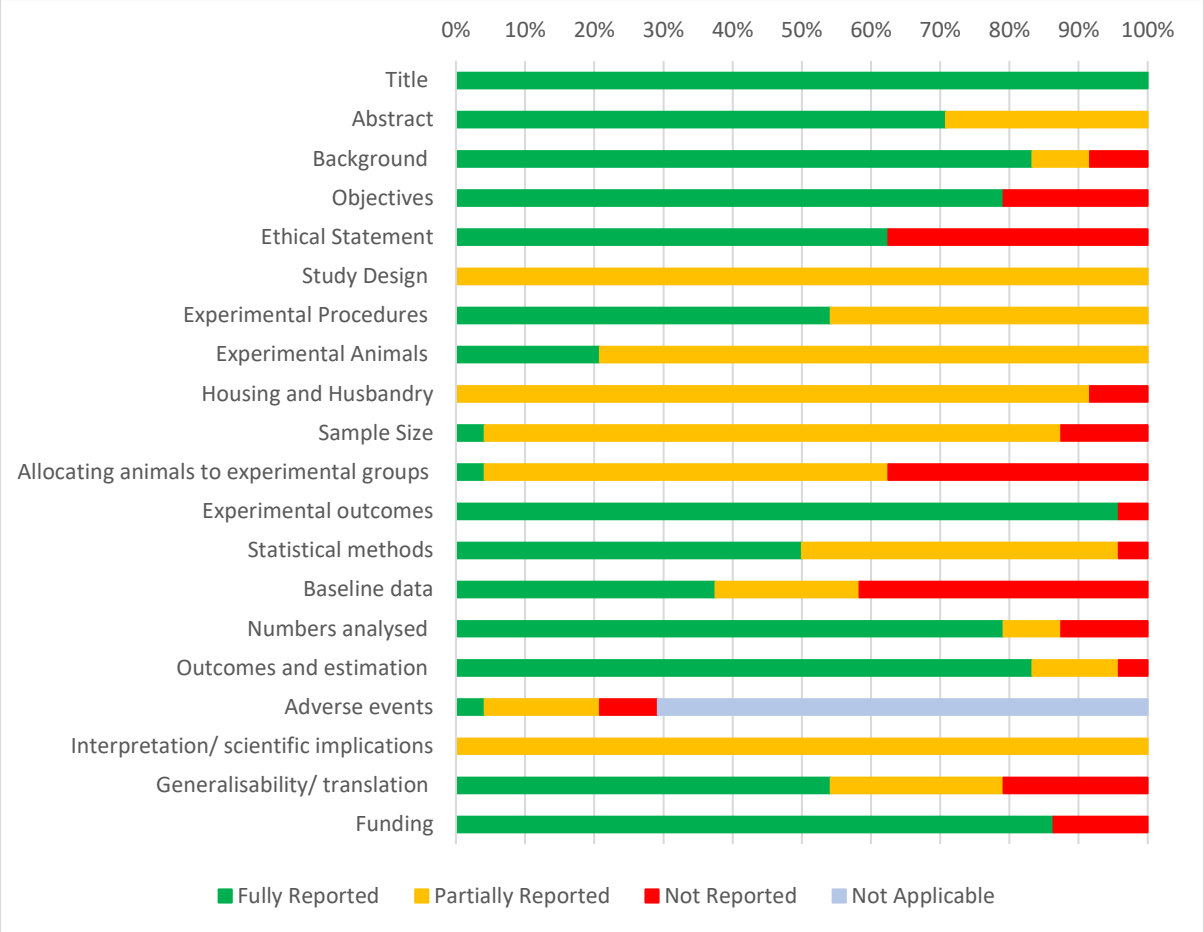

Figure 1. Adherence of the ARRIVE Guidelines Checklist per item. Percentages (0-100%) refer to the percentage of included studies reporting at each level for each item. Items are described as 'fully reported', partially reported or non-reported. For items with associated sub-items fully reported means that all sub-items were reported, partially reported that not all sub-items are fully reported, and not reported that none of the sub-items was reported.

Chapter 2: Metabolic and behavioural effects in offspring exposed to maternal sucrose consumption: a systematic review and meta-analysis of data from rodent models

Chapter 2 has been published as:

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Statement from co-authors confirming authorship contribution of the PhD candidate

The co-authors of the paper: *Metabolic and behavioural effects in offspring exposed to maternal sucrose consumption: a systematic review and meta-analysis of data from rodent models. Journal of Developmental Origins of Health and Disease, 12(4), 603–618* confirm that Heidi Louise Morahan has made the primary contribution to this study in each of the following areas:

- Conception and design of the research
- Collection and analysis of data, interpretation of the findings
- Writing of the manuscript and critical appraisal of content

In addition to the statements above, in cases where I am not the corresponding author of a published item, permission to include the published material has been granted by the corresponding author.

Heidi Louise Morahan Date: 26 September 2023

As supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

Associate Professor Kieron Rooney Date: 26 September 2023

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Metabolic and behavioural effects in offspring exposed to maternal sucrose consumption: a systematic review and meta-analysis of data from rodent models

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Sucrose; sugar-sweetened beverages; pregnancy; rodent offspring; metabolism; obesity

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Abstract

Consumption of sugar-sweetened beverages (SSBs) during pregnancy has been associated with childhood obesity. Research in which rodent dams have been given high-fat/high-sugar diets has consistently found metabolic alterations in their offspring. However, what remains unclear is the potential impact on the developing fetus of giving sugar in isolation at concentrations similar to SSBs to the mothers. Therefore, we conducted a systematic review and meta-analysis (Protocol No: 127115 on Prospero) to identify potential relationships between maternal sucrose consumption and metabolic outcomes in offspring of rodent (rat or mouse) models. We analysed studies that provided rodent mothers dams with access to sucrose solutions (8–20% w/v) prior to conception, during pregnancy and/or lactation and that reported offspring outcomes of body weight (BW), body composition and glycaemic control. Following a systematic search of four databases (PubMed, EMBASE, Web of Science and Scopus) performed on 15 January 2019, maternal and offspring data from 15 papers were identified for inclusion. Only rat studies were identified. Meta-analyses were performed on standardised mean differences for maternal and offspring BW and fasting glucose levels, with subgroup analyses of strain, sucrose concentration, exposure period and sex of offspring. A bias towards the inclusion of only data from male offspring was identified and this limited interpretation of potential sexually dimorphic outcomes. Maternal sucrose exposure was associated with an increased risk of obesity and poor glucose disposal in adult and aged offspring.

Introduction

Obesity is a significant health challenge facing modern society. The World Health Organisation has reported that prevalence has nearly tripled over the last 40 years.[1] With an estimated 1.9 billion people considered overweight or obese globally, the social and financial burdens of this epidemic are staggering.[1,2] In part, obesity has been attributed to the consumption of added sugars, with the greatest source from sugar-sweetened beverages (SSBs).[3] Epidemiological studies have found that consumption of added sugars is associated with obesity, type 2 diabetes and cardio-metabolic disease.[4-8] However, what is less well understood is the potential influence added sugars may have on the developing fetus during early life.

There is a growing recognition that the origins of obesity begin *in utero*; extensive research has consistently shown an increased risk of childhood obesity when the mother is overweight or obese[9,10] or consumes a high-fat diet.[11-13] This intergenerational link may be a factor in the amplification of the obesity epidemic.[14] Dysregulation of gene expression through DNA methylation, histone modification and mitochondrial dysfunction has been reported in obese offspring.[15,16] Whether excessive maternal sugar consumption is involved in these mechanistic links remains unclear.

Three recent epidemiological studies have found an association between maternal SSBs consumption during pregnancy and childhood adiposity.[17-19] Specifically, body mass index and fat mass were higher in children and infants under 7 years of age, independent of the child's SSBs intake. This association was stronger when mothers consumed SSBs during the second trimester.[17] However, such studies are inevitably subject to a range of confounding factors that limit their ability to identify causality. Because they can eliminate confounds, animal models have advanced our understanding of the biological mechanisms involved in maternal overnutrition,[20,21] including high-sugar diets.[22] Given SSB intake is a prime target for the prevention of excessive weight gain during pregnancy, it is important to validate the epidemiological findings[17-19] in animal models. Evidence from some rodent studies where dams were fed sucrose as part of a solid food diet during pregnancy has reported impaired metabolism in their

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offspring. These impairments have included increased body weight (BW), adiposity, hyperglycaemia, insulin resistance and altered hepatic lipid metabolism.[23-25] The translational value of these experiments is limited because the sucrose content (up to 65% of energy intake) was much higher than what humans normally consume. Thus, a more appropriate animal model for human SSB consumption during pregnancy would be the manipulation of the maternal diet with sucrose administered in solution.

The research included in the present review was confined to studies in which dams were provided with sucrose solutions at concentrations more comparable to those found in commercially available SSBs (8–20% w/v). We sought to determine whether sucrose consumption at this concentration alters offspring metabolism. The main aim was to identify possible relationships between maternal intakes of sucrose solutions and offspring outcomes for BW, body composition and glycaemic control. A secondary aim was to assess whether maternal sucrose consumption during pregnancy affects their offspring's consumption patterns.

Materials and methods

This review was reported in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA 2009) Guidelines (Supplementary Table S1). The protocol was developed with the SYstematic Review Centre for Laboratory animal Experimentation's (SYRCLE) Protocol template, Version 2.0[26] and registered on Prospero on the 18th March 2019 (Protocol number: 127115; available from https://www.crd.york.ac.uk/prospero/display_record.php?RecordID=127115) and Camarades NC-3R Systematic Review Facility (http://syrf.org.uk/protocols/).

Search strategy and study selection

The following databases were searched from inception to 15 January 2019: PubMed, EMBASE via OvidSP, Web of Science and Scopus. The extensive search strategy used keywords, medical subject headings (MeSH), EMTREE terms and related synonyms based on maternal sucrose feeding and fetal development. Previously published animal filters[27,28] were adapted to identify rodent (rat and mouse) papers and used in the final search sets for PubMed and EMBASE. No animal filters were used for Web of Science and Scopus as they are currently not available. The final search strategy for each database can be found in Supplementary Table S2. Extracted publications were combined and duplicates manually removed in Endnote reference management software (EndNote™ X8).

Papers were screened by two independent reviewers, according to pre-defined exclusion criteria. Based on title and abstract, papers were excluded if they were non-experimental, non-rodent, genetically bred or metabolically compromised rodent models, non-interventional during pregnancy or lactation or if they reported fetal but no offspring outcomes. Further exclusion criteria were applied to full-text screening. We then further excluded papers with no appropriate control, isocaloric pair-feeding models, high-fat diets (>15% total energy content), protein/calorie restricted diets, intragastric or intraperitoneal feeding models or if sucrose was administered as a solid component of the diet (i.e. chow component). Only papers providing dams with a sucrose solution with a concentration between 8 and 20 % w/v were included. No restriction was placed on publication date, but only English papers were included. Hand screening of references lists was performed to identify further publications.

Data extraction

We extracted bibliographical (author, year, title) and methodological information (experimental conditions, maternal and offspring sample size, unit of analysis) from eligible papers. Data on animal characteristics (species, strain, age, weight, offspring sex, litter standardisation), sucrose feeding (exposure timing: during pre-conception and/or pregnancy and/or lactation; concentration/s; compulsory or voluntary administration), maternal and offspring metabolic (bodyweight, body composition, glycaemic control) and behavioural outcomes (food/fluid intake and preference behaviours) were also extracted. In cross-fostering models, data were extracted for each experimental group. Outcome measures were collected as mean and standard error of mean (S.E.M.) or standard deviation (S.D.), as reported in the publications. If required, a digital ruler (Pixel Ruler version 3.1) was used for extracting graphical data. A second reviewer checked 5% of the extracted data for errors and discrepancies were corrected based on the original text. Attempts to contact the author were made if data were not available or further information was required.

Meta-analysis

The main outcome measures included for meta-analyses were maternal and offspring BW and fasting glucose levels (FGLs). Random effects meta-analyses were performed using R Studio Statistical Package, Version 3.5.1 (2018-07-02) 'Feather Spray' for standardised mean differences (SMD) and corresponding 95% confidence intervals. The *metacont* function from the *meta* package was used for standard analyses. The *rma* function from the *metafor* package was used for the meta-regression. Papers containing different interventional groups of animals,[29] including those using cross-fostering models,[30,31] were included as separate data sets and thus effectively treated as independent experiments. For studies that compared more than one data set to the same control group, a correction was made using the following equation: $N_{\text{corrected control}} = N_{\text{control}}/\text{no. of experimental groups}$.[32] The I^2 statistic was used to assess heterogeneity.

Data for maternal BW (~PND21) and data for maternal FGL (taken between pre-conception and PND21) were extracted, and separate meta-analyses were performed for these outcomes. All dams were exposed to a minimum of 4 weeks' sucrose feeding. Offspring BW data were extracted in the pre-weaning period (PND15–28) and adulthood (PND56–504). Offspring analyses included rodents exposed to sucrose prior to mating and/or post mating (i.e. pregnancy or pregnancy and lactation); however, rodents exposed to sucrose only during the lactation period (through a cross-fostering model) were excluded to analyse *in utero* effects. Offspring provided direct access to sucrose solution post-weaning were also excluded. Sub-group analyses were conducted on maternal and offspring data for sucrose concentration, strain and on sex in offspring outcomes. In addition, a sub-group analysis, separated by pre and post mating sucrose exposure, was performed on offspring BW to eliminate confounding effects of the maternal metabolic state. Meta-regression analysis was also performed to analyse the effect of offspring age on BW during adulthood. All data are presented as forest plots drawn using the forest function from the *metafor* package. Four papers included in this review were excluded from the meta-analysis for the following reasons: (i) no sample size reported; (ii) insufficient statistical reporting and (iii) insufficient data for pre-weaning or adult time points.

Study quality assessment

A comprehensive assessment of methodological quality was performed using the SYRCLE's Risk of Bias (RoB) Tool.[33] Adapted from the Cochrane RoB Tool,[34] it has been specifically designed for use in systematic reviews of animal models to assess internal validity. Briefly, the checklist relates to five categories of bias: selection (items 1–3), performance (items 4–5), detection (items 6–7), attrition (item 8), reporting (item 9) and with the final item relating to litter as the unit of analysis (item 10). Due to multiparous births in rodents, using the individual pup as an experimental unit rather than litter increases the chance of Type 1 errors and as such may cause unjustly inflated precision. Items were judged as 'low' risk of bias, 'high' risk of bias or 'unclear' if we were unable to clearly assign risk of bias. Two independent reviewers (HLM and CHL or HLM and RA) assessed the included publications, with discrepancies resolved by discussion.

In addition, all papers were assessed for reporting quality by adherence to the Animal Research: Reporting of in Vivo Experiments (ARRIVE) Guidelines Checklist.[35] Developed by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) to improve reporting standards in animal publications, our assessment aimed to identify specific areas of poor reporting to aid in evaluating the reliability of each paper. We modified and developed the ARRIVE Guidelines into evaluation descriptors and a reporting system (Supplementary Table S3). The 20-item checklist was extended to include 40 associated sub-items relevant to this review. Items and sub-items are described as fully reported (FR), partially reported (PR) or non-reported, similar to previously reported tools.[36,37]

Results

Study selection

Our initial database search identified 4678 papers for review. Following removal of duplicates (2219 papers), a total of 2459 papers were screened based on eligibility criteria applied to title and abstract (2181 papers excluded) and full text (263 papers excluded). Searching of reference lists did not yield any further inclusions. Fifteen papers (including 16 maternal groups and 19 offspring groups) of maternal sucrose exposure were identified for final inclusion in this systematic review (see Fig. 1). A full list of the 263 excluded papers in Phase 2 and reason for exclusion can be found in Supplementary Table S4.

Study characteristics and design

Although several terms for mice were included in the database search, only rat studies were identified. Eleven of the included papers used Sprague-Dawley rats,[31,38-47] three used Wistar rats[30,48,49] and one did not report strain type.[29] Sucrose was administered at either 10% w/v[29-31,48,49] or 20% w/v[29,38-47] and maternal exposure ranged from 16 to 126 days, including periods during pre-conception, gestation and lactation. The majority[29,30,32-47] employed a compulsory drinking paradigm with sucrose solution being the only source of drinking fluid. A voluntary consumption model was used in two papers, with sucrose offered in addition to drinking water.[31,48] In all papers, offspring were weaned on to chow and water. They remained on this standard diet until the end of experimental testing, except for one study.[48] Here male offspring aged ~13 weeks old were given direct access to chow and 10% sucrose solution for seven weeks. This data set was not included in offspring meta-analyses to avoid confounding the results. Study designs for maternal sucrose interventions can be found in Supplementary Table S5. A cross-fostering model was used in two papers, giving rise to three additional offspring groups.[30,31] Full details of extracted study characteristics can be found in the Supplementary Table S6.

For each study, the chow component of the diet was identical between control and treatment groups; therefore, the only difference was availability of sucrose in drinking fluid. Macronutrient composition of control diet was not always reported,[29,41,44,46,47] and, for the papers that included this information, variations were noted in energy content for fat (range 4% to 13.5%), carbohydrate (39.1% to 60%) and protein (19.3% to 28.5%). Total energy content was similar between reported chows (~ 3 to 4 kcal/g). A full breakdown of reported chow composition can be found in the Supplementary Table S7.

Maternal outcomes

A summary of maternal outcomes is presented in Table 1. Ten of the included papers reported maternal results for bodyweight, body composition, glycaemic control and consumption. Due to variations in experimental design between studies, data were not available for all of the maternal outcomes listed in Table 1.

Maternal BW and body composition

Maternal BW data were extracted from seven experimental groups.[29,31,43,45,48,49] No significant change was reported for BW in dams measured during gestation (GD21),[45] lactation (PND21)[31] or both time-points.[43,48,49] Two data sets from one study observed a significant increase in BW at PND21 when dams were fed 10% or 20% w/v sucrose during pregnancy and lactation.[29] A meta-analysis on six of the extracted data sets measured at PND21 did not show a significant effect on maternal BW from sucrose consumption (SMD = 0.76, 95% CI -0.21, 1.73, $I^2 = 77\%$), as seen in Fig. 2. Body composition in dams was seldom reported, with fat mass described in only three papers. Fat mass was assessed either directly by the Soxhlet method for total body fat content[38] or by resection of fat pad deposits[48,49] or, indirectly, by plasma leptin concentration.[49] Increased total body fat was observed in dams fed sucrose for 18 weeks from pre-conception to lactation.[38] Increased visceral and subcutaneous adipose tissue in dams fed from 4 weeks pre-conception to lactation was noted, although leptin concentration did not differ between sucrose fed dams and control dams.[49] Conversely, no significant difference was identified in retroperitoneal fat pad analysis during a shorter 10-week intervention, commencing four weeks pre-mating and ceasing at parturition.[48]

Maternal FGLs, glycaemic control and triglycerides

Maternal intervention groups received a minimum of 4 weeks' access to sucrose solutions and data were obtained for FGL in 10 experimental groups. Two data sets were taken prior to mating,[30,48] five during pregnancy[40-42,45,46] and three at the end of lactation.[29,43] Meta-analysis identified a significant increase in FGL in dams exposed to sucrose relative to control dams (SMD = 1.61; 95% CI = 0.78, 2.44; $I^2 = 80\%$), as shown in Fig. 3a. When concentration was taken into consideration, no effect was evident in 10% w/v solutions (SMD = 0.43; 95% CI= -0.68, 1.53; $I^2 = 76\%$), whereas 20% solutions resulted in a significant elevation in maternal FGL, with low heterogeneity

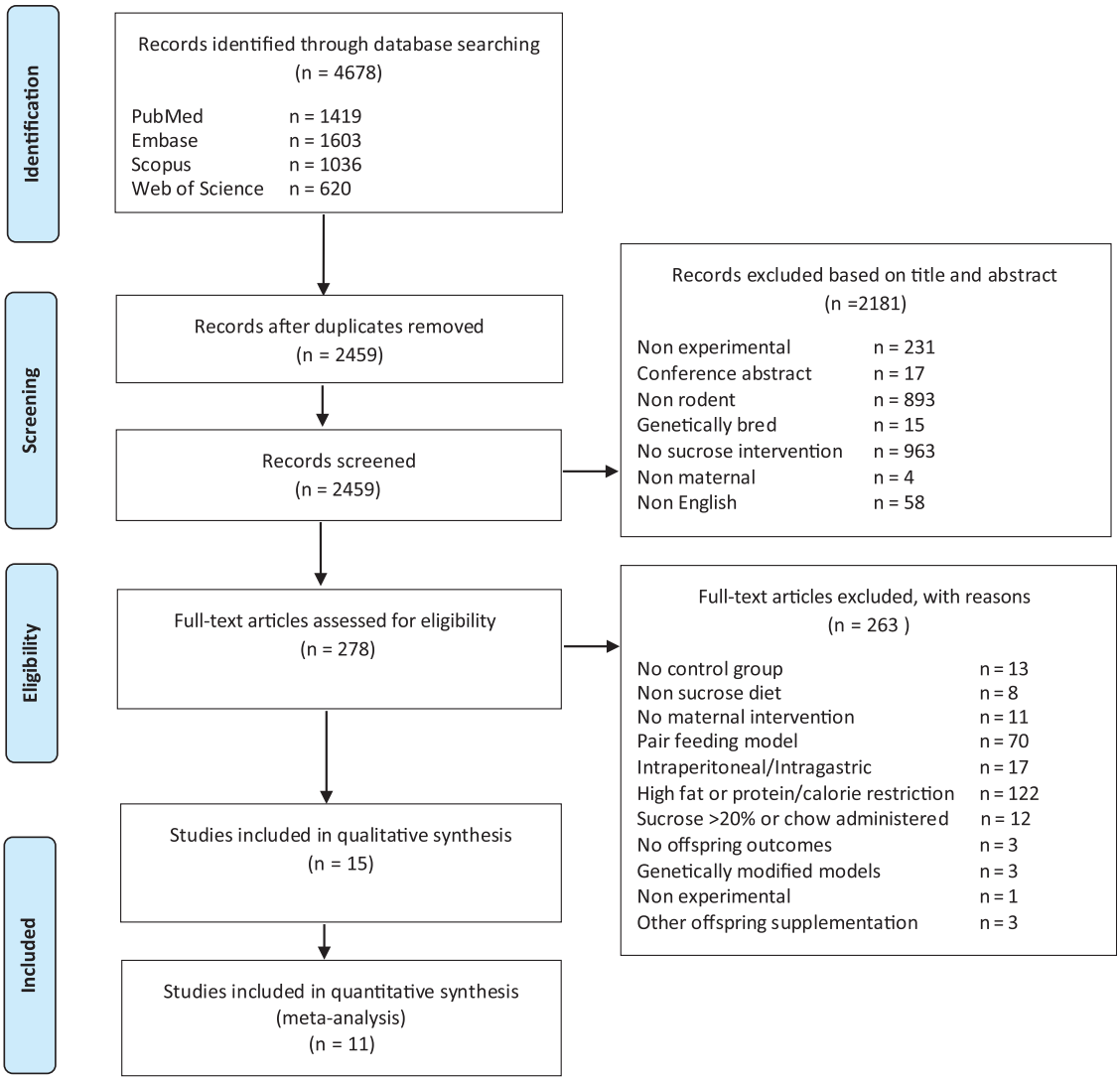

Fig. 1. PRISMA flow.

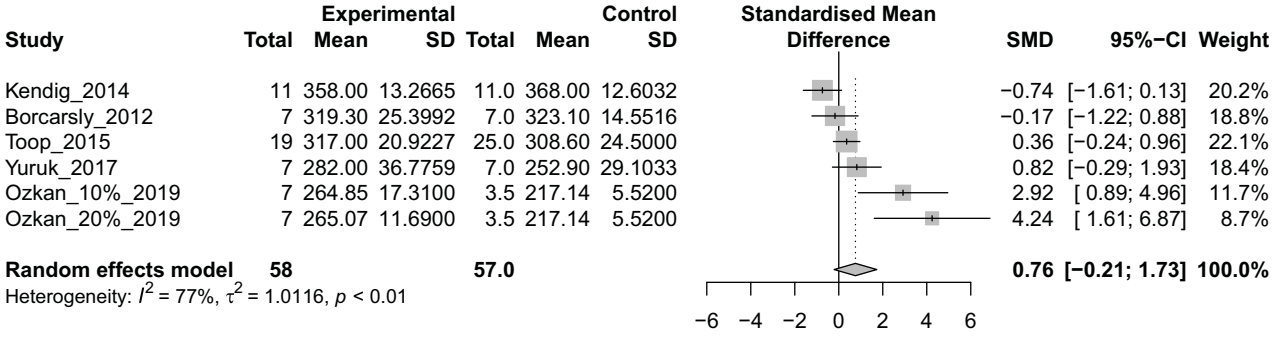

Fig. 2. Maternal body weight.

(SMD = 2.02; 95% CI = 1.51, 2.52; I^2 = 0%), see Fig. 3b. As for strain, although Wistar rats showed no significant effect (SMD = -0.07; 95% CI= -0.85, 0.71; I^2 = 56%), FGL was significantly elevated in Sprague-Dawley rats (SMD = 2.05; 95% CI = 1.51, 2.59; I^2 = 4%); see Fig. 3c. Two data sets did not report strain type.[29] It is worth noting that both studies using Wistar rats provided sucrose concentrations of 10% w/v, thus making it impossible to determine whether these failures to detect an effect of sucrose consumption on FGL were due to concentration or strain.[48,49]

Maternal fasting insulin was reported in four data sets, with three observing a significant increase relative to control[29,43], whereas one found no change.[49] Two papers reported dynamic assessment of maternal glycaemic control through glucose tolerance tests (GTT).[48,49] Reduced glucose disposal was observed following

Table 1. Summary of maternal characteristics and outcome measures of included studies. Reported outcomes extracted include maternal bodyweight prior to mating, during pregnancy and lactation, fasting blood glucose and insulin levels and consumption data

					Summary of Maternal Findings: Effect of Sucrose Exposure Relative to Control in Dams						
					Bodyweight			FBGL	FPI		
Citation	Sucrose Concentration (% w/v)	Rat Strain	N	Age at Conception (days)	Conception	GD21	PND21	(PC, G, L)	(PC, G, L)	Food Intake	Fluid Intake
Ozkan (2019)	10 20	NR NR	7 7	PND84 PND84	– –	– –	Significant ↑† Significant ↑†	Significant ↑ (L)† Significant ↑ (L)†	Significant ↑ (L)† Significant ↑ (L)†	– –	Significant ↓ Significant ↓
Kisioglu (2018)	20	SD	5-7	PND119	–	–	–	–	–	Significant ↑	No effect
Zhang (2018)	20	SD	6	–	–	–	–	Significant ↑(G)	–	–	–
Feng (2017)	20	SD	20	–	–	–	–	Significant ↑ (G)	–	No effect (DNP)	No effect (DNP)
Gu (2017)	20	SD	10	PND70–84	–	–	–	Significant ↑(G)	–	–	–
He (2017)	20	SD	11	–	–	–	–	–	–	–	–
Toop (2017)	10	WS	19	PND84	–	–	–	–	–	–	–
Yuruk (2017)	20	SD	5-7	PND98	No effect	No effect	No effect	Significant ↑(L)	Significant ↑ (L)	Data*	Data*
Wu (2016)	20	SD	NR	PND140	–	–	–	–	–	–	–
Kendig (2014)	10	WS	11	PND91–98	No effect	No effect	No effect	No effect (PC)	–	Significant ↓	Significant ↑
Toop (2015)	10	WS	19	PND91	No effect (DNP)	No effect (DNP)	No effect	No effect (PC (L)	No effect (L)	Significant ↓	Significant ↑
Kuang (2014)	20	SD	10	–	–	–	No effect	Significant ↑ (G)	–	No effect	No effect
Wu (2014)	20	SD	8	–	–	–	–	Significant ↑ (G)	–	–	–
Bocarsly (2012)	10	SD	5	–	–	–	–	–	–	–	–
Wu (2011)	20	SD	8	–	–	–	–	–	–	–	–

DNP – data not provided; FBGL – fasting blood glucose levels; FPI – fasting plasma insulin; G – gestation; GD21 – gestational day 21 (last day of pregnancy); L – lactation; N – sample size; NR – not reported; PND – postnatal day; PC – pre-conception; PND21 – postnatal day 21 (last day of lactation); SD – Sprague-Dawley; WS – Wistar; % w/v – concentration percentage weight per volume; – outcome not measured; ↑increase; ↓decrease; † data measured at PND28; * graphical data provided but unable to determine statistical effect.

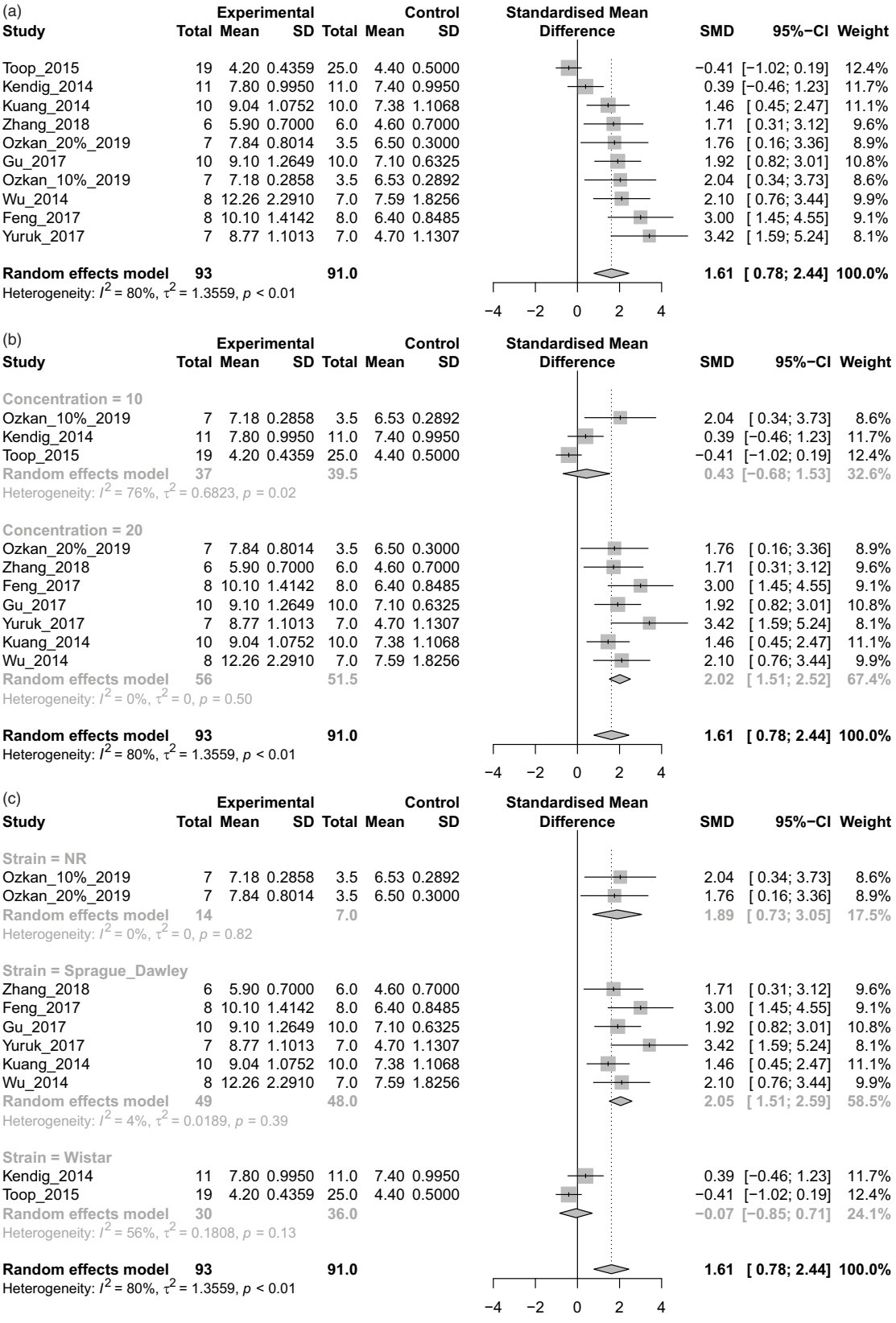

Fig. 3. (a) Maternal BGL. (b) Maternal BGL concentration. (c) Maternal BGL.

either oral[48] or intraperitoneal[49] administration of a glucose load, thus suggesting glucose intolerance. In both cases, this was measured just prior to conception, following 4 weeks of sucrose exposure. However, subsequent intraperitoneal GTT, performed at the end of lactation, observed an amelioration of this effect.[49]

Two data sets provided plasma triglyceride (TG) concentrations.[48,49] One identified a significant increase at the pre-conception time point[48] and one observed no change at the end of lactation.[49]

Maternal food and fluid intake

Significant variation in consumption was evident in eight data sets reporting maternal food and fluid intakes during the intervention period.[29,40,45,48,49] Sustained increases in sucrose solution intake compared to water were found in two groups, with a compensatory reduction of chow intake[48,49]. Other studies reported either reduced fluid intake,[29] an increase in food intake with no change in fluid[38] or no change in food or fluid intake relative to control dams.[40,45]

Relationship between maternal sucrose consumption and offspring BW and body composition

The relationship between maternal sucrose consumption and BW in pre-weaning and adult rats was not uniform across papers (Table 2). Substantial increases in offspring BW during the pre-weaning period, defined as PND1–28, were found in some studies,[29,39,45] whereas no effect was found in others[30,48] or even a significant reduction.[31] Overall, the meta-analysis on 13 experimental data sets from seven papers identified a minor elevation in pre-weaning BW (SMD = 0.81; 95% CI= 0.06, 1.57; I^2 = 81%), although significant heterogeneity was present (see Fig. 4). Further exploration by subgroup analysis showed an increase in pre-weaning BW of animals when the males and females were analysed together, compared to males and females analysed separately. An increase was also seen in rats of unknown strain compared to Sprague-Dawley and Wistar rats. It should be noted, the two data sets that did not report strain were extracted from the same publication.[29] No effect was detected on pre-weaning offspring BW for concentration. See Supplementary Fig. S8 for sub-group analysis.

BW data were available for 323 adult offspring. When maternal sucrose consumption and the impact on BW in adult offspring rats were analysed, a significant increase in BW (SMD = 0.47; 95% CI= 0.13, 0.81; I^2 = 36%) (Fig. 5a) was found with low heterogeneity. Further exploration by subgroup analysis did not show a clear effect of sex or concentration, but the increase in BW may be specific to Sprague-Dawley rats or when dams were exposed to sucrose post conception rather than prior to mating (Fig. 5b-e). Variability in the timing of BW measurement ranged from 56 to 504 days of age and, according to meta-regression modelling, the effect on BW did not depend on age, $p = 0.5944$.

Only two papers reported data on adiposity of offspring, assessed directly by resection of fat pads[30] or total body fat[38,] and their results differed. Toop *et al.*[30] reported decreases in female retroperitoneal fat mass and male total fat mass in 12-week-old rats exposed to maternal sucrose consumption during a 4-week pre-conception period, pregnancy or lactation. In contrast, Kisioglu *et al.*[38] observed an increase in offspring total body fat at the end of weaning. Although in this study, maternal sucrose exposure was significantly longer, starting 12 weeks prior to conception and finishing at the end of lactation. Furthermore, the data were not separated by sex.[38]

Relationship between maternal sucrose consumption and offspring FGLs, glycaemic control and TGs

Twelve papers provided data for fasting blood or plasma glucose concentration in offspring measured between the pre-weaning and adult period (Table 3). Thirteen experimental data sets were included in the general meta-analysis in adult offspring measured at PND84–616, with no effect observed (SMD = 0.32; 95% CI= −0.18, 0.82; I^2 = 55%); see Fig. 6. Seven of these papers provided additional data on glucose control, as indicated by static assessment of fasting plasma insulin levels,[29,30,39,43,49] Homeostatic Model of Assessment of Insulin Resistance (HOMA-IR)[39,42,43] and QUICKI estimates[43,48] or by dynamic assessment of whole body glucose tolerance through intraperitoneal[30,39,42] or oral[48] GTT. There was no significant effect on glucose disposal in offspring exposed to maternal sucrose consumption compared to control offspring aged 3 weeks,[30,43] 12 weeks[30] and approximately 13 weeks old.[48] In one study where male offspring who presented with a normal oral GTT at 13 weeks and were subsequently were offered sucrose for the following seven weeks, their QUICKI Index was elevated compared to controls.[48] Further evidence for metabolic programming in male offspring was provided by Zhang *et al.*[39], who reported hyperglycaemia at the 30-min time point during an intraperitoneal GTT in 4-month-old rats; however, HOMA-IR levels were unchanged. When intraperitoneal GTT was retested at 6 months of age, there was no longer any effect. Furthermore, He *et al.*[42] observed hyperglycaemia after 30-min during an intraperitoneal GTT, hyperinsulinaemia and elevated HOMA-IR levels for aged offspring (22 months).

Only four papers reported data on liver and/or plasma TG concentrations.[30,31,39,43] Due to limited data, a meta-analysis could not be conducted. No effect was observed in plasma and/or liver TGs in rats aged PND21 to PND180.[30,31,39] In contrast, Yuruk *et al.*[43] reported significantly higher levels for blood and liver TG concentrations measured at weaning. A full summary of offspring glycaemic outcomes is shown in Table 3.

Relationship between maternal sucrose consumption and offspring food and fluid intake

Food and fluid intake was seldom reported in offspring outcomes. Four papers reported no significant change in chow consumption for offspring exposed compared to control offspring.[30,39,42,48] Similarly, water consumption did not differ between groups, as reported in two papers[39,42] and a third observed no significant difference in sucrose consumption when 13-week-old males were given *ad libitum* access to 10% w/v sucrose for 7 weeks.[48]

Study quality assessment

Risk of bias

Results from the assessment of risk of bias are presented in Table 4. The results are presented for individual papers, and each item was assigned either low risk, high risk or unclear risk. In summary, risk of bias for the majority of papers was judged unclear. Questions 1–3 related to selection bias attributed to a lack of randomisation, baseline characteristics or concealment. Twelve of the included papers reported randomising animals to groups, yet failed to

Table 2. Summary of offspring anthropometric outcome measures. Reported outcomes extracted include offspring bodyweight at birth, the pre-weaning period and adulthood. Also included were body composition outcomes

					Summary of Offspring Anthropometric Measures: Effect of Sucrose Exposure Relative to Control			
					Bodyweight			
Citation	Rat strain	Sucrose concentration (% w/v)	Sucrose exposure	N	Birthweight	Weaning (PND1–28)	Adulthood (PND 112–504)	Body Composition
Ozkan (2019)	– –	10 20	Dams during gestation and lactation Dams during gestation and lactation	14 14	–	Significant ↑ (mixed sex) Significant ↑ (mixed sex)	– –	–
Kisioglu (2018)	SD	20	Dams 12 weeks pre-conception, pregnancy and lactation	5–7	–	–	–	Significant ↑ Measured by soxhlet technique at PND21
Zhang (2018)	SD	20	Dams during pregnancy	8–10	Significant ↑	Significant ↑	No effect ♂ (PND28–112)	–
Feng (2017)	SD	20	Dams during pregnancy	8–12 ♂	Significant ↑♂	–	No effect ♂ (PND112)	–
Gu (2017)	SD	20	Dams during pregnancy	10 ♂	Significant ↑♂	–	Significant ↑♂ (PND140)	–
He (2017)	SD	20	Dams during pregnancy	11 ♂	–	–	Significant ↑ ♂ (aged 18mths)	–
Toop (2017)	WS	10	Dams 4 weeks pre-conception, pregnancy and/or lactation∞	8 ♂ 8 ♀	–	No effect ♀[a,b,c] No effect ♂[d,e,f]	No effect ♂, ♀	Significant ↑visceral fat in ♀[a] (PND21) Significant ↓ retroperitoneal fat in ♀[b] (PND84) Significant ↓ total fat, visceral, gonadal and omental fat in ♂[c] (PND84)
Yuruk (2017)	SD	20	Dams 12 weeks pre-conception, pregnancy and lactation	5–7	No effect (mixed sex)	Significant ↑ (mixed sex)	–	–
Wu (2016)	SD	20	Dams during pregnancy	6 ♂	–	–	–	–
Kendig (2014)	WS	10	Dams 4 weeks pre-conception, pregnancy and lactation	11 ♂ 11 ♀	No effect ♂, ♀ (n=10/sex)	No effect ♂, ♀ (PND1–21)	No effect ♂, ♀ (PND56–94)	–
Toop (2015)	WS	10	Dams 4 weeks pre-conception, pregnancy and lactation	15 ♂ 15 ♀	No effect ♂, ♀	–	–	–
Kuang (2014)	SD	20	Dams during pregnancy	10 ♂	–	Significant ↑ ♂ (PND28)	Significant ↑ ♂ (PND56)	–
Wu (2014)	SD	20	Dams during pregnancy	8	Significant ↑	–	No effect ♂ (PND140)	–
Bocarsly (2012)	SD	10	Dams during pregnancy (GD6-21) and/or lactation∞	12 ♂ 12 ♀	–	Significant ↑ ♂[c,d] Significant ↓ ♀[b] (PND15)	Significant ↑♂[c,d] Significant ↑♀[a,b] (PND180)	–
Wu (2011)	SD	20	Dams during pregnancy		No effect ♂, ♀	–	No effect ♂ (PND140)	–

GD – gestational day 21; N – sample size; NR – not reported; PND – postnatal day; PC – pre-conception; PND – postnatal day; SD – Sprague-Dawley; WS – Wistar; % w/v – concentration percentage weight per volume; – outcome not measured; ↑ – increase; ↓ – decrease; ♂ - male; ♀ - female.
[a]In female interventional group exposed to sucrose during gestation period only (cross-fostering model).
[b]In female interventional group exposed to sucrose during lactation period only (cross-fostering model).
[c]In female interventional group exposed to sucrose during gestation and lactation periods (cross-fostering model).
[d]In male interventional group exposed to sucrose during gestation period only (cross-fostering model).
[e]In male interventional group exposed to sucrose during lactation period only (cross-fostering model).
[f]In male interventional group exposed to sucrose during gestation and lactation (cross-fostering model).
∞ Cross-fostering model.

adequately describe the methods (e.g. random number generator) and therefore were judged unclear. Three papers failed to describe randomisation at all.[29,31,43] Dam age and weight at the commencement of the study were considered necessary to determine whether groups were comparable, yet only six papers reported both (Q2). Concealment of the investigator to group allocation (Q3) was not reported by any papers (0/15). Similarly, randomisation of housing (0/15 papers) and blinding of caregivers (1/15 papers) were poorly

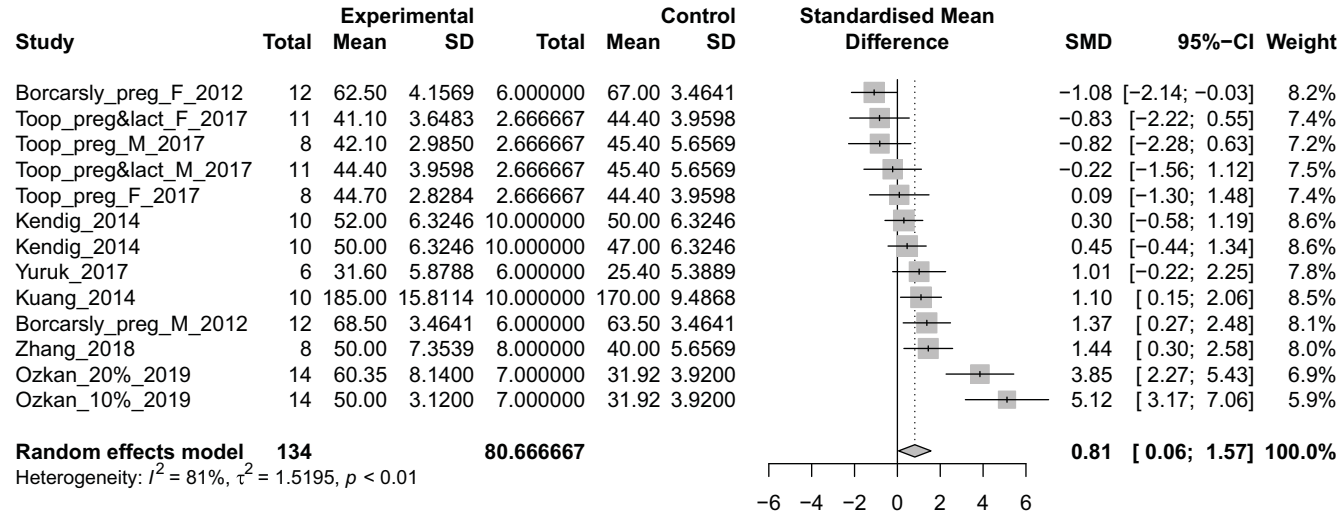

Fig. 4. Offspring body weight weaning.

performed. For detection bias, two papers reported randomly selecting animals for outcomes assessment (Q6) and blinding the assessor during outcome measurements (Q7). Data exclusion due to adverse events was addressed in three papers (Q8). The majority of studies (13/15) were assessed as free of selective outcome reporting by comparing methods and results within the paper (Question 9). Litter as a unit of analysis was considered if either (a) one animal was randomly selected to represent the litter including situations where one animal of each sex was used, (b) litter mean was used for analysis or (c) if litter was a random factor in mixed models analysis. Only five papers were judged free of using incorrect unit of analysis causing unjustly inflated precision (Q10).

Reporting adherence to ARRIVE guidelines checklist

The percentage of studies fully, partially or not reporting the 20-item checklist can be seen in Supplementary Fig. S9. In general, we observed low to moderate adherence to the guidelines for the included papers. Four items were assessed as 100% FR, including title, ethical statement, defining experimental outcomes in methods and reporting of each analysis with a measurement precision. Reporting of the abstract and background were judged as PR if the descriptor was not fully met (FR 47%; PR 53%). This was commonly observed when papers did not report species, strain or background summary in the abstract and human relevance in the introduction. Study design was not well reported (100% PR) due to an absence of blinding and reporting of litter or individual animal as unit of analysis. All papers PR detail on housing and husbandry (100% PR), with cage, bedding and housing type frequently omitted. Only two papers fully described sample size by inclusion of calculation or power analysis (13% FR; 73% PR; 13% NR). The majority of papers did not report adequate randomisation techniques and allocation to treatment groups (13% PR; 87% NR). Many failed to include testing for normality in statistical analyses (33% FR; 60% PR; 7% NR). Baseline data required for this review were maternal age and weight, with 5 out of 15 papers reporting both. Study limitations were described occasionally; however, no paper discussed scientific implications of their findings for the 3R's in animal research (100% PR), and translation to human populations was considered in just over half the included papers (53% FR; 47% NR). Individual study assessment for adherence to ARRIVE guidelines can be found in Supplementary Table S10.

Discussion

The purpose of this systematic review and meta-analysis was to identify relationships between maternal sucrose consumption and the metabolic health in offspring. Importantly, for high translational relevance, we included maternal dietary interventions where sucrose concentration closely resembled that of SSBs consumed by humans. In total, we included 15 studies for review and quantitatively synthesised data from 184 dams and 323 offspring in rat models.

Results indicate maternal sucrose consumption prior to conception and during prenatal periods did not affect maternal BW. However, a significant increase in the BW of the adult offspring was identified. A sex effect was not evident in sub-group analyses, although this conclusion was limited by a paucity of female data. Sex differences have been reported in developmental programming, although they are not commonly explored.[50,51] Literature suggests that male offspring may be more susceptible to programming of adiposity and BW than females, with the protective actions of oestrogen, maternal and paternal epigenetics and sex disparities in placental function possible mechanisms.[50-53] It is clear future studies must include female and male offspring to elucidate if sexual dimorphism exist.

A more accurate assessment of offspring adiposity can be obtained from changes in body composition rather than simple BW measurements. The latter may not indicate the contribution of prenatal food manipulation to abdominal fat.[54] Toop *et al.*[30] reported lower retroperitoneal adipose tissue in female adult offspring exposed to sucrose during pregnancy and lower relative total fat and visceral fat mass in adult males exposed during lactation. This was despite having similar BW to control offspring. This decrease in fat mass contrasts to the findings of Kisoglu *et al.*[38] who reported higher total body fat assessed by Soxhlet extraction, along with increased BW in 3-week-old pups, although sex was not considered in these data. In general, differing results limit our ability to

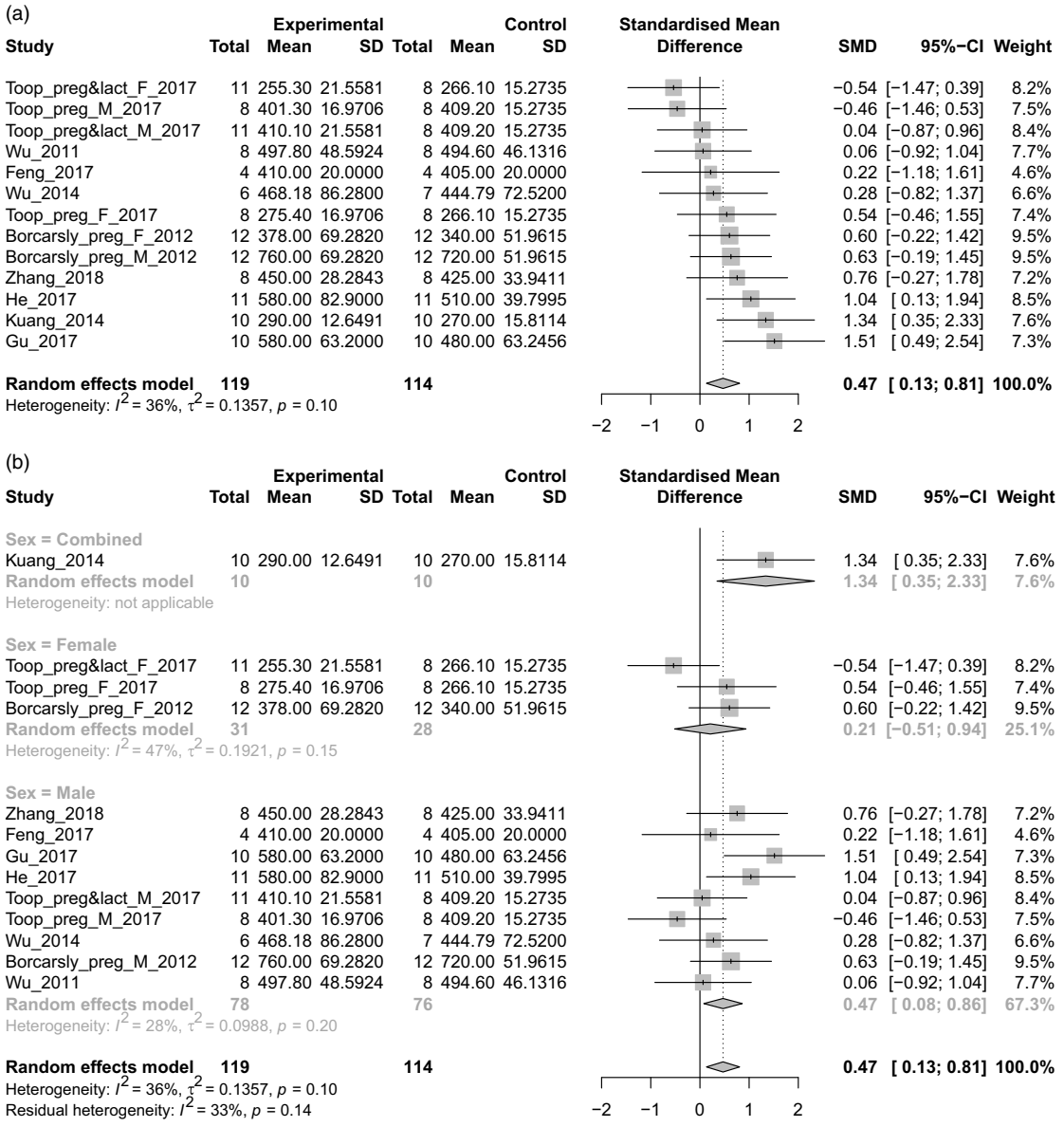

Fig. 5. (a) Offspring body weight adult. (b) Offspring body weight adult sex. (c) Offspring body weight adult concentration. (d) Offspring body weight adult strain. (e) Offspring body weight adult exposure.

interpret the potential influences prenatal sucrose exposure may have on offspring adiposity.

Hyperglycaemia and reduced glucose tolerance were evident in dams, particularly at the higher concentration of 20% w/v; however, meta-analyses did not reveal a change in the FGLs of adult offspring. When analysing outcomes for glycaemic control, we identified a number of different measures utilised, including GTT and indices from static assessment of glucose and insulin levels, such as QUICKI and HOMA-IR estimates. These were measured at varied ages in offspring. In the pre-weaning period and early adulthood, no effect was shown in male or females during OGTT[48] or intraperitoneal GTT[30,40] or in a study with combined sexes using HOMA-IR and QUICKI estimates.[43] It was only when assessing older male rats, from 4 to 18 months in age, that reduced glucose tolerance was observed with[42] and without[39] hyperinsulinaemia. Of note, Zhang *et al.*[39] found this effect was normalised when the 4-month-old offspring were re-assessed at 6 months and their data show the transient nature of glycaemic homoeostasis. Overall, results suggest that prenatal sucrose exposure did not impact the FGLs or glycaemic control in younger offspring, and it was not until rats aged that poor glucose disposal became evident. However, in many cases, prenatal exposure was confounded with an altered maternal metabolic state at the outset of pregnancy.

At the outset of this review, we included secondary behavioural outcomes for food and fluid intakes and sweet taste preferences to determine if maternal sucrose consumption elicits hyperphagia or modulate taste preferences in offspring. While consumption was

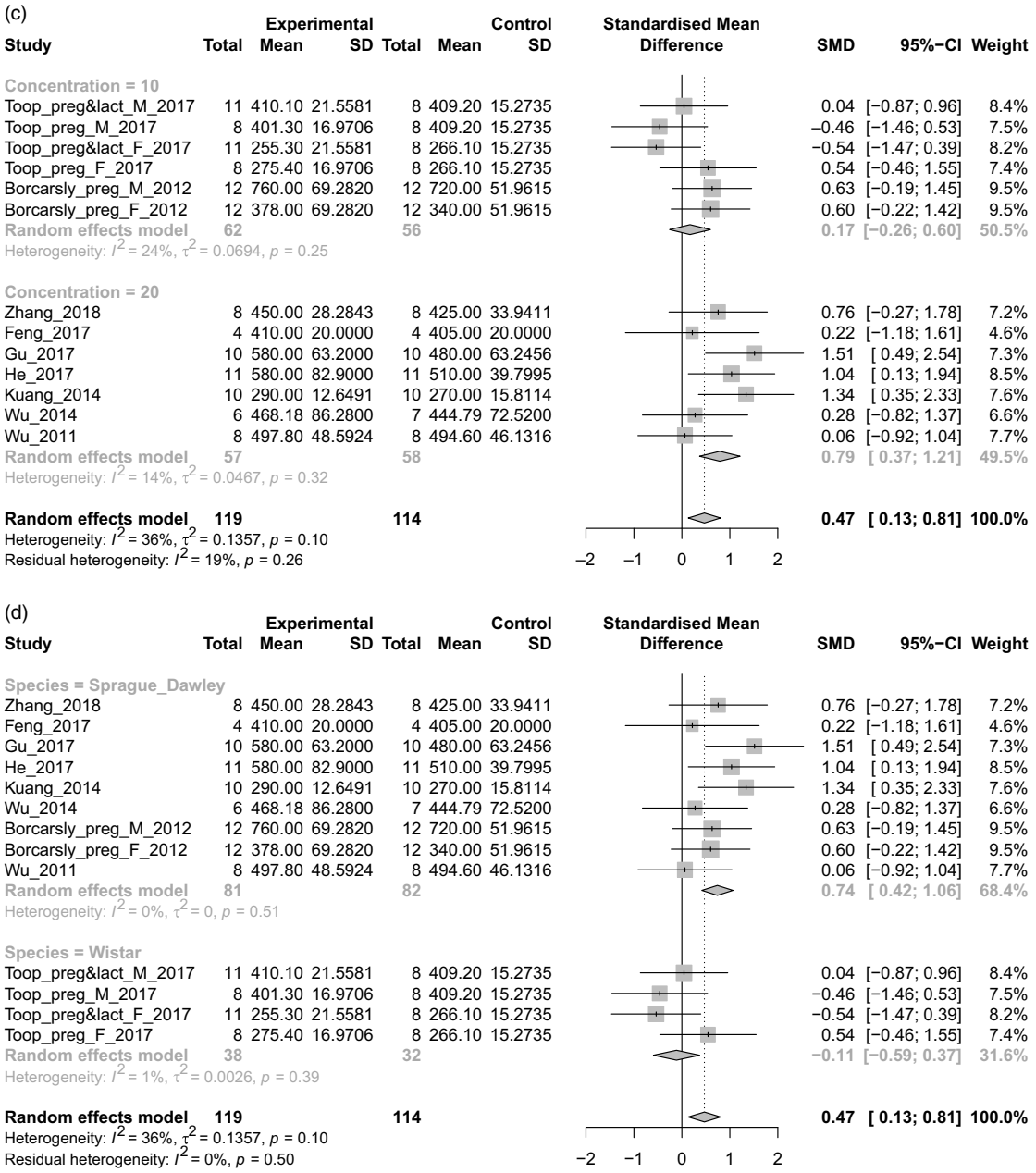

Fig. 5. *(Continued)*.

not frequently reported, studies that did report such data found no effect on offspring intakes.[30,39,42,48] No study investigating sweet preference in offspring at relevant sucrose concentrations was identified.

Cross-fostering is a valuable research tool for providing insights into the effects of sucrose exposure during crucial time-periods, i.e. lactation or pregnancy.[55] Two studies utilised this approach and both found that offspring effects were dependent on the exposure window.[30,31] Some over-nutrition models suggest lactation to be a critical time-point, displaying developmental programming of obesity.[13,56,57] Bocarsly *et al.* results support this suggestion, finding adult males were heavier in the lactation group compared to control and pregnancy groups.[31] Although Toop *et al.* observed no change to BW, exposure during the lactation period had a greater impact on adipose tissue deposition. When animals were exposed during pregnancy and lactation, poorer metabolic fatty acid profiles were also evident.[30] Notwithstanding differences between the development of human and rodent models, these results suggest health recommendations should highlight the importance of reducing SSB consumption not only during pregnancy but also throughout the lactation period.

The current review allows for exploration of mechanisms between maternal sucrose consumption and developmental programming of offspring. Some studies suggest the dysregulation of insulin signalling pathways and lipogenesis may play a role in programming offspring phenotypes.[29,30,39,43] Zhang *et al.* reported

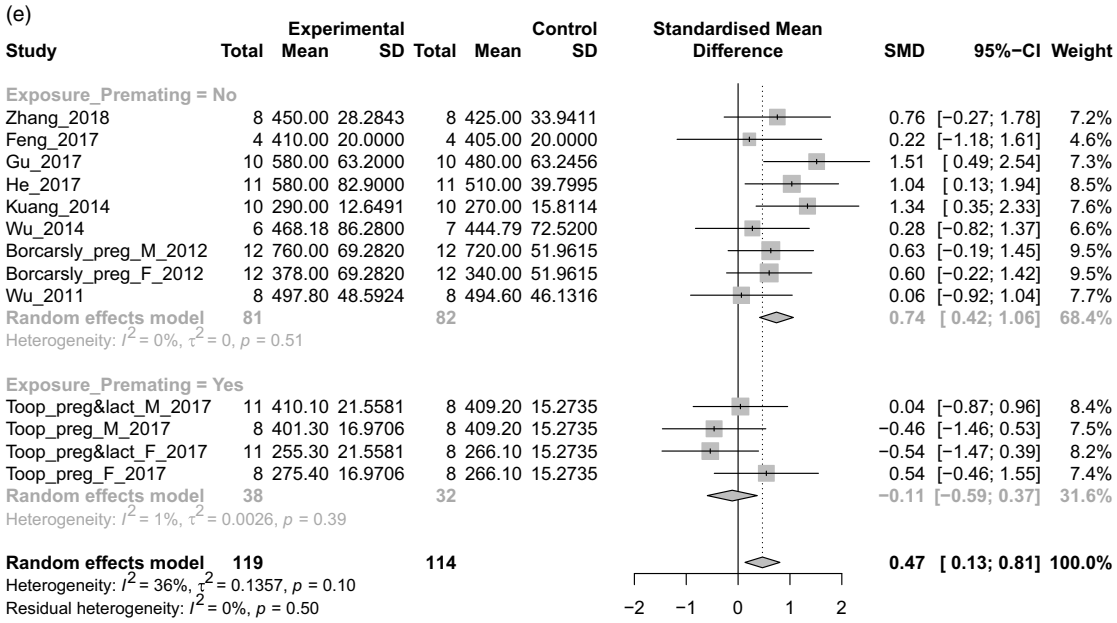

Fig. 5. *(Continued).*

a downregulation of mRNA expression of key hepatic insulin signalling molecules IRS-1, Akt and GSK-3β, although protein expression remained unchanged.[39] Adult offspring also displayed reduced glucose tolerance and enlarged pancreatic islets. Supporting these results, Ozkan *et al.* observed damage to offspring pancreatic tissue, with pups showing decreased insulin secretion and insulin receptor expression, worsening with higher sucrose concentrations.[43] Altered adipocyte programming was suggested for changes to adipose deposits in offspring with concomitant elevation of plasma free fatty acids (FFA) concentration and hepatic lipid content.[30] Yuruk *et al.* also found maternal sucrose consumption had a significant effect on increasing hepatic and liver TG and circulating NEFA levels at weaning. Other mechanisms in programming of obesity were explored, including altered appetite regulation[38] and sensitisation of reward pathways.[31] Kisogulu *et al.* found elevated offspring BWs were associated with dysregulation of satiety peptides leptin and ghrelin, although free fructose appeared to have a more significant effect than sucrose.[38] Of note, this study did not report offspring food intake. From the limited data on offspring food or fluid intake, no effect from maternal sucrose consumption was seen.[30,39,42,48] Taken together, a possible interplay of programming in metabolic pathways and appetite regulation may have detrimental effects on offspring phenotypes in rat models. Whether these mechanisms differ between rat and human systems remain unclear.

A fundamental reason for performing animal studies is to translate the outcomes to human health. There are calls amongst the academic community that rodent experimentation should more closely mimic the dietary and feeding behaviours of humans,[58] although some studies do not adhere to this principle. In our recent systematic review investigating metabolic and behavioural effects of prenatal exposure to non-nutritive sweeteners (NNS), we found many studies provided animals with NNS dosages that were not physiologically relevant to human consumption, hampering translational capacity.[37] The current review builds upon this research by only including sucrose concentrations found in commercially available SSBs. Nevertheless, it is important to acknowledge other factors can limit rodent-to-human translation when investigating maternal high-sugar diets. For example, overdrinking of sucrose solution with a compensatory reduction in chow intake was
commonly observed in dams.[48,49] In this case, it is possible that the observed offspring effects may be impacted by maternal protein restriction rather than, or in addition, to the excess sugar. Protein undernutrition appears to be important in the programming of metabolic disorders.[59] Given the majority of pregnant women exceed the minimum recommendations for protein intake,[60] one must question whether this rodent model adequately reflects human consumption patterns.

While the effects identified in this review are not as pronounced as those reported for offspring exposed during prenatal life to high-fat/high-sugar diets,[12,13,20] it is apparent the isolated effects of sucrose may contribute to metabolic developmental programming. Thus, recommendations to reduce intake of SSBs prior to conception and during pregnancy remain valid.

Strengths and limitations

Common practice in research of developmental programming is to target outcomes in males only.[12,15,32] This is evident in our review; over two-thirds of the offspring investigated were male. A number of studies reported results for both sexes combined together.[29,43] Sexual dimorphism has frequently been observed for both human and animal metabolic programming;[61,62] however, limited data meant that no firm conclusions could be reached on sex-specific differences. Future studies should include both males and females

Table 3. Summary of offspring glycaemic outcome measures. Reported outcomes extracted include offspring fasting plasma or blood glucose levels, fasting plasma insulin levels and other assessments for glycaemic control

						Summary of Offspring Glycaemic Measures: Effect of Sucrose Exposure Relative to Control			
Citation	Rat Strain	Sucrose Concentration (% w/v)	Sucrose Exposure	N	FGL	FPI	Method	Glycaemic Measure	Age Measured
Ozkan (2019)	– –	10 20	G, L G, L	14 14	Significant ↑ (mixed sex) Significant ↑ (mixed sex)	Significant ↑ (mixed sex) Significant ↑ (mixed sex)	– –	– –	PND28 PND28
Zhang (2018)	SD	20	G only	7 - 9♂	No effect ♂	No effect ♂	IPGTT HOMA-IR	Significant ↑♂ at 30 min time-point (PND112) No effect ♂ (PND168) No effect ♂	PND112 PND168 PND112
Feng (2017)	SD	20	G only	8–12 ♂	Significant ↑♂	–	–	–	PND112
Gu (2017)	SD	20	G only	10 ♂	Significant ↑♂	–	–	–	PND140
He (2017)	SD	20	G only	8 ♂	No effect ♂	Significant ↑♂	IPGTT HOMA-IR	Significant ↑♂ at 30 min time-point Significant ↑♂	PND504
Toop (2017)	WS	10	4 weeks PC, G, and/or L∞	5–11 ♂ 5–11 ♀ 5–11 ♂ 5–11 ♀	Significant ↓ ♂[f] Significant ↓ ♀[c] No effect ♂[d,e,f] No effect ♀[a,b,c]	No effect ♂ [d,e,f] No effect ♀ [a,b,c] No effect ♂ [d,e,f] No effect ♀ [a,b,c]	IPGTT IPGTT	No effect ♂ [d,e,f] No effect ♀ [a,b,c] No effect ♂ [d,e,f] No effect ♀ [a,b,c]	PND21 PND84
Yuruk (2017)	SD	20	12 weeks PC, P, L	6–7	No effect (mixed sex)	No effect (mixed sex)	HOMA-IR QUICKI	No effect (mixed sex) No effect (mixed sex)	PND21
Wu (2016)	SD	20	G only	6 ♂	No effect ♂	–	–	–	PND616
Kendig (2014)	WS	10	4 weeks PC, P, L Male offspring fed sucrose PND91142	7 ♂ 7 ♀ 10 ♂	No effect ♂ No effect ♀ Significant ↑ ♂†		OGTT QUICKI	No effect ♂ No effect ♀ ↓ Insulin sensitivity ♂†	PNDP89–94 PND142
Toop (2015)	WS	10	4 weeks PC, P, L	6 ♂ 5 ♀	No effect ♂, ♀	No effect ♂, ♀	–	–	PND1
Wu (2014)	SD	20	G only	8	No effect ♂	–	–	–	PND140
Wu (2011)	SD	20	G only		No effect ♂	–	–	–	PND140

FGL – fasting glucose level; FPI – fasting plasma insulin; G – gestation; IPGTT – intraperitoneal glucose tolerance test; N – sample size; NR – not reported; OGTT – oral glucose tolerance test; PND – postnatal day; PC – pre-conception; PND – postnatal day; SD – Sprague-Dawley; WS – Wistar; % w/v – concentration percentage weight per volume; – outcome not measured; ↑ – increase; ↓ – decrease; ♂ - male; ♀ - female.
[a]In female interventional group exposed to sucrose during gestation period only (cross-fostering model).
[b]In female interventional group exposed to sucrose during lactation period only (cross-fostering model).
[c]In female interventional group exposed to sucrose during gestation and lactation periods (cross-fostering model).
[d]In male interventional group exposed to sucrose during gestation period only (cross-fostering model).
[e]In male interventional group exposed to sucrose during lactation period only (cross-fostering model).
[f]In male interventional group exposed to sucrose during gestation and lactation (cross-fostering model).
† In male interventional group when sucrose was added to their diet from PND91 to 142 compared to control offspring.
∞ Cross-fostering model.

in their study design, with analyses exploring sex effect and sex by treatment interactions.

A comprehensive assessment of the study and reporting quality identified a high risk of bias and low or moderate adherence of reporting guidelines for many of the included studies. Generally, studies lacked appropriate reporting on randomisation and blinding techniques. This drawback has the potential to affect the internal bias of a study and may promote overestimation of the effects of the intervention.[63] A further concern, relevant to rodents having multiparous births, is the effect of litter on statistical analysis. For conclusions to be valid, each dam and its litter should be considered one experimental unit. If individual offspring are counted as its own experimental unit, this gives rise to an increase in Type 1 errors.[13,64] In over two-thirds of the studies, the experimental unit was not made clear. As such, we could not determine if statistical analyses in these studies used inflated sample sizes, which may lead to false-positive results.

The strengths of this review include detailed assessment of the quality of the studies it covers and meta-analyses, with sub-group analyses to explore heterogeneity in study design. Importantly, this

Table 4. Summary of risk of bias assessment according to SYRCLE's tool (Hooijmans et al., 2014). Results are presented for individual papers with questions judged as 'low' risk of bias, 'high' risk of bias or 'unclear' if unable to clearly assign risk, according to pre-defined signalling questions (hoojimans). Five types of bias and assessment of litter as a unit of analysis are included for assessment

	Question and Bias Type									
Citation	Q1 Selection	Q2 Selection	Q3 Selection	Q4 Performance	Q5 Performance	Q6 Detection	Q7 Detection	Q8 Attrition	Q9 Reporting	Q10 Litter as a Unit of Analysis
Ozkan (2019)	Unclear	High	High	Unclear	Unclear	Unclear	Low	Low	Low	High
Kisioglu (2018)	Unclear	Low	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Low	Low
Zhang (2018)	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Low	Unclear
Feng (2017)	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	High	Unclear
Gu (2017)	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	High	Unclear
He (2017)	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Low	Low
Toop (2017)	Unclear	High	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Low	Low
Yuruk (2017)	Unclear	Low	Unclear	Unclear	Unclear	Unclear	Unclear	Low	Low	Unclear
Wu (2016)	Unclear	Low	Unclear	Unclear	Low	Unclear	Low	Unclear	Low	Unclear
Kendig (2015)	Unclear	Low	Unclear	Unclear	Unclear	Low	Unclear	Low	Low	Low
Toop (2015)	Unclear	Low	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Low	Low
Kuang (2014)	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Low	Unclear
Wu (2014)	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Low	Unclear
Bocarsley (2012)	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Low	Unclear
Wu (2011)	Unclear	Low	Unclear	Unclear	Unclear	Low	Unclear	Unclear	Low	Unclear

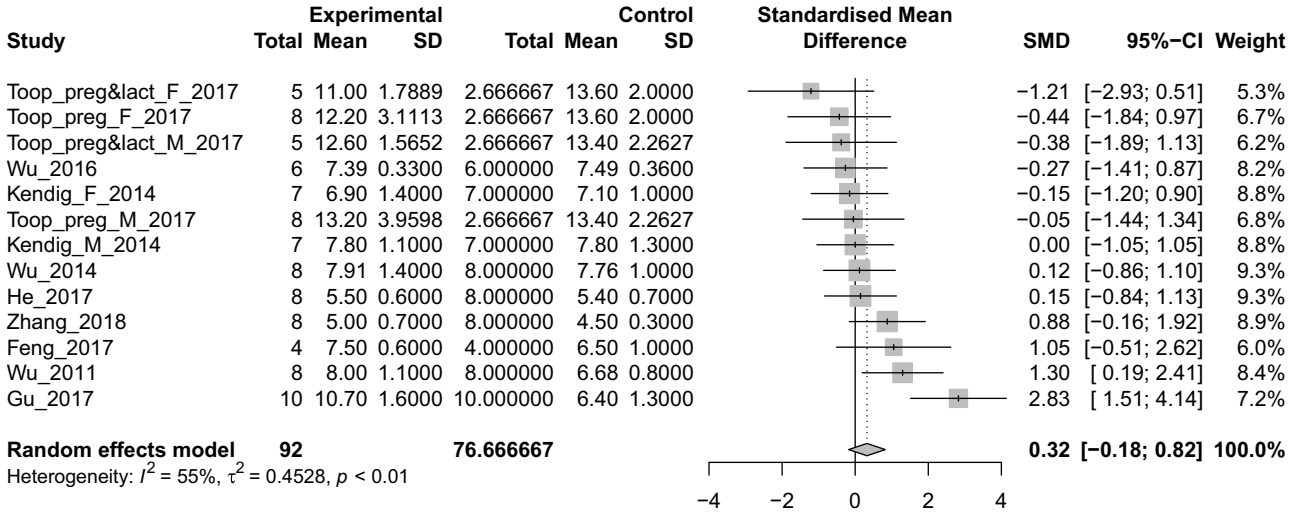

Fig. 6. Offspring BGL adult.

review offers insights for translational relevance by including sucrose concentrations that mimic aspects of the human diet when consuming SSBs.

Conclusion

Sucrose consumption prior to conception or during pregnancy, at concentrations similar to that of SSBs, elicits hyperglycaemia and adiposity with no change to bodyweight in dams. A paucity of female offspring data limited our capacity to identify potential sexually dimorphic responses in body composition and glycaemic control; therefore, future studies must include both sexes. We also revealed study design and reporting weaknesses, predisposing many of the papers to bias. Although our data did not mirror results from epidemiological studies for significant risks of childhood obesity, our review identified a risk of obesity and poor glucose control in adult offspring. These effects were not as pronounced as those obtained from research using maternal high-fat/high-sugar diets. Current recommendations to reduce consumption of SSBs during pregnancy and lactation remain

valid, not only for the metabolic health of mothers but also for that of future generations.

Supplementary mterial. For supplementary material for this article, please visit https://doi.org/10.1017/S2040174420000823

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Conflicts of interest. None.

Ethical standards. The authors assert this research was conducted and reported in accordance with the Preferred Reporting Items for Systematic Review and Meta-Analyses (PRISMA) Statement.

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PRISMA 2009 Checklist

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	3, 4
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	4
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	2, 4
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	5
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	5
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	S2.
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	5
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	6
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	6, 7
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	7, 8
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	7
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	7

PRISMA 2009 Checklist

Section/topic	#	Checklist item	Reported on page #
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	7
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	7
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	8, Fig 1., S4
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	9, S5, S6,
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	15, 16, S9, S10
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	10-14, Fig. 2-5
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	Fig. 2-5
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	Table 4
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	Fig 3, 5, S8.
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	16, 17, 18
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	19
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	20
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	20

From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi:10.1371/journal.pmed1000097

(1) PubMed database was searched using the following combination of Medical Subject Headings (MeSH) terms and relevant free terms in title and/or abstract [tiab]. In addition, an adaptation from the SYRCLE PubMed animal filter[1] designed to maximise retrieval of all animal experiments in rodent models was used.

(Epigenesis, Genetic[Mesh:noexp] OR Embryonic and Fetal Development[Mesh:noexp]) OR Epigenomics[Mesh] OR Embryonic Development[Mesh:noexp] OR Fetal Development[Mesh:noexp] OR Fetal Weight[Mesh] OR Fetal Nutrition Disorders[Mesh] OR Gene-Environment Interaction[Mesh] OR Phenotype[Mesh:noexp] OR "Foetal development"[Title/Abstract] OR "Fetal development"[Title/Abstract] OR "Foetal programming"[Title/Abstract] OR "Fetal programming"[Title/Abstract] OR Epigenetic[Title/Abstract] OR Offspring[Title/Abstract] OR "Phenotyp* programming"[Title/Abstract] OR "Phenotyp* development"[Title/Abstract] OR "Developmental programming"[Title/Abstract] OR "Epigenetic inheritance"[Title/Abstract]) AND (Nutritive Sweeteners[Mesh:noexp] OR Dietary Sugars[Mesh:noexp] OR Dietary Sucrose[Mesh] OR Sucrose[Mesh:noexp] OR Sugars[Mesh:noexp] OR Fructose[Mesh] OR Sweetening Agents[Mesh] OR "sweet* diet*"[Title/Abstract] OR "Nutritive sweeten*"[Title/Abstract] OR "High sugar diet*"[Title/Abstract] OR "HSD"[Title/Abstract] OR "added sugar*"[Title/Abstract] OR sucrose[Title/Abstract] OR fructose[Title/Abstract] OR sugar*[Title/Abstract]) AND ("Mothers"[Mesh] OR "Maternal Exposure"[Mesh] OR Maternal Nutritional Physiological Phenomena[Mesh] OR "Prenatal Exposure Delayed Effects"[Mesh] OR "Pregnancy"[Mesh:noexp] OR "Prenatal Nutritional Physiological Phenomena"[Mesh] OR Lactation[Mesh:noexp] OR Weaning[Mesh] OR Maternal[Title/Abstract] OR Gestat*[Title/Abstract] OR "in-utero"[Title/Abstract] OR "inutero"[Title/Abstract] OR pregnan*[Title/Abstract] OR offspring*[Title/Abstract] OR prenatal[Title/Abstract] OR perinatal[Title/Abstract] OR lactat*[Title/Abstract] OR Mother*[Title/Abstract] OR embryo*[Title/Abstract] OR intrauterine[Title/Abstract] OR "pre-gestation"[Title/Abstract] OR "early exposure"[Title/Abstract] OR "early life exposure"[Title/Abstract] OR wean*[Title/Abstract])

(In addition to the adapted animal filter[1])

AND ("animal experimentation"[MeSH Terms] OR "models, animal"[MeSH Terms] OR
"invertebrates"[MeSH Terms] OR "Animals"[Mesh:noexp] OR "animal population groups"[MeSH Terms] OR "rodentia"[MeSH Terms]
OR animals[tiab] OR animal[tiab] OR rodentia[Tiab] OR rodent[Tiab] OR rodents[Tiab] OR mice[Tiab] OR mus[Tiab] OR mouse[Tiab] OR murine[Tiab] OR woodmouse[tiab] OR rats[Tiab] OR rat[Tiab] OR murinae[Tiab] OR muridae[Tiab])

(2) Embase was searched using the following combination of Subject Headings and relevant keywords in addition to an adapted SYRCLE Embase animal filter[2].

(exp maternal nutrition/ OR maternal exposure/ OR exp prenatal exposure/ OR pregnancy/ OR progeny/ OR exp perinatal period/ OR lactation/ OR mother/ OR

gestation period/ OR Maternal.tw. OR Gestat*.tw. OR In utero.tw. OR Pregnan*.tw. OR Offspring.tw. OR Prenatal.tw. OR Perinatal.tw. OR Lactat*.tw. OR Mother*.tw. OR Embryo*.tw. OR Intrauterine.tw. OR Pre-gestat*.tw. OR (Early adj 2 exposure).tw. OR Wean*.tw.) AND (high-sucrose diet/ OR nutritive sweetener/ OR sugar intake/ OR sugar/ OR sucrose/ OR fructose OR Sweet* diet*.tw. OR Nutritive sweeten*.tw. OR High sugar diet*.tw. OR HSD.tw OR Added sugar*.tw. OR Sucrose.tw OR fructose.tw. OR Sugar*.tw) AND (Exp fetus development/ OR epigenetics/ OR fetus growth/ OR exp prenatal growth/ OR ecp ecological genetics/ OR exp perinatal development/ OR exp prenatal development/ OR development/ OR phenotypic plasticity/ OR F$etal development.tw. OR F$etal programming.tw. OR Epigenetic.tw. OR Offspring.tw. OR Phenotyp* programming.tw. OR Phenotyp* development.tw. OR Developmental programming.tw. OR Epigenetic inheritance.tw.)

(In addition to the adapted animal filter[2])

AND (exp animal experiment/ or exp animal model/ or exp experimental animal/ or exp transgenic animal/ or exp male animal/ or exp female animal/ or exp juvenile animal/ OR animal/ OR rodentia.tw. OR rodent.tw. OR rodents.tw. OR murinae.tw. OR mouse.tw. OR mice.tw. OR mus.tw. OR musculus.tw. OR murine.tw. OR woodmouse.tw. OR apodemus.tw. OR rat.tw. OR rats.tw. OR rattus.tw. OR norvegicus.tw.)

(3) Scopus and Web of Science will be searched using relevant keywords as seen below:

(maternal OR gestat* OR "In utero" OR "inutero" OR pregnan* OR offspring OR prenatal OR perinatal OR lactat* OR mother* OR embryo* OR intrauterine OR "Pre-gestat*" OR "early exposure" OR "early life exposure" OR wean*) AND ("Sweet* diet*" OR "Nutritive sweeten*" OR "High sugar diet*" OR HSD OR "Added sugar*" OR sucrose OR fructose OR sugar*) AND "Fetal development" OR "Foetal programming" OR epigenetic OR offspring OR "Phenotyp* programming" OR "Phenotyp* development" OR "Developmental programming") AND (rodentia OR rodent OR rodents OR murinae OR mouse OR mice OR mus OR musculus OR murine OR woodhouse OR apodemus OR rat OR rats OR rattus OR norvegicus OR "animal experiment" OR "animal model" OR "experimental animal" OR "transgenic animal" OR "male animal" OR "female animal" OR "juvenile animal" OR animal)

References

1. Hooijmans, C. R., Tillema, A., Leenaars, M., Ritskes-Hoitinga, M. Enhancing search efficiency by means of a search filter for finding all studies on animal experimentation in PubMed. Laboratory Animals, 2010; 44, 170–175. https://doi.org/10.1258/la.2010.009117

2. de Vries, R. B. M., Hooijmans, C. R., Tillema, A., Leenaars, M., Ritskes-Hoitinga, M. Letter to the Editor. Laboratory Animals, 2014; 48, 88–88

Supplementary 3. ARRIVE Descriptors

Study Quality Assessment: ARRIVE Guidelines Reporting Evaluation Descriptors

ARRIVE Checklist	Item/ sub-items	Evaluation	Descriptor for Evaluation
Title	1	FR / PR / NR	Accurate and concise description of article content
Abstract	2	FR / PR / NR	Accurate summary of background, research objectives including details of species/strain, key methods, principal findings and conclusions
			Introduction
Background	3a.	R / NR	Sufficient scientific background (relevant references) for motivation/context of study and explain experimental approach and rationale
	3b.	R / NR	Why rodent model was chosen and study's relevance to human biology
Objectives	4	FR / PR / NR	Objectives and hypotheses accurately described
			Methods
Ethical statement	5	FR / PR/ NR	Ethical review permissions/licences and relevant guidelines for care and use of animals in research
Study design	6a.	R / NR	Number of experimental and control groups
	6b.	R / NR	Randomisation measures applied during study
	6c.	R / NR	Blinding measures applied during study
	6d.	R / NR	Experimental unit reported i.e. individual animal or litter
Experimental procedures	7a.	R / NR	Description of how the experiment was performed (including procedures and equipment/suppliers used)
	7b.	R / NR	Details of timing of experimental procedures performed (i.e. time of day or timing of light/dark cycle or time according to study flow chart)
	7c.	R / NR	Details on where each experimental procedure/s was carried out (e.g. drinking cage for sweetener preference testing, Water Maze testing, home cage)
	7d.	R / NR	Rationale for why procedures were performed
Experimental animals	8a.	R / NR	Detail of rodent species
	8b.	R / NR	Details of strain
	8c.	R / NR	Details of source of rodent
	8d.	R / NR	Detail of age of dam
	8e.	R / NR	Detail of weight of dam
	8f.	R / NR	Details of health or test status (i.e. experimentally naïve, virgin)
Housing and husbandry	9a.	R / NR	Details of cage type and dimensions for each stage of experimental study
	9b.	R / NR	Details of bedding material/environmental enrichment

Supplementary 3. ARRIVE Descriptors

	9c.	R / NR	Details of housing during each stage of experimental study (i.e. group or individually housed)
	9d.	R / NR / NA	Details of number of cage companions if group housed
	9e.	R / NR	Details of facility (i.e specific pathogen free)
	9f.	R / NR	Details of light/dark cycle timing
	9g.	R / NR	Details of temperature/humidity
	9h.	R / NR	Details of food and water (composition, source, access or delivery mode)
	9i.	R / NR	Details of welfare-related assessments and interventions prior, during or after experiment; not related to adverse event
Sample size	10a.	R / NR	Details of total number of rodents used (both breeding programme and offspring) for each experiment and experimental group
	10b.	R / NR	Explanation for how the number of rodents was arrived at (i.e. sample size calculation, power analysis)
	10c.	R / NA	Number of independent replications performed (Not applicable if none reported)
Allocating animals to experimental groups	11a.	R / NR	Details of how rodents were allocated to experimental groups (randomisation or weight/sex matching)
	11b.	R / NR	Details of the order in which rodents in each experimental group were treated and assessed
Experimental outcomes	12	FR / PR / NR	Clearly defined the primary and secondary experimental outcomes assessed
Statistical methods	13a.	R / NR	Details of statistical methods used for each analysis
	13b.	R / NR	Clearly defined unit of measurement for each data set i.e. individual animal or litter
	13c.	R / NR	Testing for normality was performed
Results			
Baseline data	14	FR / PR / NR	Details of baseline characteristics and health status of rodents in each experimental group
Numbers analysed	15a.	R / NR	Reported the number of rodents in each group included in each analysis
	15b.	R / NR / NA	Reported and explained why rodents excluded from analysis, if any (Not applicable if no exclusions)
Outcomes and estimations	16	FR / PR / NR	Results reported for each analysis carried out with a measure of precision (i.e. standard error or confidence interval)
Adverse events	17a.	R / NR / NA	Details of all adverse events in each experimental group reported (Not applicable if no adverse events reported but numbers analysed in each intervention group was consistent with total randomised animals)
	17b.	R / NR / NA	Reported any modification to experimental protocols made to reduce adverse events (Not applicable if no adverse events)
Discussion			
Interpretation/scientific implications	18a.	R / NR	Results interpreted, taking into account the study's objectives and hypotheses, current theory and other relevant studies
	18b.	R / NR	Discuss any limitation of the study, including potential source of bias, animal model limitation and imprecision associated with results
	18c.	R / NR	Described implications of methods/ findings of the study for the 3R's of the use of animals in research

Supplementary 3. ARRIVE Descriptors

Generalisability/translation	19	FR / PR / NR	Commented on whether the findings are likely to translate to other species or systems, including relevance to human biology
Funding	20	FR / PR / NR	Listed funding sources (including grant number/s) and the role of the funder/s in the study

Abbreviations: FR Fully Reported; R Reported; PR Partially Reported; NR Not Reported; NA Not applicable

Supplementary S4. Excluded papers

Excluded papers from full-text screening (phase 2) with reason

Reason for Exclusion	*Number of studies (n)*	*Excluded Studies*
No appropriate non-sucrose control group for comparison:	*n* = 13	Cagiano, R., et al. (2002). "Genetic factors involved in the effects of developmental low-level alcohol induced behavioral alterations in rats." Neuropsychopharmacology **26**(2): 191-203. Ceccanti, M., et al. (2016). "Paternal alcohol exposure in mice alters brain NGF and BDNF and increases ethanol-elicited preference in male offspring." Addiction Biology **21**(4): 776-787. Coccurello, R., et al. (2016). "Paternal alcohol exposure in mice alters brain NGF and BDNF and increases ethanol-elicited preference in male offspring." Addiction Biology **21**(4): 776-787. Dunlop, M., et al. (1981). "The effects of maternal carbohydrate (sucrose) supplementation on the growth of offspring of pregnancies with habitual caffeine consumption." Biol Neonate **40**(3-4): 196-198. Gentry, G. D., et al. (1995). "Effects of prenatal maternal ethanol on male offspring progressive-ratio performance and response to amphetamine." Neurotoxicology and Teratology **17**(6): 673-677. Gentry, G. D. and L. D. Middaugh (1988). "Prenatal ethanol weakens the efficacy of reinforcers for adult mice." Teratology **37**(2): 135-144. Isaac, W. L., et al. (2009). "D-cycloserine and early ethanol exposure in developing rats." Psychological reports **105**(2): 472-476. Kakihana, R., et al. (1980). "Endocrine effects of meternal alcoholization: plasma and brain testosterone, dihydrotestosterone, estradiol, and corticosterone." Alcohol Clin Exp Res **4**(1): 57-61. Leena, H., et al. (1987). "Effect of prenatal alcohol exposure on neonatal sleep-wake behaviour and adult alcohol consumption in the AA and ANA rat lines." Alcohol and Alcoholism **22**(3): 231-240. Monjan, A. A. and W. Mandell (1980). "Fetal alcohol and immunity: Depression of mitogen-induced lymphocyte blastogenesis." Neurobehavioral Toxicology and Teratology **2**(3): 213-215. Perez, V. J., et al. (1983). "Exposure to ethanol during pregnancy in mice: potential importance of dose for the development of tolerance in offspring." Physiology and Behavior **30**(3): 485-488. Ruff, J. S., et al. (2015). "Compared to sucrose, previous consumption of fructose and glucose monosaccharides reduces survival and fitness of female mice." Journal of Nutrition **145**(3): 434-441.
Non sucrose interventional study	*n* = 8	Altshuler, G. and A. Ornoy (1986). "Thickness of renal glomerular capillary basement membrane in the offspring of diabetic rats fed a regular or high-sucrose diet." Cells Tissues Organs **126**(4): 237-239.

Supplementary S4. Excluded papers

		Belpoggi, F., et al. (2006). "Results of long-term carcinogenicity bioassays on Coca-Cola administered to Sprague-Dawley rats." Living in a Chemical World: Framing the Future in Light of the Past **Annals of the New York Academy of Sciences. 1076**: 736-752. Bezpalko, L., et al. (2015). "Inflammatory response in visceral fat tissue and liver is prenatally programmed: Experimental research." Journal of Physiology and Pharmacology **66**(1): 57-64. Lee, W. C., et al. (2016). "Maternal Fructose Exposure Programs Metabolic Syndrome-Associated Bladder Overactivity in Young Adult Offspring." Scientific reports **6**: 34669. Ruff, J., et al. (2014). "A novel method for detecting dietary (and other) toxicities: Quantifying the adverse effects of added sugar consumption." FASEB Journal. Conference: Experimental Biology **28**(1 SUPPL. 1). Ruff, J. S., et al. (2013). "Human-relevant levels of added sugar consumption increase female mortality and lower male fitness in mice." Nature Communications **4 (no pagination)**(2245). Singh, S. P., et al. (1984). "Effects of ethanol ingestion on maternal and fetal glucose homeostasis." J Lab Clin Med **104**(2): 176-184. Tanaka, H., et al. (1982). "Hypoglycemia in the fetal alcohol syndrome in rat." Brain and Development **4**(2): 97-103.
No maternal intervention exposure during pre-gestation and/or gestation and/or lactation	*n* = 11	Cicek, F. A., et al. (2014). "Di-peptidyl peptidase-4 inhibitor sitagliptin protects vascular function in metabolic syndrome: possible role of epigenetic regulation." Mol Biol Rep **41**(8): 4853-4863. Cordero, P., et al. (2013). "Maternal methyl donors supplementation during lactation prevents the hyperhomocysteinemia induced by a high-fat-sucrose intake by Dams." International Journal of Molecular Sciences **14**(12): 24422-24437. Cordero, P., et al. (2013). "Transcriptomic and epigenetic changes in early liver steatosis associated to obesity: effect of dietary methyl donor supplementation." Mol Genet Metab **110**(3): 388-395. David, E. S., et al. (1995). "Dietary induction of intestinal fructose absorption in weaning rats." Pediatric Research **37**(6): 777-782. Hilakivi, L., et al. (1987). "Effect of prenatal alcohol exposure on neonatal sleep-wake behaviour and adult alcohol consumption in the AA and ANA rat lines." Alcohol and alcoholism (Oxford, Oxfordshire) **22**(3): 231-240 Quiclet, C., et al. (2017). "Maternal exercise modifies body composition and energy substrates handling in male offspring fed a high-fat/high-sucrose diet." Journal of Physiology **595**(23): 7049-7062.

Supplementary S4. Excluded papers

		Quiclet, C., et al. (2015). "Effect of maternal exercise during gestation on high-fat high-sucrose diet-fed offspring: Body composition, glucose tolerance and energy substrates management." Clinical Nutrition **1)**: S35. Quiclet, C., et al. (2016). "Effects of maternal exercise before and during gestation or during lactation on offspring metabolic health: Body composition pancreatic function and energy substrates management." Clinical Nutrition **35 (Supplement 1)**: S126. Ramirez, I. and J. L. Fuller (1976). "Genetic influence on water and sweetened water consumption in mice." Physiology and Behavior **16**(2): 163-168. Sognnaes, R. F. (1948). "Caries-conducive effect of a purified diet when fed to rodents during tooth development." Journal of the American Dental Association **37**(6): 676-692. Villegas-Romero, M., et al. (2018). "Short-term exposure to high sucrose levels near weaning has a similar long-lasting effect on hypertension as a long-term exposure in rats." Nutrients **10 (6) (no pagination)**(728).
Pair feeding models	*n = 70*	Anandam, N., et al. (1980). "In utero alcohol heightens juvenile reactivity." Pharmacology Biochemistry and Behavior **13**(4): 531-535. Angelogianni, P. and C. Gianoulakis (1989). "Prenatal exposure to ethanol alters the ontogeny of the beta-endorphin response to stress." Alcoholism: Clinical and Experimental Research **13**(4): 564-571. Angelucci, F., et al. (1997). "Prenatal exposure to ethanol causes differential effects in nerve growth factor and its receptor in the basal forebrain of preweaning and adult rats." Journal of Neural Transplantation & Plasticity **6**(2): 63-71. Barbaccia, M. L., et al. (2007). "Cognitive impairment and increased brain neurosteroids in adult rats perinatally exposed to low millimolar blood alcohol concentrations." Psychoneuroendocrinology **32**(8-10): 931-942. Barnes, D. E. and D. W. Walker (1981). "Prenatal ethanol exposure permanently reduces the number of pyramidal neurons in rat hippocampus." Brain Res **227**(3): 333-340. Becker, H. C., et al. (1988). "Effect of prenatal ethanol exposure on response to abrupt reward reduction." Neurotoxicology and Teratology **10**(2): 121-125. Belenichev, I. F., et al. (2016). "Pharmacological modulation of heat shock protein 70 (hsp70) - Dependent mechanisms of endogenous neuroprotection in conditions of prenatal chronic alcoholism by cerebrocurin and tiocetam." Klinik Psikofarmakoloji Bulteni **26**(2): 103-108. Bond, N. W. (1980). "Postnatal alcohol exposure in the rat: Its effects on avoidance conditioning, Hebb-Williams maze performance, maternal behavior, and pup development." Physiological Psychology **8**(4): 437-443.

Supplementary S4. Excluded papers

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Sucrose >20% or chow administered	*n* = 12	Samuelsson, A. M., et al. (2013). "Sucrose feeding in mouse pregnancy leads to hypertension, and sex-linked obesity and insulin resistance in female offspring." Frontiers in Physiology **4 FEB (no pagination)**(Article 14). Choo, E. and R. Dando (2018). "No detriment in taste response or expression in offspring of mice fed representative levels of sucrose or non-caloric sucralose while pregnant." Physiology and Behavior **184**: 39-45.

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No offspring outcomes	*n* = 3	Munilla, M. A. and E. Herrera (2000). "Maternal hypertriglyceridemia during late pregnancy does not affect the increase in circulating triglycerides caused by the long-term consumption of a sucrose-rich diet by rats." J Nutr **130**(12): 2883-2888. Oliveros, L., et al. (1995). "Effect of sucrose feeding during pregnancy on rat maternal and fetal liver lipid and glycogen metabolism." Bioscience, biotechnology, and biochemistry **59**(3): 412-416. Shigeo, K. (1972). "Experimental study on the calcium and phosphorus metabolism Effect of the calcium and phosphorus deficient diet, on rat's fetus." Folia Pharmacologica Japonica **68**(3): 377-384.

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Genetically modified models	*n* = 3	Eberle, C., et al. (2012). "Maternal Immunization Affects In Utero Programming of Insulin Resistance and Type 2 Diabetes." PLoS ONE **7 (9) (no pagination)**(e45361). Isganaitis, E., et al. (2009). "In utero exposure to insulin resistance results in accelerated weight gain and insulin resistance in offspring." Diabetes. Conference: 69th Annual Meeting of the American Diabetes Association. New Orleans, LA United States. Conference Publication: **58**(SUPPL. 1A). Ooshima, T., et al. (1988). "Maternal transmission and dental caries induction in Sprague-Dawley rats infected with Streptococcus mutans." Microbiology and Immunology **32**(8): 785-794.
Non experimental or conference abstract	*n* = 1	Shaw, J. H. (1949). "Nutrition and dental caries." Federation Proceedings **8**(2): 536-545.
Other offspring supplementation	*n* = 3	Ciftci, Z. Z., et al. (2016). "The effects of beneficial bacteria on oral pathogens and caries development." Journal of Clinical Gastroenterology **50 (Supplement 2)**: S216. Dunlop, M., et al. (1981). "The effects of maternal carbohydrate (sucrose) supplementation on the growth of offspring of pregnancies with habitual caffeine consumption." Biology of the Neonate **40**(3-4): 196-198. Fortino, M. A., et al. (2017). "Could post-weaning dietary chia seed mitigate the development of dyslipidemia, liver steatosis and altered glucose homeostasis in offspring exposed to a sucrose-rich diet from utero to adulthood?" Prostaglandins Leukotrienes and Essential Fatty Acids **116**: 19-26.

Supplementary Table 5. Study design and maternal sucrose intervention summary

Citation	*Species*	*Strain*	*n (sucrose dams)*	*Sucrose concentration (% w/v)*	*Administration of sucrose solution*	*Duration of maternal exposure*		
						PG	*G*	*L*
Ozkan 2019	Rat	NR	7	10	Added to drinking water *ad libitum*	-	Y	Y
			7	20	(5 days per week)		Y	Y
Kisioglu 2018	Rat	Sprague Dawley	5-7	20	added to drinking water *ad libitum*	84	Y	Y
Zhang 2018	Rat	Sprague Dawley	6	20	added to drinking water *ad libitum*	-	Y	-
Feng 2017	Rat	Sprague Dawley	20	20	added to drinking water *ad libitum*	-	Y	-
Gu 2017	Rat	Sprague Dawley	10	20	added to drinking water *ad libitum*	-	Y	-
He 2017	Rat	Sprague Dawley	11	20	added to drinking water *ad libitum*	-	Y	-
Toop 2017	Rat	Wistar	19	10	added to drinking water *ad libitum* (cross fostering model)	28	Y	Y
Yuruk 2017	Rat	Sprague Dawley	5-7	20	added to drinking water *ad libitum*	84	Y	Y
Wu 2016	Rat	Sprague Dawley	NR	20	added to drinking water *ad libitum*	-	Y	-
Kendig 2015	Rat	Wistar	11	10	in addition to chow and water *ad libitum*	28	Y	-
Toop 2015	Rat	Wistar	19	10	added to drinking water *ad libitum*	28	Y	Y
Kuang 2014	Rat	Sprague Dawley	10	20	added to drinking water *ad libitum*	-	Y	-
Wu 2014	Rat	Sprague Dawley	8	20	added to drinking water *ad libitum*	-	Y	-
Borcarsly 2012	Rat	Sprague Dawley	5	10	in addition to chow and water *ad libitum* (cross-fostering model)	-	GD5-21	-
Wu 2011	Rat	Sprague Dawley	8	20	added to drinking water *ad libitum*	-	Y	-

Abbreviations: G – gestation; GD – gestational days; ml – millilitres; M; Molar; *n* – number of animals; NR – not reported; L – lactation; PG – pre-gestation (in days); PND – post natal days; w/v – weight per volume

Coder Initials	HLM	HLM	HLM	HLM	HLM	HLM	HLM	HLM	HLM	HLM	HLM	HLM	HLM	HLM	HLM
Date Started	4/03/2019	4/03/2019	4/03/2019	4/03/2019	4/03/2019	4/03/2019	4/03/2019	4/03/2019	4/03/2019	4/03/2019	4/03/2019	4/03/2019	4/03/2019	4/03/2019	4/03/2019
Date Completed	4/03/2019	4/03/2019	4/03/2019	4/03/2019	4/03/2019	4/03/2019	4/03/2019	4/03/2019	4/03/2019	4/03/2019	4/03/2019	4/03/2019	4/03/2019	4/03/2019	4/03/2019
Study Identification															
Study ID	Ozkan 2019	Kisioglu 2018	Zhang 2018	Feng 2017	Gu 2018	He 2017	Toop 2017	Yuruk 2017	Wu 2016	Kendig 2015	Toop 2015	Kuang 2014	Wu 2014	Borcarsly 2012	Wu 2011
Author/s	Ozkan, H., Topsakal, S., Cemen, O.	Kisioglu, K., Nerigiz-Unal, R.	Zhang, P., Zhu, D., Zhang, Y., Li, L., Chen, X., Zhang, W., Shi, R., Tao, J., Han, B., Xu, Z.	Feng, X., Xiuwen, Z., Zhang, W., Xiang, L., He, A., Liu, B., Shi, R., Wu, J., Zhu, D., Sun, M., Xu, Z.	Gu, X., He, A., Fan, X., Shi, R., Feng, X., Bo, L., Jiang, L., Li, N. , Li, Y., Wu, J., Yang, Y., Gao, Q., Xu, Z.	He, A., Zhang, Y., Yang, Y., Li, Y., Feng, X., Wei, B., Zhu, D., Liu, Y., Wu, L., Zhang, L., Xu, Z., Sun, M.	Toop, C.R., Muhlhausler, B.S., O'Dea, K., Gentili, S.	Yuruk, A.A., Nergiz-Unal, R.	Wu, L., Shi, A., Zhu, D., Bo, L., Zhong, Y., Wang, J., Xu, Z., Mao, C.	Kendig, M.D., Ekayanti, W., Stewart, H., Boakes, R.A., Rooney, K.	Toop, C.R., Muhlhausler, B.S., O'Dea, K., Gentili, S.	Kuang, H., Sun, M., Lv, J., Li, J., Wu, C., Chen, N., Bo, L., Wei, X., Gu, X., Liu, Z., Mao, C., Xu, Z.	Wu, C., Li, J., Bo, L., Gao, Q., Zhu, Z., Li, D., Shigang, L., Sun, M., Mao, C., Xu, Z.	Borcarsly, M.E., Barson, J.R., Hauca, I.M., Hoebel, B.G., Leibowitz, S.F., Avena, N.M.	Wu, L., Mao, C., Liu, Y., Shi, A., Xu, F., Zhang, L., Xu, Z
Year of Publication	2019	2018	2018	2017	2017	2017	2017	2017	2016	2015	2015	2014	2014	2012	2011
Title of Publication	Investigation of the diabetic effects of maternal high-glucose on rats	Potential effect of maternal dietary sucrose or fructose syrup on CD36, leptin, and ghrelin-mediated fetal programming of obesity	Synergetic Effects of Prenatal and Postnatal High Sucrose Intake on Glucose Tolerance and Hepatic Insulin Resistance in Rat Offspring	Maternal high-sucrose diets altered vascular large-conductance Ca^{2+}-activated K+ channels via reactive oxygen species in offspring rats	Regulation of cerebral arterial BKCa channels by angiotensin II signaling in adult offspring exposed to prenatal high sucrose diets	Prenatal high sucrose intake affected learning and memory of aged rat offspring with abnormal oxidative stress and NMDARs/Wnt signaling in the hippocampus	Impact of perinatal exposure to sucrose or high fructose corn syrup (HFCS-55) on adiposity and hepatic lipid composition in rat offspring	Maternal dietary free or bound fructose diversely influence developmental programming of lipogenesis	High sucrose intake during gestation increases angiotensin II type 1 receptor-mediated vascular contractility associated with epigenetic alterations in aged offspring rats	Metabolic effects of access to sucrose drink in female rats and transmission of some effects to their offspring	Consumption of sucrose, but not high fructose corn syrup, leads to increased adiposity and dyslipidaemia in the pregnant and lactating rat	Hippocampal apoptosis involved in learning deficits in the offspring exposed to maternal high sucrose diets	High-sucrose diets in pregnancy alter angiotensin II-mediated pressor response and microvessel tone via the PKC/Ca v 1.2 pathway in rat offspring	Effects of perinatal exposure to palatable diets on body weight and sensitivity to drugs of abuse in rats	Altered dispogenic response and expression of angiotensin receptors in the offspring exposed to prenatal high sucrose
Journal	Biomedicine & Pharmacotherapy	Nutritional Neuroscience	Molecular Nutrition and Food Research	Biology of Reproduction	Bioscience Reports	Brain Research	Journal of Physiology	Lipids in Health and Disease	Peptides	PLoS One	Journal of developmental origins of health and disease	Journal of Nutritional Biochemistry	Hypertension Research	Physiology & Behaviour	Peptides
Volume/Pages	110/ 609-617		Volume 62 Issue 5 No pxeination	Volume 96 Issue 6 no1085-1095	Volue 37 Issue 3 No examination	Volume 1669 pp114-121	Volume 595, Issue 13 pp 4379-4388	Volume 16 Issue 226 No examination	Volume86 pp133-144	Volume 10 Issue 7	Volume 6 Issue 1 pp 38-46	Volume 25 Issue 9 pp985-990	Volume 37 Issue 9 pp818-823	Volume 107 pp568-575	Volume 32 Issue 1 pp 104-111
Language	English	English	English	English	English	English	English	English	English	English	English	English	English	English	English
Location (Country)	Turkey	Turkey	China	China	China	China	Australia	Turkey	China	Australia	Australia	China	China	United States	China
Study Design															
Primary aim of study	Examine the effects of the exposure of high - does sucrose to rats and pups during pregnancy and lactation	Determine the effects of HFCS or sucrose on the peptide-mediated appetite in fetal programming of obesity	Determine whether prenatal high sucrose intake alleviateds the detrimental effects of high postnatal sugar consumption in the offspring	How ROS may influence functions of large-conductance Ca2+ - activated K+ channels in the offspring exposed to prenatal high sucrose	Determine if high sucrose intake in pregnancy affected central vascular functions in middle cerebral arter of offspring	Determine the effect of prenatal high sucrose diets on hippocampal NMDARs/Wnt signalling	Determine the impact of exposure to maternal consumption of sucrose or HFCS-55 during prenatal and/orsuckling periods on offspring	Determine the impact of maternal dietary free or bound fructose in added sugars on developmental programming of lipogenesis	Determine if maternal high sucrose diet in pregnancy can further deteriorate the Ang II-induced cardiovascular response in aged offspring	Examine the metabolic consequences for female rats having unrestricted sucrose and the effects on their offspring	Investigate and compare the impact of maternal consumption of sucrose or HFCS-55 during pregnancy and lactation on the metabolic health of the dam and her offspring	Determine the influence of maternal high sucrose diets on behaviour and hippocampal neurons in the young offspring	Determines the influence of prenatla high sucrose diet on Ang II-mediated pressor reponse and determine the underlying mechanism	Determine the effects of fat and suger rich diets in utero and during pre-weaning period on body weight and responses to drugs of abuse	Determine the water and salt intake as well as expression of AT1 and AT2 receptors in the brain and kidney in the adult offspring prenatally exposed to high sucrose
Study design	10, 20 & 30% sucrose vs Control	Maltodextrin vs Sucrose vs Fructose vs HFCS vs Control	Sucrose vs Control	Sucrose vs Control	Sucrose vs Control	Sucrose vs Control	Sucrose vs HFCS-55 vs Control; cross fostering model during lactation - CC (con-con); SS (suc-suc); SC (suc-con); CS (con-suc)	Maltodextrin vs Sucrose vs Fructose vs HFCS vs Control	Sucrose vs Control	Sucrose vs Control	Sucrose vs HFCS-55 vs Control	Sucrose vs Control	Sucrose vs Control	Sucrose vs HFCS vs Control; Cross fostering model - in utero sucrose, pre-weaning sucrose	Sucrose vs Control
Experimental conditions	Individually housed dam with two pups and administered compulsory drinking of allocated sucrose solution and standard chow	Individually housed; assigned to one of 5 groups plus standard chow	Dam and offspring housed together until weaning; assigned sucrose or control plus standard chow	Dam and offspring housed together until weaning; assigned sucrose or control plus standard chow	Dam and offspring housed together until weaning; assigned sucrose or control plus standard chow	Housing not reported; assigned to sucrose or control plus standard chow	Individually housed; assigned to sucrose, HFCS-55 or control and administered compulsory drinking of allocated solution plus standard chow	Individually housed; assigned to one of 5 groups plus standard chow	Individually housed and assigned to either sucrose or control plus standard chow	Group housed for pre-gestation dietary intervention; Individually housed with litter on assigned sucrose in addition to water and chow;	Individually housed; assigned to sucrose, HFCS-55 or control and administered compulsory drinking of allocated solution plus standard chow	Housing not reported; assigned to sucrose or control plus standard chow	Housing not reported; assigned to sucrose or control plus standard chow	Individually housed; assigned to sucrose, HFCS in addition to water and standard chow	Housing not reported; assigned to sucrose or control plus standard chow
Control	Standard chow and water	standard chow and water	standard chow and water	standard chow and water	standard chow and water	standard chow and water	standard chow and water	standard chow and water	standard chow and water	standard chow and water	standard chow and water	standard chow and water	standard chow and water	standard chow and water	standard chow and water
Number of animals in control group (dams)	7	5	6	20	10	11	25	7	NR	11	25	10	7	5	8
Number of animals in sucrose group (dams)	10% - 7 20% - 7		7	6	20	10	11	19	7	NR	11	19	10	8	5	8
Number of animals in control group (offspring)	14	6 to 7	8 to 10	Male n = 3 to 5	males n =10	n= 11 male post weaning to 18mth	CC males n=32 females n=32	6 to 7 per group		6	male n=50 female n=34	At 3 weeks: male n=8 female n=8 At 12 weeks: males n=8 female n=8		10	8	12	8
Number of animals in sucrose group (offspring)	10% - 14 20% - 14	6 to 7	8 to 10	Male n = 3 to 5	males n =11	n= 11 male post weaning to 18mth	SS males n=44; females n=44 SC males n=32; females n=32 CS males n=32; females n=32	6 to 7 per group		6	male n=48 female n=32	At 3 weeks: male n=8 female n=11 At 12 weeks: males n=5 female n=5		10	8	in utero sucrose males n=10 females n=12 Pre-weaning sucrose male n= 10 female n=12		8
Unit of measurement	unclear	litter	unclear	individual animal	individual animal	litter	litter	litter	litter	litter	litter	individual	?	?	?		
Animal Model Characteristics															
Rodent species	rat	rat	rat	rat	rat	rat	rat	rat	rat	rat	rat	rat	rat	rat	rat
Rodent strain (as stated by author)	NR	sprague dawley	sprague dawley	sprague dawley	sprague dawley	sprague dawley	wistar	sprague dawley	sprague dawley	wistar	wistar	sprague dawley	sprague dawley	sprague dawley	sprague dawley
Maternal age at conception (weeks +/- SD)	12 weeks	17 weeks	NR	NR	10 - 12 weeks	NR	12 weeks	14 weeks	25 weeks	13-14 weeks	13 weeks	NR	NR	NR	NR
Maternal weight at intervention (grams +/- SD)	NR	NR	250-280g	220-250g	NR	250-280g	216-221g	50-60g	200-250g	300-350g	NR	250-280g	NR	NR	NR
Litter standardisation	NR	no standardisation reported	no standardisation reported	Standardised to 3-5 male offspring only	Standardised to male offspring only	standardised to 1 male offspring per litter	standardised to four males and four females per litter at birth	no standardisation reported	no standardisation reported	standardised to 5 males:3 females	two males & two females per litter culled at partution	no standardisation reported	no standardisation reported	Standardised to 8 pups per litter	no standardisation reported
Dietary Intervention Characteristics															
How did study define dosage?	% w/v	% w/v	% w/v	% w/v	% w/v	% w/v	% w/v	% w/v	% w/v	% w/v	% w/v	% w/v	% w/v	% w/v	% w/v
Concentration/s used?	10% and 20% sucrose	20%	20%	20%	20%	20%	20%	10%	20%	20%	20%	10%	20%	20%	10%	20%
Timing and duration of maternal dietary intervention															
Pre-gestation	no	12 weeks	-	no	no	no	4 weeks	12 weeks	no	4 weeks	4 weeks	no	no	no	no
Gestation	yes	Yes	Yes	yes	yes	yes	yes SS and SC; no CS	yes	yes	yes	Yes	yes	yes	yes - in utero sucrose; no pre-weanine sucrose	yes
Lactation	yes	Yes	no	no	no	no	yes SS and CS; no SC	yes	no	yes	Yes	no	no	yes - pre-weaning sucrose; no in utero sucrose	no
Administration of maternal dietary intervention	added to drinking water as only source of drinking fluid ad libitum 5 days per week (water given other 2 days)	Administered to drinking water as only source of drinking fluid ad libitum	Administered to drinking water as only source of drinking fluid ad libitum	Administered to drinking water as only source of drinking fluid ad libitum	Administered to drinking water as only source of drinking fluid ad libitum	Administered to drinking water as only source of drinking fluid ad libitum	Administered to drinking water as only source of drinking fluid ad libitum	Administered to drinking water as only source of drinking fluid ad libitum	Administered to drinking water as only source of drinking fluid ad libitum	administered as a solution ad libitum in addition to standard chow and water	Administered to drinking water as only source of drinking fluid ad libitum	Administered to drinking water as only source of drinking fluid ad libitum	Administered to drinking water as only source of drinking fluid ad libitum	Administered to drinking water as only source of drinking fluid ad libitum	Administered to drinking water as only source of drinking fluid ad libitum
Additional maternal intervention/s other than sucrose	-	HFCS, Fructose, Maltodextrin (vehicle)	-	-	-	-	-	HFCS, Fructose, Maltodextrin (vehicle)	-	-	HFCS-55	-	-	-	
Offspring dietary intervention/s	-	-	half offspring fed sucrose between 4-6 months age	-	-	-	-	-	-	+/- activity	-	-	-	-	
Timing and duration of offspring dietary intervention	-	-	8 weeks of feeding	-	-	-	-	-	-	males - sucrose feeding PND 95-150	-	-	-	-	
Additional offspring intervention/s other than sucrose	-	-	-	-	-	-	-	-	-	wheel running	-	-	-	-	
List of all relevant measurable outcomes in study	**Maternal and offspring:** water consumption, body weight, BGL, serum insulin levels	**Maternal and offspring:** Feed and drink intake, total body fat accumulation, leptin and ghrelin, daily energy intake and energy contributions of macronutrients	**Maternal outcomes:** BGL at delivery **Offspring outcomes:** Body weight, food and fluid intake, FBGL, GTT, TG, plasma insulin, HOMA-IR	**Maternal and offspring:** body weight and blood glucose levels	**Maternal** : plasma glucose levels Offspring outcomes: body weight, plasma glucose	**Offspring:** food and fluid intake, body weight and plasma glucose, plasma insulin, OGTT, HOMA-IR	**Offspring:** bodweight, visceral adipose mass, IPGTT (3 & 12 weeks), FBGL, plasma glucose, TG, TC, HDL, LDL, FFA, plasma insulin, leptin, hepatic lipid concentration,	**Maternal and offspring:** Maternal Feed and drink intake, maternal and offspring body weight, plasma glucose and insulin, HOMA-IR, QUICKI, blood and hepatic TG, NEFA	**Offspring:** Plamsa glucose concentration	**Maternal outcomes:** Body weight, chow intake, solution intake, total energy intake, OGTT, FBGT, plasma triglycerides, retorperitoneal fat pads **Offspring outcomes:** Body weight gain, OGTT, solution intake, chow intake, QUICKI, FBGL, retroperitoneal fat	**Maternal outcomes:** Chow and fluid intake, IPGTT at baseline, pre-pregnancy and end of lactation, plasma glucose, insulin, NEFA, TC, HDL, LDL, leptin. **Offspring outcomes:** pup weight, plasma glucose, insulin, NEFA (all taken 24hrs after birth)	**Maternal outcomes:** chow and fluid intkae, body weight and plasma glucose **Offspring outcomes:** Body weight	**Maternal outcomes:** **blood** glucose **Offspring outcomes:** body weight, blood glucose	**Maternal outcomes:** chow and fluid intake, body weight **Offspring outcomes:** pre-weaning body weight, serum TG, chow intake	**Offspring outcomes:** body weight, plamsa glucose concentration
Sponsorship/Funding	Scientific Projects Commission of the University of Pamukkale	Supported by the Hacettepe University Scientific Research Projects Coodination Unit, Turkey	Supported by Natural Fund of Jiangsu Province,	Grant suppor by National Science Foundation of China	Grant support by National Science Foundation of China	Supported by Ministry of Science and Technology of China	Supported by Career Development award NHMRC	Supported by the Hacettepe University Scientific Research Projects Coodination Unit. Turkey	Grant support by National Science Foundation of China	Funded by ARC Discovery Grant	Funded by Career Development award from the NHMRC	Supported by National Key Scientific Research Projects	Supported by NSFC grants	Supported by AA-019623, DA-03123, AA-12882	Pantially supported by NIH Grant and NSFC grant
Author/s conflict of interest	No conflicts of interest reported	No conflicts of interest reported	No conflict of interest reported	Not stated	No conflict of interest reported	No conflict of interest reported	No conflict of interest reported	No conflict of interest reported	No conflict of interest reported	No conflict of interest reported	No conflict of interest reported	Not stated	Not stated	Not stated	Not stated

Supplementary File 7. Chow Composition

Study ID	***Chow Supplier***	***Macronutrient Chemical Composition****
Ozkan 2019	Not reported	Not reported
Kisioglu 2018	Nukleon Laboratory Systems	13% fat (corn oil) 60% carbohydrate (starch and dextrose) 24% protein (casein) Energy content 4.2 kcal/ g feed
Zhang 2018	Slaccas Laboratory Animal Centre, Shanghai China (Catalog number: M01)	13.8% fat 60.5% carbohydrate 25.7% protein
Feng 2017	Slaccas Laboratory Animal Centre, Shanghai China	4.0% fat 39.1% carbohydrate 19.3% protein 0.6% sodium chloride 1% calcium 0.70% phosphate 0.68% potassium
Gu 2017	Not reported	Not reported
He 2017	Slaccas Laboratory Animal Centre, Shanghai China	4.0% fat 39.1% carbohydrate 19.3% protein 0.6% sodium chloride 1% calcium 0.70% phosphate 0.68% potassium
Toop 2017	Speciality Feeds, Glen Forrest Western Australia, Australia	4% fat 60% carbohydrate 20% protein Energy content 14 kJ /g
Yuruk 2017	Nukleon Laboratory Systems Inc., Ankara, Turkey	13% fat (corn oil) 60% carbohydrate (corn starch and dextrose) 24% protein (casein) Energy content 4.2 kcal/g (gross energy)
Wu 2017	Not reported	Not reported

Supplementary File 7. Chow Composition

Kendig 2015	Speciality Feeds, Glen Forrest Western Australia, Australia	4% fat 60% carbohydrate 20% protein Energy content 14.2 kJ/g
Toop 2015	Speciality Feeds, Glen Forrest Western Australia, Australia	4% fat 60% carbohydrate 20% protein Energy content 14 kJ /g
Kuang 2014	Slaccas Laboratory Animal Centre, Shanghai China	4.0% fat 39.1% carbohydrate 19.3% protein 0.6% sodium chloride 1% calcium 0.70% phosphate 0.68% potassium
Wu 2014	Not reported	Not reported
Borcarsely 2012	Purina rodent chow	13.5% fat 58% carbohydrate 28.5% protein Energy content 3.02 kcal/g
Wu 2011	Not reported	Not reported

* As reported verbatim from publication

Supplementary Fig S8. Offspring body weight weaning subgroup analysis

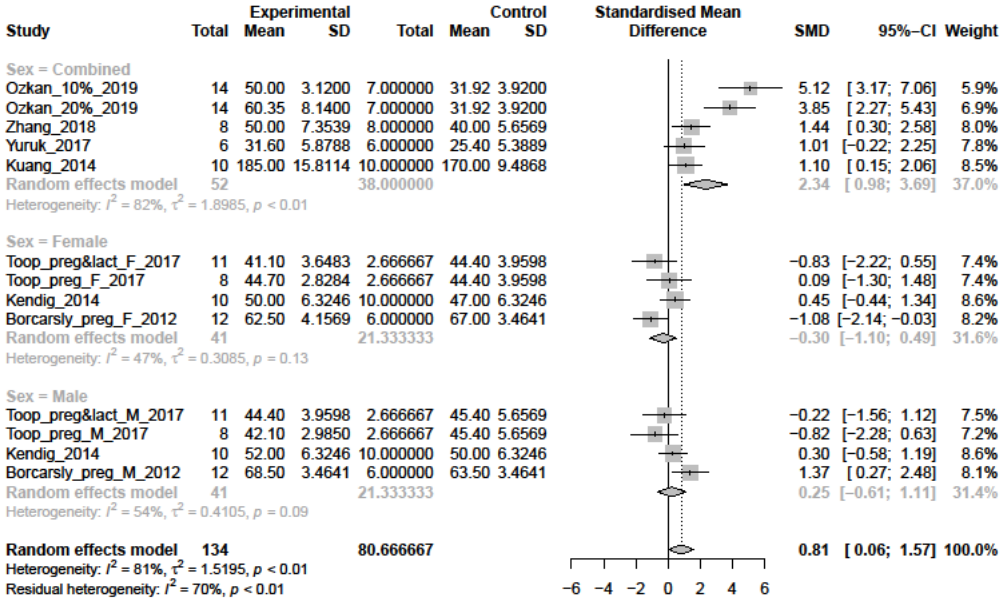

Fig 1. Offspring body weight during pre-weaning period. Offspring BW measured during PND1-28 subgroup analysed by sex (n = 7 studies; 13 experimental groups).

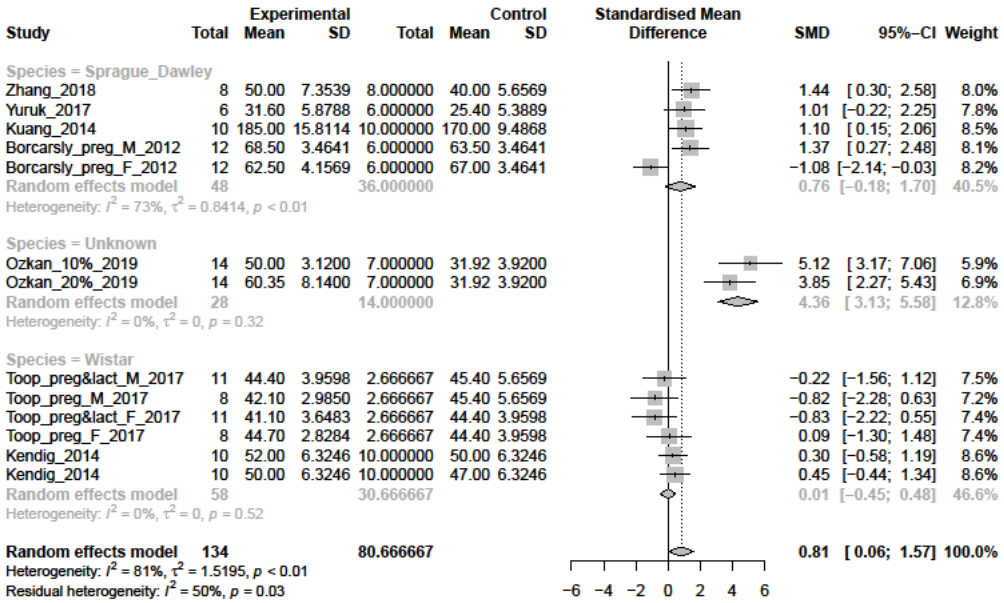

Fig 2. Offspring body weight during pre-weaning period. Offspring BW measured during PND1-28 subgroup analysed by strain (n = 7 studies; 13 experimental groups).

Supplementary Fig S8. Offspring body weight weaning subgroup analysis

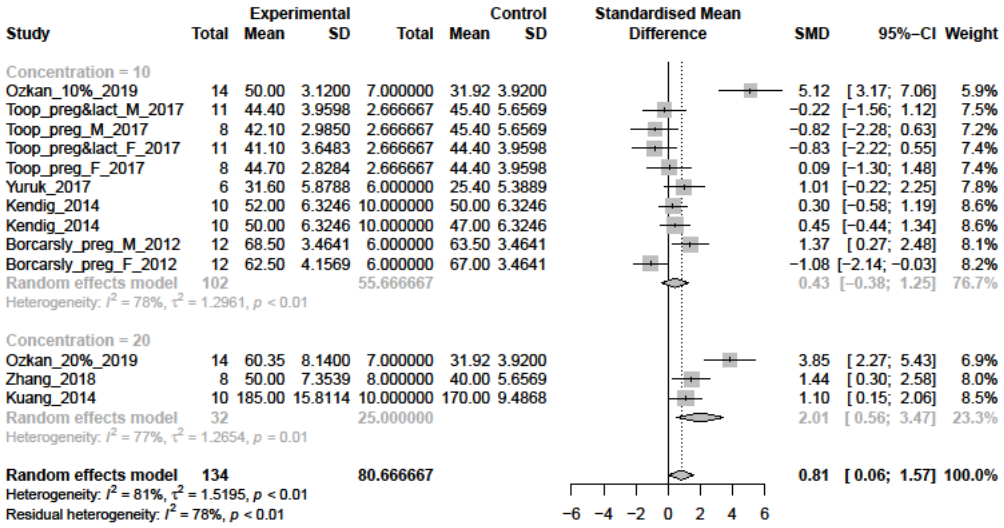

Fig 3. Offspring body weight during pre-weaning period. Offspring BW measured during PND1-28 subgroup analysed by concentration (n = 7 studies; 13 experimental groups).

Supplementary S9. Adherence of ARRIVE Guidelines Checklist per item

Figure 1. Adherence of the ARRIVE Guidelines Checklist per item. Percentages (0-100%) refer to the percentage of included studies reporting at each level for each item. Items are described as 'fully reported', partially reported or non-reported. For items with associated sub-items fully reported means that all sub-items were reported, partially reported that not all sub-items are fully reported, and not reported that none of the sub-items was reported.

		Item	*Ozkan 2019*	*Kisioglu 2018*	*Zhang 2018*
	Title	1	**FR**	**FR**	**FR**
	Abstract	2	**PR**	**FR**	**FR**
Introduction	Background	3	**PR**	**FR**	**PR**
	Objectives	4	**PR**	**PR**	**PR**
	Ethical Statement	5	**FR**	**FR**	**FR**
Methods	Study Design	6	**PR**	**PR**	**PR**
	Experimental Procedures	7	**PR**	**PR**	**PR**
	Experimental Animals	8	**PR**	**PR**	**PR**
	Housing and Husbandry	9	**PR**	**PR**	**PR**
	Sample Size	10	**PR**	**FR**	**PR**
	Allocating animals to experimental groups	11	**NR**	**PR**	**NR**
	Experimental outcomes	12	**FR**	**FR**	**FR**
	Statistical methods	13	**PR**	**FR**	**NR**
Results	Baseline data	14	**PR**	**PR**	**PR**
	Numbers analysed	15	**FR**	**FR**	**FR**
	Outcomes and estimation	16	**FR**	**FR**	**FR**
	Adverse events	17	**N/A**	**FR**	**N/A**
Discusssion	Interpretation/ scientific implications	18	**PR**	**PR**	**PR**
	Generalisability/ translation	19	**NR**	**NR**	**FR**
	Funding	20	**FR**	**FR**	**FR**

Abbreviations FR Fully Reported;
Categorised FR if all items or sub
Categorised PR if only some sub-
Categorised R if sub-items report
Categorised NR if items or all sub

Feng 2017	*Gu 2018*	*He 2017*	*Toop 2017*	*Yuruk 2017*	*Wu 2016*	*Kendig 2015*	*Toop 2015*	*Kuang 2014*	*Wu 2014*
FR	FR	FR	FR	FR	FR	FR	FR	FR	FR
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PR	NR	PR	PR	FR	PR	PR	PR	PR	PR
NR	NR	NR	NR	NR	NR	PR	NR	NR	NR
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N/A	N/A	N/A	N/A	FR	N/A	FR	N/A	N/A	N/A
PR	PR	PR	PR	PR	PR	PR	PR	PR	PR
NR	NR	NR	FR	FR	NR	FR	FR	FR	NR
FR	FR	FR	FR	FR	FR	FR	FR	FR	FR

PR Partially Reported; R Reported; NR Not Reported

›-items from reporting evaluation descriptors reported

items from reporting evaluation descriptors reported e.g. 4/6 sub-items for Item 8

ted

›-items not reported

Borcarsly 2012	*Wu 2011*	Fully Reported	Partially Reported	Not Reported	Not Applicable
FR	**FR**	100	0	0	0
PR	**PR**	47	53	0	0
FR	**FR**	47	53	0	0
FR	**FR**	73	27	0	0
FR	**FR**	100	0	0	0
PR	**PR**	0	100	0	0
FR	**PR**	27	73	0	0
PR	**PR**	27	73	0	0
PR	**PR**	0	100	0	0
PR	**NR**	13	73	13	0
NR	**NR**	0	13	87	0
FR	**FR**	100	0	0	0
PR	**PR**	33	60	7	0
PR	**NR**	33	60	7	0
FR	**NR**	87	0	13	0
FR	**FR**	100	0	0	0
N/A	N/A	20	0	0	80
PR	**PR**	0	100	0	0
FR	**FR**	53	0	47	0
FR	**FR**	100	0	0	0

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Not Reported
Not Applicable

Chapter 3: An Ecological Validity Model for the Prevention of Obesity: Non-Nutritive Sweetener Consumption in Rats and the Effects of Switching from Sugar-Sweetened to Diet Beverages

Chapter 3 has been published as:

Morahan, H., & Rooney, K. (2022). An Ecological Validity Model for the Prevention of Obesity: Non-Nutritive Sweetener Consumption in Rats and the Effects of Switching from Sugar-Sweetened to Diet Beverages. *Nutrients*, *14*(13), 2758.

Statement from co-authors confirming authorship contribution of the PhD candidate

The co-authors of the paper: *An Ecological Validity Model for the Prevention of Obesity: Non-Nutritive Sweetener Consumption in Rats and the Effects of Switching from Sugar-Sweetened to Diet Beverages. Nutrients, 14(13), 2758* confirm that Heidi Louise Morahan has made the primary contribution to this study in each of the following areas:

- Conception and design of the research, ethics approval, recruitment
- Collection and analysis of data, interpretation of the findings
- Writing of the manuscript and critical appraisal of content

In addition to the statements above, in cases where I am not the corresponding author of a published item, permission to include the published material has been granted by the corresponding author.

Heidi Louise Morahan Date: 26 September 2023

As supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

Associate Professor Kieron Rooney Date: 26 September 2023

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MDPI

Article

An Ecological Validity Model for the Prevention of Obesity: Non-Nutritive Sweetener Consumption in Rats and the Effects of Switching from Sugar-Sweetened to Diet Beverages

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Abstract: Reducing consumption of sugar-sweetened beverages (SSBs) has been encouraged due to its strong association with obesity. In parallel, consumption of "diet" or non-nutritive sweetened (NNS) beverages has significantly increased. This has led to burgeoning numbers of animal studies investigating metabolic consequences of NNS beverage consumption. However, most animal study designs do not reflect the way humans consume NNS drinks, thus reducing translational capacity. The present experiment aimed to find an ecologically valid model of NNS consumption and evidence of metabolic recovery following a switch from sucrose to NNS in female and male Sprague Dawley rats. The main behavioural outcome was consumption of commercially available NNS beverages during preference and acceptance testing, with changes to consumption following chronic sucrose consumption as a secondary outcome. The main metabolic outcome was retroperitoneal fat pad mass at culling, with body weight gain and fasting blood glucose levels (FBGLs) as secondary outcomes. In a two-phase experiment, behavioural tests were performed before and after 4 weeks of ad libitum access to 10% w/v sucrose. During Phase 2, the rats were given ad libitum access to assigned commercial NNS drinks for a further 4 weeks, with controls provided access to water only. FBGLs were measured at the end of Phases 1 and 2. Female and male rats accepted commercially available NNS beverages, although the volumes consumed varied considerably. Following the switch from sucrose to NNS (containing no sucrose), no group difference was observed in retroperitoneal fat mass, body weight change or FBGLs, suggesting both sexes exhibited limited metabolic recovery. These findings demonstrate that an ecologically valid model for NNS consumption can be developed for some commercially available NNS beverages to further enhance translational capacity.

Keywords: non-nutritive sweetener; sugar-sweetened beverages; metabolism; obesity; behaviour; translational model

Citation: Morahan, H.; Rooney, K. An Ecological Validity Model for the Prevention of Obesity: Non-Nutritive Sweetener Consumption in Rats and the Effects of Switching from Sugar-Sweetened to Diet Beverages. *Nutrients* **2022**, *14*, 2758. https://doi.org/10.3390/nu14132758

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1. Introduction

Global prevalence of obesity has dramatically increased over past decades and represents a major health challenge for both high- and low-income countries [1]. Worldwide, over 13% of the adult population are obese, and approximately 8% of deaths are the result of obesity as an attributing risk factor [2]. In 2016, it was estimated more than 1.9 billion adults and 340 million children and adolescents were considered overweight or obese, which is defined as abnormal or excessive fat accumulation to the degree that health may be impaired [3]. The World Health Organisation (WHO) and other international institutions classify an adult with a body mass index (BMI) between 25.0 and 29.9 kg/m^2 as overweight, $\geq$30.0 kg/m^2—as obese (for both sexes) [3,4]. Although the cause of obesity may be complex in nature, excessive consumption of added sugars has been widely recognised as a key contributor to its increasing prevalence [5,6]. Accordingly, global and public health authorities have strongly focused on reducing sugar-sweetened beverage (SSB) consumption [7,8] as these drinks represent the single largest source of added dietary sugars [9]. As

such, non-nutritive sweetened (NNS) or "diet" beverages have been commonly advocated as a healthy alternative to SSBs. This is due to the fact they contain no or low calories, thus reducing energy intake while maintaining sweetness [10].

Despite the expected benefits of NNS consumption, evidence from some animal studies suggests NNSs may be associated with adverse metabolic effects, including obesity and disruption of glycaemic homeostasis [11–13]. In contrast, works of others have concluded NNSs show limited or no metabolic effects in mice and rats [14–16]. As the scientific debate continues, so too do animal studies. Rodents are frequently used in NNS research to further understand certain aspects of pathophysiology that may not be feasible in humans, such as invasive and end-of life investigations. In 2016, a large systematic review investigating low-energy sweetener (LES) consumption and body weight (BW) identified over sixty studies using either rat or mouse models [17]. Although this review did not formally assess study quality using a risk-of-bias tool, the authors concluded that most studies did not reflect the way humans consume LES beverages and questioned translational relevance. They found significant differences between the design of animal studies and human behaviours in their feeding patterns and in the volumes consumed. Supporting these findings, we also identified several design flaws in studies included in our recent systematic review investigating maternal NNS consumption in rodents. These included, but were not limited to, excessive NNS dosages (often exceeding daily intake limits for humans), inappropriate feeding models and poor sweetener choice [18]. We found that included studies were largely dominated by investigations of a single sweetener dissolved in water, commonly saccharin or aspartame. However, diet beverages consumed by humans generally contain two or more sweeteners blended to improve palatability. Moreover, saccharin makes up only a small portion of the NNSs in diet drinks consumed by humans [19], limiting the ability of such studies to assess relative risk to human health. We, therefore, considered how to improve study design investigating NNSs to better foster the translation of animal outcomes to human outcomes.

It is important future animal studies deliver enhanced translational capacity to ultimately reduce wastage of animals and resources [20,21]. Some encourage change to the way researchers design aspects of the animal diet that more adequately mimics human consumption, thus giving potential insights into human obesity [22]. We, therefore, wondered whether a more ecologically valid model of NNS consumption could be developed, that is, a consumption model in rodents that is more generalized to real-life settings. Consequently, this study aimed to test whether rats would drink commercially available NNS beverages commonly consumed by humans.

In addition to the above aim, the current study extended on previous work by our laboratory that tested the potential effect of NNSs to either prevent or mediate recovery from sugar damage [23]. That experiment used female Sprague Dawley rats and modelled a switch away from SSB consumption to an NNS solution. During the initial 4- or 8-week stage, all the rats were given unrestricted access to 10% w/v sucrose (representative of SSB concentration) in addition to water and chow. In the subsequent stage, two groups of rats undertook a dietary "switch" where sucrose was replaced with saccharin or water for a further 4 or 7 weeks. Metabolic measures for BW, insulin sensitivity and fat pad mass showed near complete recovery.

The present study design differs by providing both female and male rats with a dietary switch to commercially available beverages sweetened with NNSs, further mimicking human patterns of changing behaviour from drinking SSBs to diet beverages. Moreover, we added a test group that switched from sucrose to a reformulated blend of reduced sucrose and an NNS (Sprite™) to determine if a reduced sucrose/NNS mixture might also improve metabolic status. The primary metabolic measure to determine recovery was fat pad mass at culling.

It is common for NNS consumption to be tested in naïve animals, that is, rodents that have not been previously exposed to anything sweet [15,23]. Again, this does not appear to be reflective of how NNSs are consumed in the human population. Therefore, a secondary

question was whether the consumption of commercially available drinks sweetened with NNS differed if animals had prior chronic exposure to sugar-sweetened drinks. The question we sought to answer was, would rats reduce or increase their consumption? This measure was assessed by performing preference and acceptance testing prior to and following sucrose feeding.

The experiment was conducted over two phases. In Phase 1, all the rats had access to a sucrose solution in addition to chow and water for a period of 4 weeks. We tested preference and acceptance of four different NNS beverages (and sucrose) before and after sucrose exposure. In Phase 2, the rats were allocated to the groups with four NNS beverages or water for a further 4-week period. In addition to fat mass, other metabolic measures included BW, BW gain and fasting blood glucose levels.

In summary, the primary aims of this study were twofold. First, to determine if rats consume commercially available NNS beverages, so an ecologically valid model for NNS consumption could be developed. Second, to determine if commercially available beverages sweetened with NNSs impact metabolic recovery from the damage caused by excess consumption of sugars.

2. Materials and Methods

2.1. Animals

Eighty experimentally naïve Sprague Dawley rats (40 females; 40 males) were block-randomised by weight and assigned using a computerised random number generator (RNG) to one of five groups (control, Diet Coke, Sprite, Cordial and Kombucha). Concealment of allocation to the groups was not possible due to the nature and delivery of dietary interventions. The animals were group housed (n = 4 rats/sex/cage) for a 7-day acclimatisation and handling period. They were maintained under controlled temperatures of 22–23 °C with humidity of 30–50% and standard 12:12 h reverse light/dark cycle (lights off at 10:00 h). The cages were randomly housed within the laboratory, and environmental enrichment was provided via cardboard tunnels and wood blocks for gnawing. Animals received ad libitum access to standard chow (Specialty Feeds®, Glen Forrest, WA, Australia; 14.2 kJ/g; 20% protein, 4% fat and 60% carbohydrate) and fresh tap water (Sydney water) throughout the study unless stated otherwise in the General Procedures. Approval was granted by the University of Sydney Animal Ethics Committee (No. 2019/1502). The protocol was registered in the preclinical trials registry (registry ID PCTE0000147; available from https://www.preclinicaltrials.eu/ Accessed on 23 March 2022).

2.2. Sample Size

A power analysis performed in G*Power (3.1.92, Heinrich Heine University Düsseldorf, Düsseldorf, Germany) was calculated using previous data from 24 h saccharin intake tests in naïve Sprague Dawley rats [23]. In addition, the effect size from fat pad mass data in Sprague Dawley rats following chronic sucrose feeding was also evaluated [23]. As such, a sample size of eight animals per treatment group was determined as optimal (α = 0.05; power of 95%).

2.3. Apparatus

Large racked acrylic individual ventilated cages (IVCs) were used for group housing (58 × 31 × 36 cm) with corncob bedding changed twice weekly. Smaller acrylic "drinking chambers" with metal wire lids were used for preference and acceptance testing (36 × 20 × 18 cm). Drinking solutions were accessible via plastic drinking bottles with metal spouts (500 mL for group cages and 100 mL for drinking chambers) inserted at designated openings on left and right sides. Chow was not available in the drinking chambers. Fluid intake was measured by weighing drink bottles on an electric balance to the nearest 0.1 g. Any visible spillages were recorded. Chow and fluid consumption per cage and individual bodyweight were measured every three days throughout the experiment.

2.4. Test Drinks

Four commercially available test drinks were chosen based on broad population consumption and common NNS combinations. The selected test drinks were Diet Coke™ (The Coca-Cola Company, Atlanta, GA, USA), Sprite™ (The Coca-Cola Company, Atlanta, GA, USA), Cottee's Cordial No Added Sugar™ (Woolworths Ltd., Bella Vista, Australia) and Remedy Kombucha™ (Remedy Drinks, Melbourne, Australia). The beverages were decarbonated using vigorous agitation on a magnetic plate stirrer, and Cordial was prepared as per manufacturer's instructions of a 1-in-10 dilution. Solutions were prepared daily and offered at room temperature. Preference and acceptance experiments (Days 1–4 and Days 33–36) used solutions purchased on the same day to reduce potential batch discrepancies. The composition of beverages as described on the ingredients label is shown in Table 1. Additives were reported on labels as sweetener or food additive numerical codes. For the purpose of this table, numerical codes were converted to compound names according to the Food Additives and Sweetener Numerical List [24].

Table 1. Components and nutritional information of the commercially available drinks administered during preference and acceptance testing and the four-week period following chronic sucrose feeding.

	Diet Coke™	**Sprite™**	**Cottee's Cordial Raspberry (No Added Sugar)™**	**Remedy Kombucha Ginger and Lemon™**
Sweetener(s) *	aspartame acesulfame K	sucrose stevia	cyclamate acesulfame K sucralose	stevia erythritol
Other ingredients *	water, colour (caramel IV), caffeine, phosphoric acid, citric acid	water, citric acid, sodium citrate, flavour, potassium sorbate	water, citric acid, flavour, sodium carbomethylcellulose, sodium benzoate, sodium metabisulphites, colour (carmoisine)	water, kombucha culture, black tea, green tea, ginger, lemon
Nutritional information (per 100 mL)				
Energy (kCal)	0.5	36	0.25	7
Energy (kJ)	1.9	151	2	29
Fat (g)	0	0	0	< 0.1
Protein (g)	0.05	0	0	< 0.1
Carbohydrates (g)	0.1	8.6	0	1.5
including sugars	0	8.6	0	0
Sodium (mg)	1.0	6.7	12	< 5

* Components reported as per manufacturers' nutritional information labels.

2.5. General Procedures

The experiment was conducted across two phases. Phase 1 was designed to assess preference and acceptance of test drinks and determine if a period of sucrose exposure will affect palatability and behavioural responses. Phase 2 switched experimental groups from sucrose to their assigned test drinks to assess the levels of potential metabolic recovery. The timeline of all the procedures is shown in Figure 1. BW was measured every three days during sucrose and NNS exposure, fluid intake—daily.

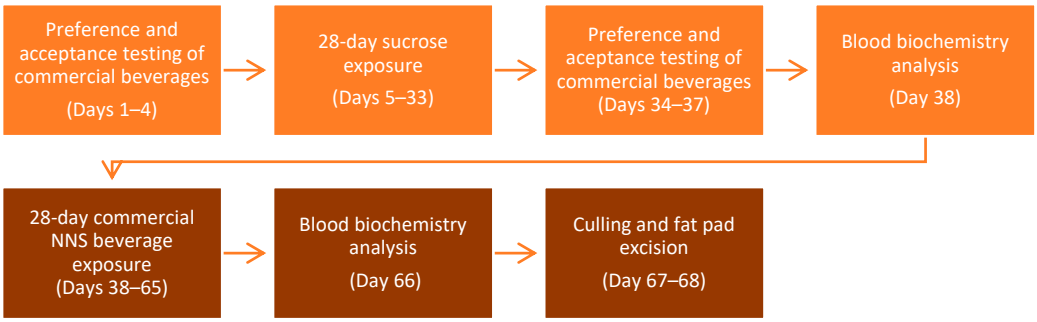


Figure 1. General timeline of all the procedures during the two phases of the experiment.

2.5.1. Phase 1

Preference and acceptance testing of NNS drinks pre-chronic sucrose exposure: Prior to testing, the rats were acclimatised to individual drinking chambers and trained to drink from two bottles as previously described [25]. On Days 1 and 2, the animals undertook a 10 min preference test over two consecutive days (13:00 h) with water offered in one bottle and the assigned test drink in the other. The control group received 10% w/v sucrose. Water was placed on the left and the test drink was placed on the right for the first test session and vice versa for the second test session to account for potential position preferences. The bottle positions were counterbalanced across test sessions for all the groups. Following testing, the rats were returned to home cages and received 30 min water access. On Days 3 and 4, the rats undertook a 4 h acceptance test to measure the overall intake of the test drink. Acceptance was defined as an intake greater than 1 mL as this was also considered the error for spillage. Similarly to the preference testing, the animals were offered two bottles, one containing water and the other containing the test drink. Following behavioural testing, the animals received free access to water and chow.

Twenty-eight-day sucrose exposure (Days 5–33): Over the next four weeks, all the groups received ad libitum access to 10% w/v sucrose solution in addition to one bottle of water and chow. As previously reported [23], a four-week sucrose feeding model induces metabolic derangements in female rats, allowing identification of metabolic recovery or deterioration following a switch to the test drink during Phase 2. Due to large sucrose intake, bottles were measured and refilled daily. On the last day (Day 33), the rats were restricted to 30 min fluid access in preparation for preference testing which commenced the following day.

Preference and acceptance testing of NNS drinks post-chronic sucrose exposure: To observe if NNS consumption differed following chronic exposure to sugar-sweetened drinks, the preference and acceptance testing was repeated (Days 34–37).

Fasting glucose and insulin (Day 38): Following overnight fasting, the animals were randomly selected (using an RNG) for fasting blood glucose and plasma insulin and triglyceride testing by a blinded experimenter. Briefly, blood samples were taken from the lateral tail vein by removing the tail tip with a sterile scalpel. Blood glucose was determined with a hand-held glucometer (OneTouch Verio©, LifeScan IP Holdings, LLC, Malvern, PA, USA). An additional 100 µL of blood were collected and diluted 1:1 with 0.9% saline in an Eppendorf tube and centrifuged at 10,000 RPM at 4 °C for 20 min. Plasma was extracted and stored at −80 °C until further analysis of fasting insulin and triglyceride levels using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (ALPCO Diagnostics, Salem, NH, USA).

2.5.2. Phase 2

Twenty-eight-day NNS beverage exposure (Days 38–65): On the same day as blood sampling, the animals started 28 days of ad libitum drinking of one of the four test drinks (Diet Coke, Sprite, Cordial or Kombucha) or water (control) in addition to water and chow. Bottles were measured and refilled daily.

Fasting glucose, insulin and retroperitoneal fat mass (Day 66–68): Repeated blood sampling was taken using the exact methods as previously described (Section 2.5.1). The rats had free access to chow and water for 24 h prior to culling by lethal injection of 300 mg/mL sodium pentobarbitone (Valabarb®, Jurox, Rutherford, Australia). The animals were randomly divided (using a computerised RNG) between two cohorts, and retroperitoneal fat pads were excised and weighed by a blinded assessor over two consecutive days. Obesity was defined as a significant increase in fat pad mass relative to the control group, as currently there is no standard anthropometrical guideline defining rodent adiposity.

2.6. Data Analysis

All data are presented as the means ± SEM. Statistical analyses were performed using IBM SPSS Statistics version 28.0 (Armonk, NY, USA) with a significance level set at $p < 0.05$. Normality was assessed using the Shapiro–Wilk test for one-way ANOVAs and for violations of Mauchly's test of sphericity in repeated and mixed measures; the Greenhouse–Geisser correction was used. The preference and acceptance tests were analysed using one-way ANOVAs with post-hoc pairwise comparisons applying Tukey's HSD or the Games–Howell correction. To determine if chronic sucrose exposure changed an animal's preference and acceptance to the test drinks, data before and after sucrose exposure were analysed using a 5 × (2) mixed ANOVA with Group as the between-subjects factor and Test as the within-subjects factor. During the 4-week sucrose exposure in Phase 1 when all the animals received identical treatment, repeated-measures ANOVAs were used to analyse changes in BW, chow and fluid consumption and total energy intake. Energy intake was calculated by converting chow (1 g = 3.4 kCal) and sucrose (1 g = 0.4 kCal) consumption into kilocalories. In Phase 2, when the groups were fed different test drinks, the data were analysed using 5 × (9) or 5 × (10) mixed ANOVAs with Group as the between-subjects factor and Days as the within-subjects factor. Changes in fasting glucose and insulin from the end of Phase 1 to the end of Phase 2 were analysed using 5 × (2) mixed ANOVAs with Group as the between-subjects factor and Test as the within-subjects factor. One-way ANOVAs with post-hoc comparisons were used to analyse FBGLs, FPI and retroperitoneal fat mass.

3. Results

3.1. Two-Bottle Preference Tests

Preference scores were calculated as the percentage of the consumed test drink relative to the total volume consumed (sum of the test drink and water) as a measure of preference for one beverage over water. An average was taken across the testing performed over two consecutive days, and data are shown for preference before and after sucrose exposure in Figure 2. Of note, both sexes displayed similar patterns of preference across most groups. As predicted, sucrose and Sprite were highly preferred over water, whereas the preference for Diet Coke was significantly lower, but still greater than that for Cordial and Kombucha, relative to water. Chronic sucrose exposure did not alter preferences for either sex.

3.1.1. Test 1 Pre-Sucrose Exposure (Days 1–2)

Analysis of the preference scores found the main effect, indicating there was a significant difference for the test drink groups (female: $F(4,35) = 94.10$, $p < 0.001$; male: $F(4,35) = 167.58$, $p < 0.001$). Pairwise comparisons (Tukey's HSD correction) revealed female preference for sucrose and Sprite was significantly higher than all other conditions (ps < 0.001), but did not differ from each other ($p > 0.05$), whereas in male rats, sucrose preference was greater than that for Sprite ($p < 0.024$). In both sexes, the preference scores for Diet Coke were greater than for Cordial and Kombucha (ps < 0.001), with no difference detected in the latter two conditions in either sex, $F > 1$.

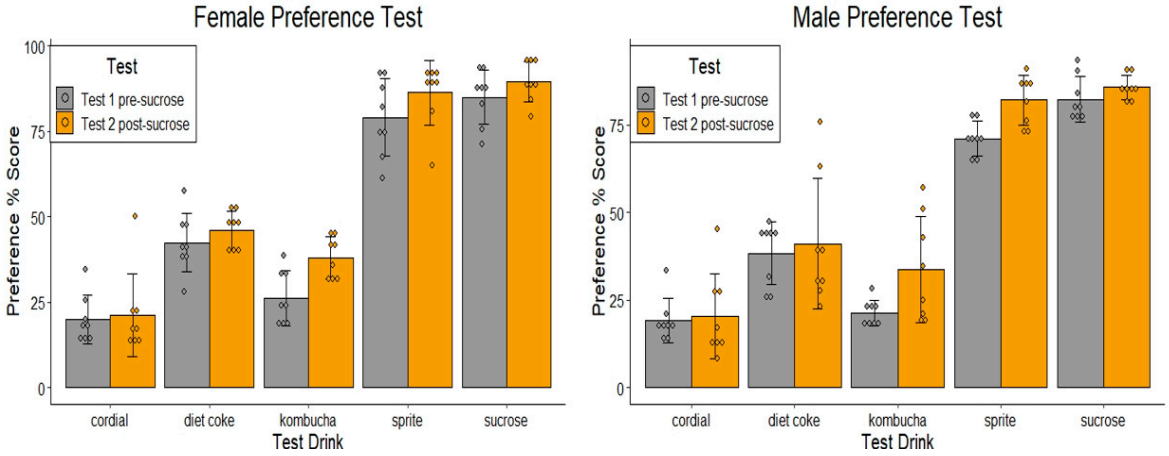


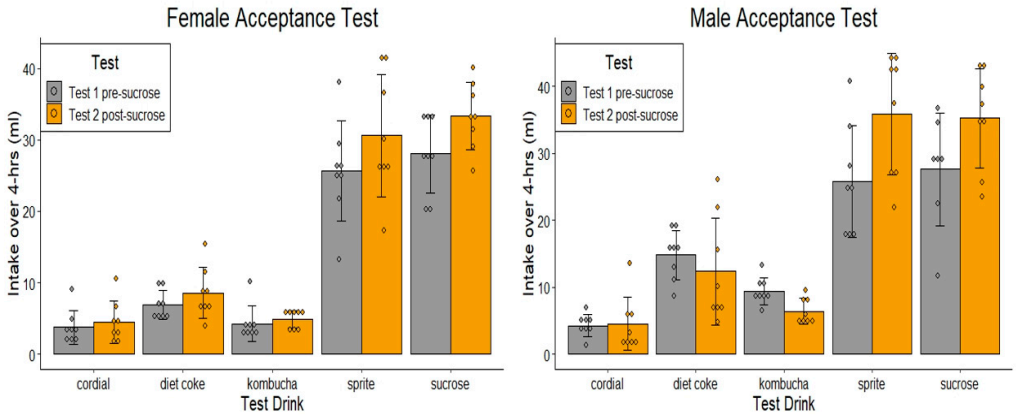
Figure 2. Preference tests performed in female and male naïve animals (Test 1 pre-sucrose) and following 4 weeks of sucrose exposure (Test 2 post-sucrose) during Phase 1. Mean % (± SEM) preference of the assigned test drink over water during 10 min two-bottle preference tests. The control group received 10% *w*/*v* sucrose (n = 8/group).

3.1.2. Test 2 Post-Sucrose Exposure (Days 34–35)

Mean scores provided in repeated preference tests following 28-days of sucrose exposure were analysed using 5 × (2) mixed ANOVAs. In females and males, there was no significant Group × Test interaction detected (ps > 0.05), indicating preference from Test 1 to Test 2 did not differ between groups (see Figure 2).

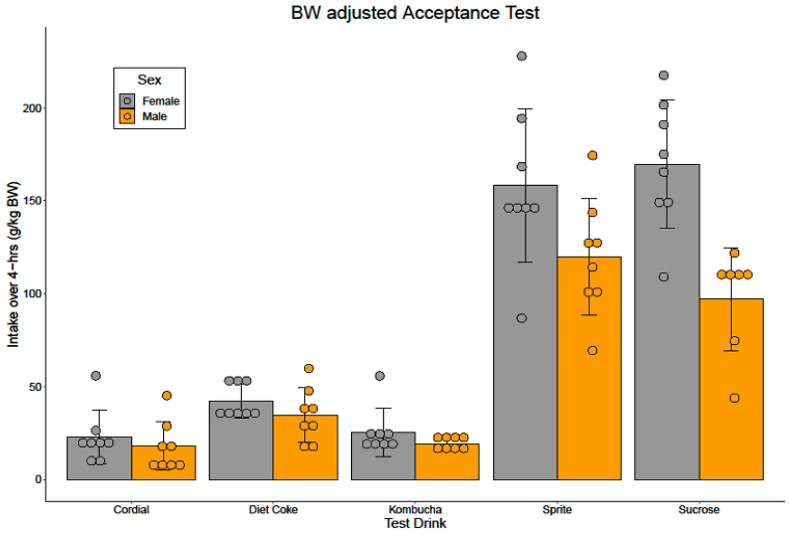
3.2. Four-Hour Acceptance Testshis

Intake of the test drinks over a 4 h period before and after sucrose exposure is shown in Figure 3. As expected, sucrose and Sprite intakes were significantly higher than that of other test groups for both sexes. An interesting observation from the data was that, although naïve female and male rats drank similar volumes of sucrose and Sprite during their tests, when adjusted for BW, females drank significantly more than males, whereas the body weight-adjusted intakes for the test drinks that did not contain sucrose (Diet Coke, Cordial and Kombucha) did not differ between sexes.



(**a**)

Figure 3. *Cont.*



(**b**)

Figure 3. (**a**) Acceptance tests performed in female and male naïve animals (Test 1 pre-sucrose) and following 4 weeks of sucrose exposure (Test 2 post-sucrose) during Phase 1. Mean intake (mL ± SEM) of the test drinks during a 4 h acceptance test showed female and male rats drank significantly higher amounts of sucrose and Sprite compared to Diet Coke, Cordial and Kombucha, $p < 0.001$, which had similar intakes. Sucrose and Sprite intakes increased following 4 weeks of sucrose exposure ($p < 0.05$) in both sexes. (**b**) BW-adjusted acceptance test in female and male naïve animals during Phase 1. Mean intake (g/kg BW) was significantly higher in female rats in the sucrose and Sprite groups ($p < 0.05$) ($n = 8$/sex/group; $n = 7$ males in the sucrose group).

3.2.1. Test 1 Pre-Sucrose Exposure (Days 3–4)

One-way ANOVAs revealed a group difference in test drink intake (females: $F(4,35) = 61.27$, $p < 0.001$; males: ($F(4,34) = 25.58$, $p < 0.001$). One dataset from the male sucrose group was excluded from analyses due to a drink bottle blockage. Pairwise comparisons confirmed the intakes of sucrose and Sprite were significantly higher than those in the other groups (ps < 0.004), although they did not differ from each other (ps > 0.05). This was evident for both sexes. In females, consumption of Diet Coke, Cordial and Kombucha did not differ over a 4 h period (ps > 0.05), whereas males drank more Diet Coke than Cordial ($p < 0.005$) (see Figure 3a). When the intakes were adjusted for BW (intake g/kg BW), a 5 × (2) (test drink × sex) ANOVA identified the main effect of sex, where female rats drank more sucrose ($F(1,68) = 39.00$, $p < 0.001$) and Sprite ($F(1,69) = 37.20$, $p < 0.001$) than male rats. There was no sex effect on weight-adjusted intakes in the Diet Coke, Cordial or Kombucha groups, (ps > 0.39) (see Figure 3b).

3.2.2. Test 2 Post-Sucrose Exposure (Days 36–37)

Following 4 weeks of sucrose exposure, analysis using 5 × (2) mixed ANOVAs observed a Group × Test interaction (females: $F(4,35) = 2.96$, $p < 0.033$; males: $F(4,34) = 6.78$, $p < 0.001$). Post-hoc comparisons revealed a simple main effect for Group, indicating rats drank more sucrose (females: $F(1,7) = 10.75$, $p = 0.014$; males: $F(1,6) = 9.61$, $p = 0.021$) and Sprite (females: $F(1,7) = 8.23$, $p = 0.024$; males: $F(1,7) = 27.67$, $p = 0.001$) during Test 2 compared to Test 1 (See Figure 3a). No difference was detected for the other conditions (ps > 0.05).

3.3. Body Weight and Weight Gain

BW data are presented in Figure 4. During Phase 1, mean BW increased significantly from 184.4 ± 2.3 g to 260.0 ± 4.7 g in females (linear trend: $F(1,35) = 759.90$, $p < 0.001$) and from 305.9 ± 2.9 g to 476.9 ± 7.3 g in males (linear trend: $F(1,35) = 918.19$, $p < 0.001$).

One-way ANOVAs found no difference in BW or weight gain on the final day of Phase 1 for either sex (Fs < 1). At the end of Phase 1, the animals were fluid- and chow-restricted in preparation for behavioural and physiological testing. This was reflected in a small BW reduction on the first day of NNS exposure (NNS1), although this soon recovered. Similarly, BW increased linearly during Phase 2 (females: $F(1,35) = 554.00$, $p < 0.001$; males: $F(1,35) = 2131.87$, $p < 0.001$). However, analyses failed to detect a Day × Group interaction (ps > 0.26), indicating that the rate of weight gain did not differ between the test drink groups. Baseline BWs during test group allocation are displayed in Tables 2 and 3.

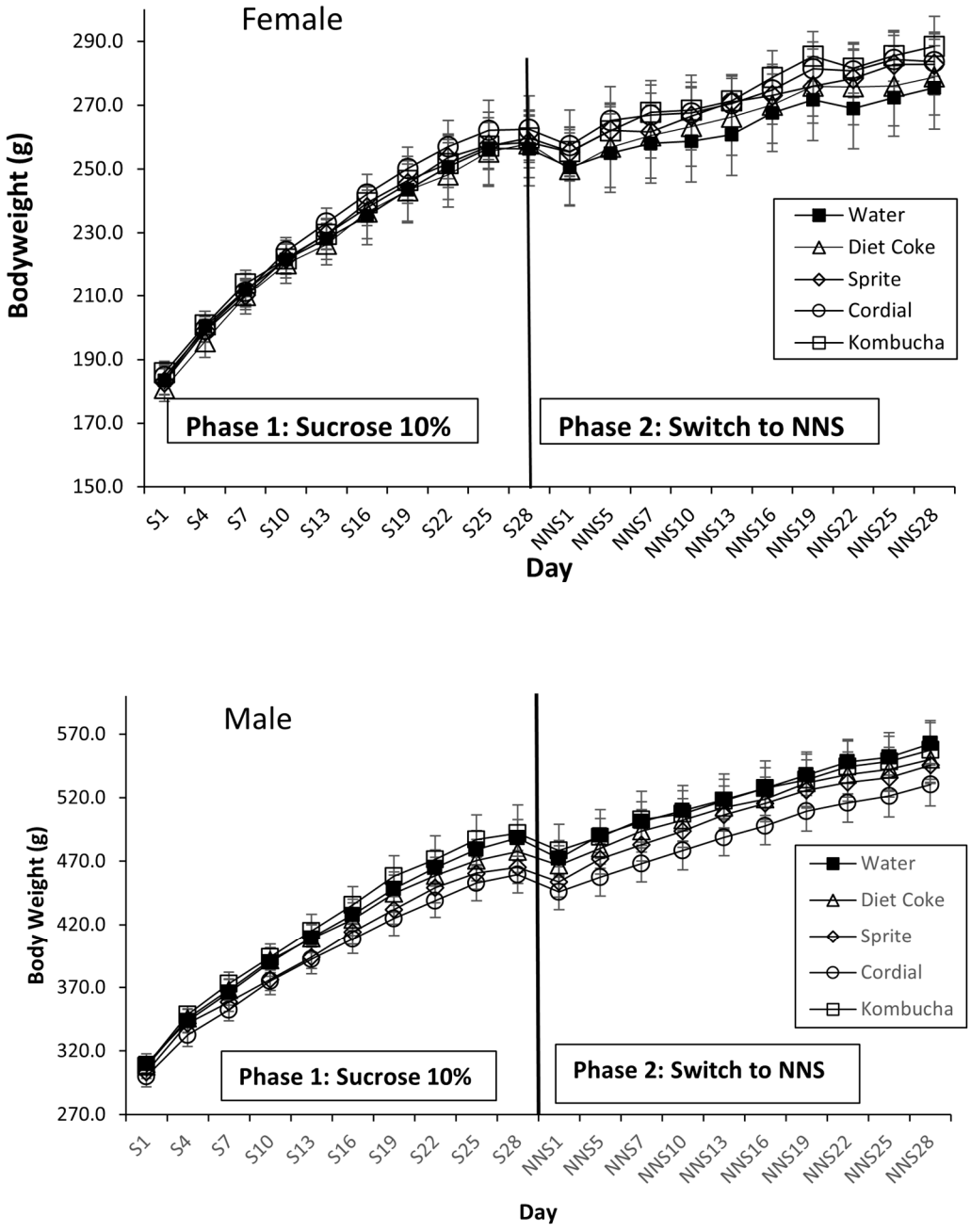


Figure 4. BW gain for the groups during Phase 1 sucrose exposure and following a switch to NNS exposure in Phase 2 in female and male rats. During Phase 1, all the rats received 28 days of 10% sucrose in addition to water and chow ($n = 40$). In Phase 2, the rats received 28 days of their assigned NNS test drink in addition to water and chow ($n = 8$/group). No significant effect on BW was observed at any point ($p > 0.05$). S = sucrose; NNS = non-nutritive sweetener.

Table 2. Female group means ± SEM for sucrose exposure in Phase 1 and NNS exposure in Phase 2.

Phase 1 Sucrose Exposure	***Control* (*n* = 8)**	***Diet Coke* (*n* = 8)**	***Sprite* (*n* = 8)**	***Cordial* (*n* = 8)**	***Kombucha* (*n* = 8)**
Baseline BW (g)	142.6 ± 4.4	141.9 ± 4.1	141.7 ± 4.1	142.1.1 ± 3.7	142.4 ± 3.6
Chow intake (g/rat/day)	11.4 ± 0.4	11.5 ± 0.3	11.3 ± 0.4	12.8 ± 0.5	12.2 ± 0.4
Sucrose (mL/rat/day)	78.6 ± 1.4	81.5 ± 1.3	89.9 ± 3.0	75.6 ± 4.0	72.4 ± 2.4
Water (mL/rat/day)	0.7 ± 0.2	0.5 ± 0.1	0.7 ± 0.01	0.7 ± 0.01	0.7 ± 0.01
Total energy intake (kcal/rat/day)	70.3 ± 1.3	70.6 ± 1.1	74.6 ± 0.9	73.6 ± 1.1	70.5 ± 1.2
Fasting blood glucose (mM)	4.7 ± 0.1	4.7 ± 0.1	4.8 ± 0.1	5.0 ± 0.1	4.7 ± 0.1
Phase 2 NNS exposure					
Chow intake (g/rat/day)	14.0 ± 0.7 [b]	14.7 ± 0.2	11.4 ± 0.4 *	15.3 ± 0.4	15.8 ± 0.5 [a]
Test drink (mL/rat/day)	N/A	17.5 ± 1.5	50.9 ± 1.6	6.8 ± 0.6	9.6 ± 0.4
Water (mL/rat/day)	21.4 ± 0.4	10.5 ± 0.7	2.6 ± 0.3	18.8 ± 0.6	20.3 ± 0.8
Total fluid intake (mL/rat/day)	21.4 ± 0.4	28.0 ± 0.9	53.5 ± 1.7	25.7 ± 0.4	30.0 ± 0.9
Total energy intake (kcal/rat/day)	47.3 ± 0.9	49.9 ± 0.7	56.7 ± 0.7	51.3 ± 0.7	53.8 ± 0.8
Fasting blood glucose (mM)	4.6 ± 0.1	4.8 ± 0.1	5.1 ± 0.1 *	4.7 ± 0.1	4.7 ± 0.1
Fasting plasma insulin (ng/mL)	NR	NR	NR	NR	NR
Retroperitoneal fat mass (g)	13.0 ± 0.9	17.6 ± 2.2	16.0 ± 1.7	17.8 ± 2.5	18.2 ± 2.3

Abbreviations: SEM—standard error of mean; n—sample size; BW—body weight; g—grams; mL—millilitres; kcal—kilocalorie; mM—millimolar; NNS—non-nutritive sweetener; N/A—not applicable; ng—nanogram; NR—not reportable. * Significantly different from all the other groups, $p < 0.05$. [a] Significantly different from [b]. Note: The control group in Phase 1 was provided sucrose and water, in Phase 2—water only.

Table 3. Male group means ± SEM for sucrose exposure in Phase 1 and NNS exposure in Phase 2.

Phase 1 Sucrose Exposure	***Control* (*n* = 8)**	***Diet Coke* (*n* = 8)**	***Sprite* (*n* = 8)**	***Cordial* (*n* = 8)**	***Kombucha* (*n* = 8)**
Baseline BW (g)	247.6 ± 5.9	243.9 ± 5.0	248.4 ± 3.4	248.1 ± 5.2	244.6 ± 4.6
Chow intake (g/rat/day)	22.5 ± 0.6	22.0 ± 0.5	20.6 ± 0.7	21.9 ± 0.6	21.9 ± 0.5
Sucrose (mL/rat/day)	101.7 ± 4.7	85.2 ± 1.5	90.1 ± 4.1	77.0 ± 1.6	119.9 ± 7.4
Water (mL/rat/day)	0.8 ± 0.1	0.8 ± 0.03	1.0 ± 0.1	1.0 ± 0.2	1.1 ± 0.2
Total energy intake (kcal/rat/day)	115.9 ± 1.4	108.1 ± 1.8	105.2 ± 2.0	104.6 ± 2.3	122.6 ± 1.9
Fasting blood glucose (mM)	4.8 ± 0.1	5.1 ± 0.1	4.9 ± 0.1	5.2 ± 0.2	5.2 ± 0.1
Fasting plasma insulin (ng/mL)	0.7 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	1.0 ± 0.1
Phase 2 NNS exposure					
Chow intake (g/rat/d)	25.9 ± 0.2	24.6 ± 0.2	20.9 ± 0.2 *	24.7 ± 0.3	24.8 ± 0.2
Test drink (mL/rat/day)	N/A	26.0 ± 1.8	77.5 ± 1.8	9.0 ± 0.7	12.6 ± 1.2
Water (mL/rat/day)	33.5 ± 0.4	13.5 ± 0.7	3.6 ± 0.3	24.1 ± 0.6	31.9 ± 0.6
Total fluid intake (mL/rat/day)	33.5 ± 0.4	39.5 ± 1.3	81.1 ± 2.0	33.1 ± 0.4	44.5 ± 1.2
Total energy intake (kcal/rat/day)	88.1 ± 0.8	83.7 ± 0.7	99.0 ± 1.0 *	84.0 ± 1.0	85.1 ± 0.9
Fasting BGL (mM)	5.5 ± 0.1	5.3 ± 0.1	5.2 ± 0.1	5.2 ± 0.1	5.2 ± 0.2
Fasting plasma insulin (ng/mL)	0.5 ± 0.1 [b]	0.8 ± 0.1	1.0 ± 0.1 [a]	0.9 ± 0.1	0.8 ± 0.1
Retroperitoneal fat mass (g)	25.1 ± 1.8	24.1 ± 1.8	23.9 ± 3.1	19.3 ± 1.5	21.4 ± 2.9

Abbreviations: SEM—standard error of mean; n—sample size; BW—body weight; g—grams; mL—millilitres; kcal—kilocalorie; mM—millimolar; NNS—non-nutritive sweetener; N/A—not applicable; ng—nanogram. * Significantly different from all the other groups, $p < 0.05$. [a] Significantly different from [b]. Note: The control group in Phase 1 was provided sucrose and water, in Phase 2—water only.

3.4. Consumption and Energy Intake

Because rats were group-housed, consumption data were obtained by dividing the overall cage fluid or chow intake by the number of animals per cage (n = 4) and reporting as mean g/rat/day or mL/rat/day. The power to detect group differences in consumption was limited as there were only two cages/sex/test group. Thus, some statistical analyses were not reported, and the mean group intakes are presented in Table 2 (for females) and Table 3 (for males).

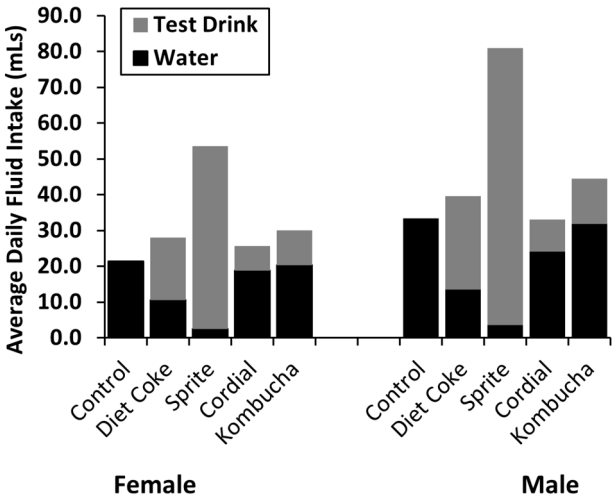
3.4.1. Phase 1: Sucrose and Chow Consumption, Energy Intake

As expected, all the rats drank almost exclusively the sucrose solution during 28 days of exposure (mean water intake = 0.8 mL/day). In both sexes, sucrose intake steadily increased over days (female linear trend: $F(1,35) = 141.39$, $p < 0.001$; male linear trend: $F(1,35) = 191.86$, $p < 001$). In contrast, chow intake decreased over the same period (female linear trend: $F(1,35) = 880.75$, $p < 0.001$; male linear trend: $F(1,35) = 343.32$, $p < 0.001$), indicating a compensatory effect for the increased energy contribution from sucrose by reducing chow consumption. During Phase 1, the average daily energy intake was 72.3 kCal/rat/day for females, with sucrose contributing an average of 31.8 kCal/rat/day (44.0% of the total daily energy intake), whereas the daily energy intake for males was significantly greater ($p < 0.05$), with an average of 111.3 kCal/rat/day and sucrose contributing 37.2 kCal/rat/day (33.4% of the total daily energy intake).

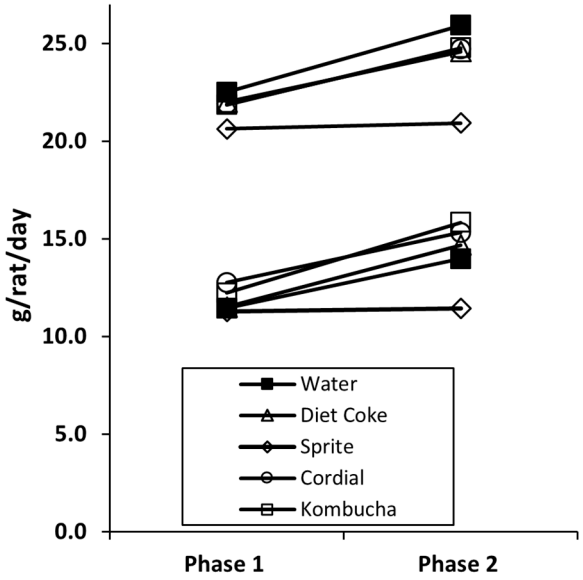
3.4.2. Phase 2: NNS Drink and Chow Consumption, Energy Intake

Following the switch from sucrose to NNS, an interesting observation was that the total average daily fluid intake (water plus test drink) for most NNS test drink groups remained higher than in the control group where only water was offered, suggesting a pattern of over-drinking. This was particularly evident in the Sprite group (females: 53.5 $\pm$ 1.7 mL/rat/day; males: 81.1 $\pm$ 1.3 mL/rat/day) compared to the control group (females: 21.4 $\pm$ 0.4 mL/rat/day; males: 33.5 $\pm$ 0.4 mL/rat/day) (see Figure 5a). The percentages of test drink contribution to the average daily fluid intake recorded for females and males, respectively, for Diet Coke were 62.5% and 65.8%, 95.1% and 95.6% for Sprite, 26.5% and 27.2% for Cordial, 32.0% and 28.3% for Kombucha.

The mean chow consumption during Phase 2 is shown in Tables 2 and 3. Across measurement periods in Phase 2, 5 $\times$ (9) ANOVA analyses were performed. In females, this failed to detect any Group $\times$ Day interaction during NNS exposure ($F < 1$). However, a significant main effect for Group was revealed, with pairwise comparisons indicating the Sprite group ate significantly less than other groups ($p = 0.012$) and the Kombucha group consumed more chow than the control and Sprite groups (ps < 0.036). In males, an interaction between Day and Group ($F(4,35) = 6.44$, $p < 0.001$) and the main effect of Group ($F(4,35) = 14.93$, $p < 0.001$) were detected. Further analysis found the Sprite group ate less than all the other groups (ps < 0.01). We also compared the average daily chow intake between Phases 1 and 2 using separate 5 $\times$ (2) ANOVAs. A significant interaction between Group and Phase was detected (females: $F(1,39) = 15.92$, $p < 0.005$; males: $F(1,39) = 8.52$, $p < 0.001$), indicating a difference in chow intake between the groups from Phase 1 to Phase 2. Post-hoc analyses revealed chow consumption increased in all the groups (female $p < 0.046$; male $p < 0.012$), except for the Sprite group where consumption remained unchanged (See Figure 5b). Similar analyses were conducted to determine if the total daily energy intake differed between Phases 1 and 2. A significant reduction in energy intake was seen in all the groups, with the exception of the male Sprite group which did not change ($p > 0.05$).



(**a**)



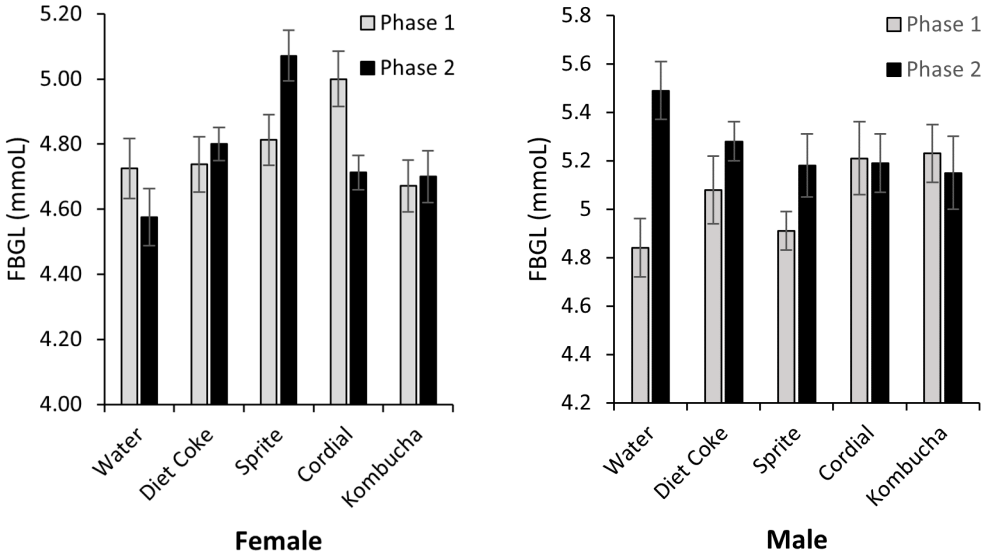
(**b**)

Figure 5. Phase 2 consumption data for the mean total daily fluid intake in Phase 2 (**a**) and chow intake (**b**). Sprite was highly consumed during 28 days of NNS exposure, followed by Diet Coke, Kombucha, and Cordial. Chow consumption increased for all the groups except Sprite, where it remained unchanged (n = 8/group).

3.5. Fasting Blood Glucose and Plasma Insulin

Fasting blood glucose levels are shown in Figure 6. At the end of Phase 1, when all the animals were treated in an identical way, one-way ANOVA analyses confirmed there was no significant difference in FBGLs between the groups (females: $p = 0.24$; males: $p = 0.13$). Subsequently, when the rats were switched to the allocated test drinks for a further 28 days (Phase 2), 5 × (2) mixed ANOVA analyses found Group × Phase interactions (females: $F(4,35) = 4.71$, $p = 0.04$; males: $F(4,35) = 3.31$, $p = 0.021$). In females, further analyses revealed a simple main effect for Group ($F(4,35) = 3.99$, $p < 0.009$) and Phase ($F(1,7) = 11.46$, $p = 0.012$), with the pairwise comparison identifying the Sprite group had both significantly higher FBGLs than all the other groups in Phase 2 (ps = 0.04) and increased between the tests ($p = 0.012$). No difference was detected between the latter conditions. Similarly, a simple main effect for Phase was identified in males, with post-hoc analyses finding

control (F(1,7) = 14.61, $p = 0.007$) and Sprite (F(1,37) = 6.91, $p = 0.034$) FGBL values increased between the tests. No other interactions or effects were detected.



(**a**) (**b**)

Figure 6. Fasting blood glucose levels measured at the end of Phases 1 and 2 for female (**a**) and male rats (**b**). Female Sprite group's FBGLs increased between the tests ($p = 0.012$) and were significantly higher than in the other groups ($p = 0.04$). Male Sprite and control groups' FBGLs increased between the tests ($p < 0.34$) ($n = 8$/sex/group).

Due to technical issues, female plasma insulin samples could not be analysed. Mean plasma insulin data for males are displayed in Table 3. Two plasma samples, one Sprite and one Diet Coke, were haemolysed and could not be analysed; therefore, these male groups had $n = 7$ for insulin analyses. Analysis of change from Phase 1 to Phase 2 detected no significant Phase × Group interaction or main effect of Phase (ps > 0.10). A one-way ANOVA conducted on male insulin data at the end of Phase 2 identified the main effect of Group (F(4,33) = 4.66, $p = 0.004$). Post-hoc analysis revealed the Sprite group had significantly higher insulin values than the control group ($p = 0.002$) but did not differ from the Diet Coke or Cordial groups (ps > 0.17). A trend was noted for the Sprite group insulin data to be greater than in the Kombucha group ($p = 0.052$).

3.6. Retroperitoneal Fat Pad Mass

Retroperitoneal fat mass was adjusted for BW (g/kg) and is shown in Tables 2 and 3. One-way ANOVAs were conducted separately for female and male cohorts. No significant difference of fat mass at culling was detected: females: F(4,35) = 1.14, $p = 0.36$; males: F(4,35) = 1.12, $p = 0.36$.

4. Discussion

The main aim of this experiment was, first, to determine if rats would drink commercially available NNS beverages commonly consumed by humans, thus to develop an ecologically valid animal model for future obesity research. In terms of acceptance of these "diet" drinks, the results were largely positive, with both female and male rats drinking the NNS beverages offered. However, the volume consumed and preference for different test beverages varied considerably. As for the second aim, we assessed whether switching from SSBs to NNS beverages could prevent or mediate metabolic recovery from sugar damage. Earlier assessments conducted in female rats switched from sucrose to saccharin suggest almost complete recovery is possible [23]. However subsequent replication studies failed

to confirm this [26]. Given the use of rarely consumed NNSs in the diet of humans limits translational capacity of that work [19], we undertook a different study design to better mimic human NNS consumption patterns. Below, we discuss our findings for the related outcome measures in more detail.

Developing an ecological validity model of NNS consumption in rats is dependent on whether animals accept and prefer the offered beverages. We had predicted, and our results confirmed, that animals would consume Sprite on the basis that it contains sucrose and stevia, both highly preferred by rodents [27] and containing a higher energy content for greater satiety. Some studies have reported on metabolic effects of Diet Coke consumption in rodents; however, behavioural testing and detailed beverage intake assessments were not performed [28,29]. In regard to the present study, our preference and acceptance results established Sprite was highly preferred and accepted, followed by Diet Coke, whereas both Kombucha and Cordial had very low intakes. Of note, considerable individual differences were seen in some animals, mainly in the Cordial, Diet Coke and Kombucha groups. Such variability was expected as differences in behavioural responses to flavoured solutions have been well-reported [30,31]. Despite this, the average fluid intakes during Phase 2 remained consistent with the drinking patterns displayed in the behavioural testing. That is, the rats consumed more Sprite than Diet Coke and more diet coke than Kombucha and Cordial. Another interesting observation was the trend of over-drinking when the rats were offered both water and NNS beverages; however, this was not as evident in the beverages that did not contain sucrose. Caution should be taken when drawing conclusions on this observation as it may be specific to rodents and we cannot generalise that this effect is representative of human drinking behaviours.

In line with enhancing translational outcomes, the present study design also explored whether consumption of commercially available drinks sweetened with NNSs differed if animals have had prior chronic exposure to sugar-sweetened drinks. Repeated preference and acceptance test post-sucrose feeding failed to confirm this effect. Only male rats in the Sprite group and the control group (given sucrose in the behavioural testing) increased intakes during acceptance testing after four weeks of sucrose exposure. In summary, while each of the commercially available NNS beverages was accepted by the rats (previously defined as intakes greater than 1 mL), we determined that sugar-free Cordial and Kombucha were not reliable ecologically valid models for NNS consumption due to low intake volumes.

With regard to metabolic outcomes after switching from sucrose to NNS beverages, our primary outcome measure was a change in body fat, specifically retroperitoneal fat pad mass. Separate studies have reported conflicting results. Previous work by our laboratory demonstrated female rats showed an improved metabolic profile, including retroperitoneal and visceral fat pads, following a switch from chronic sucrose feeding to saccharin or water [23]. However, in more recent experiments where sucrose-fed rats were switched to water alone, both sexes showed persistence of adiposity, concluding only a partial recovery was made [26]. It was not unexpected for males to display persisting body fat given previous observations [32], but our current experiment supports the conclusion that females also showed incomplete recovery where adiposity remained similar between the control and test drink groups, as seen in Tables 2 and 3. This is consistent with measures of body weight and weight gain, where no detectable change between the groups was noted at the end of Phase 2. We must also take into account that, although rodent fat pad mass is commonly used as a measure for human adiposity, there may be limitations when comparing fat distribution and function between species [33].

As for other secondary metabolic measures, blood marker results demonstrate only the rats that continued to drink sucrose for a further 4 weeks, albeit at a reduced concentration (i.e., the Sprite group), showed an increase in fasting blood glucose levels in both sexes. For females, this value was higher than in all the other groups, suggesting the detrimental effect of sucrose was even greater. Unfortunately, female plasma insulin samples could not confirm this. Gender differences in blood glucose and plasma insulin levels have been

reported, with females more susceptible to poorer outcomes [34]. Male insulin data were inconsistent, with the Sprite group higher than controls but not differing from other groups that switched to NNS beverages. This suggests the model of changing from sucrose to diet drinks does not mediate recovery in males, although there is the possibility we did not have adequate power to conclude this result. A secondary consideration is whether recovery in rats was not achieved due to NNS adversely effecting metabolism; however, the possibility is reduced due to low intake volumes in the Kombucha and Cordial groups. Some studies have reported changes to the gut microbiome following NNS consumption, which may be associated with poor glycaemic outcomes [12,35], whereas others have contradicted this [36]. We cannot confirm or refute this result as it was outside the scope of our research aims. Moreover, a limitation of this current study is the lack of inclusion of a water/chow group, so determination of sucrose-induced metabolic damage during Phase 1 could not be presented.

An important feature of this study is the modelling of human consumption to enhance translational outcomes, which has historically been poor [37,38]. As such, we attempted to develop an improved ecologically valid model to inform further studies and prevent an unnecessary use of animals under the guise of informing human health. Commercially available diet beverages contain other ingredients, as listed in Table 1, and we cannot be certain that these components do or do not have physiological interactions that affect metabolism. However, it is important to recognise that a translational consumption model in animals should mimic human consumption.

Despite our attempts to enhance translation through an ecologically relevant experimental design, another important consideration is the question of predictability of animal-to-human outcomes, specifically in the context of obesity. Of course, we cannot be certain of translational success of our results; however, prior to developing the study design, factors were identified to address external validity. The selection of an appropriate animal model to simulate human disease is a critical step in animal studies [39]. Our meta-analysis in maternal NNS consumption provided quantitative data showing rat models displayed effects in body weight changes compared to mouse models, although, on balance, much of these data were predisposed to bias [18]. Moreover, Sprague Dawley rats are commonly used in obesity-related research as they have been shown to display diet-induced adiposity similar to humans [40]. Concurring with this, prior work conducted in our laboratory which provided female and male Sprague Dawley rats ad libitum 10% sucrose solution in addition to chow and water showed metabolic changes similar to those produced in humans by a poor diet [32]. Taken together, best efforts were made to select an appropriate animal model so the results from this study would be more relevant when considering strategies to prevent human obesity, such as replacing SSBs with "diet" beverages. Although we identified limited metabolic recovery following the switch, we cannot be certain full recovery could not be achieved given a longer period of NNS consumption. Of considerable interest for future investigations is the recent trend whereby manufacturers have reduced sugar content in beverages, as was the case with Sprite™. Our findings suggest chronic sucrose consumption at reduced concentrations does not attenuate the deleterious effects of sugars. However, our study did not explore the potential metabolic differences between 10% sucrose and a reduced sucrose model, which may be a direction for future research.

In conclusion, this experiment shows that an ecologically valid model for the consumption of NNSs can be developed, with female and male rats consuming Diet Coke and Sprite in larger volumes than sugar-free Cordial and Kombucha. In addition, we found both sexes displayed limited metabolic recovery after switching from sucrose to "diet" beverages.

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Institutional Review Board Statement: The study was conducted in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes, 8th Edition, 2013. The animal study protocol was approved by the Animal Ethics Committee of The University of Sydney (protocol No. 2019/1502). The protocol was preregistered in the preclinical trials registry (registry ID PCTE0000147; available from https://www.preclinicaltrials.eu/, accessed on 23 March 2022).

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Chapter 4: "It seems really hard to justify killing animals": A qualitative study of early career researchers conducting animal experiments

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Statement from co-authors confirming authorship contribution of the PhD candidate

The co-authors of the paper: *"It seems really hard to justify killing animals": A qualitative study of early career researchers conducting animal experiments* confirm that Heidi Louise Morahan has made the primary contribution to this study in each of the following areas:

- Conception and design of the research, ethics approval, recruitment
- Collection and analysis of data, interpretation of the findings
- Writing of the manuscript and critical appraisal of content

In addition to the statements above, in cases where I am not the corresponding author of a published item, permission to include the published material has been granted by the corresponding author.

Heidi Louise Morahan Date: 26 September 2023

As supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

Associate Professor Kieron Rooney Date: 26 September 2023

“It seems really hard to justify killing animals”: A qualitative study of early career researchers conducting animal experiments

4.1 Abstract

While best practice methodology in animal research aims to address reproducibility and translational issues, awareness and implementation remains low. Preclinical systematic reviews have highlighted many flaws, including issues with internal validity and reporting. With early career researchers (ECRs) heavily involved in all aspects of animal experiments, it is crucial we understand what shapes their research practices. Semi-structured interviews were conducted with thirteen ECRs, including research masters, PhD, and postdoctoral academics. Data were collected and analysed concurrently using constant comparison techniques and an iterative approach. Findings revealed low-level awareness of best practice recommendations but a desire to engage in dedicated workshops on designing and reporting animal experiments. Current laboratory practices and previous literature were main influences on research practice, more than institutional training. An unexpected finding was the discovery of ethical and emotional dilemmas ECRs faced when working with animals. This highlights the need for a multifaceted approach to better support junior researchers, both emotionally and practically, to encourage responsible science.

4.2 Introduction

Every year, millions of animals are used for the purpose of scientific research.[1] A primary goal of animal research, especially in medical sciences, is to help scientists better understand human health and develop cures for disease. Whilst these models have made important contributions to human health, in recent years there has been growing concern over the quality of animal research, raising questions about the robustness of findings and translational success.[2-4] For ethical and economic reasons, scientific communities have made concerted efforts to improve the quality of preclinical research and reduce the use of animals.[5-8] Despite the use of non-animal alternatives gaining traction, researchers remain reluctant to move away from animal studies.[9] Until that time, it is imperative we continue to re-evaluate research practices to promote best practice methodologies in an effort to improve translational capacity and ensure the animals' lives are respected.

We know poor experimental design, poor reporting, or both, contribute to irreproducibility and translational failures in animal research.[10] In recent times, preclinical systematic reviews (SRs) have uncovered significant issues with internal validity. Furthermore, they have highlighted that many studies are designed without careful consideration of animal to human translation.[11,12] For example, a recent SR found animals were frequently given dietary interventions that lacked relevance for human behaviour.[14] Despite increased awareness, significant improvement in research practices remains elusive and the quality of reporting standards stubbornly low.[15,16] Evidence suggests researchers lack an understanding of the consequences of poor reporting and further, how this can influence the design of experiments.[10]

Early career researchers are at the coalface of preclinical research. Their animal experiments form a large body of original research included in publications. ECRs are heavily involved in study design, analysis, and manuscript preparation. As such, it is crucial they have access to relevant training and resources to ensure they can perform high-quality animal experiments.

Yet, ECRs report they lack formal training on important topics, such as designing or analysing experiments, and that institutional preparatory courses do not extend beyond animal ethics and welfare.[17]

There has been little research conducted on what shapes the abilities of less experienced researchers to design and report animal experiments. We can identify only one such study, where stakeholders were interviewed on their experiences in design and analysis in animal work.[18] Of 131 interviewees, 34 were post-graduate academics; however, the paper's overarching recommendation for 'massive-scale education efforts' did not elucidate relevant issues specific to this population. Given this, we wondered what experiences encountered by ECRs might influence their decisions when designing and reporting animal studies. We were also interested in exploring ECR's lived experiences to identify if other barriers exist that may contribute to the irreproducibility crisis.

Therefore, the primary aim of this study was to explore influences on ECR's abilities to design and report high quality animal studies and identify any areas that could be targeted for future development.

4.3 Methods

A qualitative approach was adopted to gain a rich understanding of what factors influence ECRs when making important decisions in animal experiments. Methods were drawn from constructive grounded theory enquiry, including iterative processes, constant comparison, and researcher reflexivity to enhance rigour. This approach was chosen as it is grounded in the reality of participants, allowing researchers to make sense of experiences through systematic, rigorous processes.[19] Ethics approval was obtained from The University of Sydney Human Ethics Committee (2022/472). The protocol was pre-registered on Open Science Framework

Registries,[20] and methods are reported in line with Consolidated criteria for Reporting Qualitative research Guideline.[21] (Supplementary 1). The researcher reflexivity statement can be found in Supplementary 2.

4.3.1 Recruitment

Participants were recruited by advertising through email invitations sent to animal laboratories, heads of department and group leaders within Australian universities. Flyers were disseminated globally via relevant newsletters and social media platforms. Post-graduate (honours, masters, or PhD students) and post-doctoral academics (<5 years) with current or previous experience in animal experiments were invited to participate. Passive snowballing recruitment was also used. Thirteen researchers interested in participating in the study independently contacted the research team and were given an information sheet. All agreed to participate and provided informed consent prior to being interviewed.

4.3.2 Data collection and analysis

Semi-structured in-depth interviews were completed via a video conferencing platform. Interviews were conducted by the primary investigator (HM) and ranged between 46 to 78 minutes in length. They were audio-recorded, transcribed verbatim and imported into NVivo software [QSR International 2020] for data management and coding. A flexible interview guide was designed to ensure participants could focus on areas relevant to their experiences and for the interviewer to ask follow-up questions.[19] (Supplementary 3).

Using principles of constant comparison,[19] data collection and analysis were performed concurrently to allow for inductive development of concepts and themes. Transcripts were initially coded line-by-line to identify the main idea within each segment of data, constantly

comparing each data segment with previously developed codes. Segments sharing similar ideas were grouped together under a single initial code. To ensure rigour, the first three transcripts were independently coded by two authors (HM, KR) who then compared to reach consensus on early coding decisions. Subsequent transcripts were coded by HM. Initial codes were compared and those reflecting similar underlying concepts were drawn together into focused codes (Supplementary 4). Reflexive discussions were undertaken (HM, KR) to ensure emerging codes were reflective of data until saturation achieved. We defined saturation as the point in which no relevant new codes could be found in the data and when issues began to be repeated.

Further data were collected during final stages of analysis through an in-depth member checking technique.[22] Consenting participants were given the opportunity to build on conceptual development of themes to enhance data credibility. A synthesised summary from emerging themes was sent to ten consenting participants (Supplementary 5). Five ECRs responded, two were unavailable for comment and three did not respond. Most respondents strongly agreed or agreed with the summary and data were integrated into the findings. Results are presented as themes and relevant subthemes and include illustrative quotes to support interpretations.

4.4 Results

4.4.1 Participants

Of the thirteen ECR's interviewed, two were research masters' students, six were PhD candidates and five were postdoctoral academics. Twelve currently worked at Australian universities, however described experiences from previous work in animal laboratories in Canada, France, United Kingdom, or the United States. One ECR worked at a Belgian

university. They used a range of animal models, including sheep (n=1), fish (n=1), rats (n=3), mice (n=3), or both rats and mice (n=5). Further information on participant demographics is included in Table 1.

Table 1. Demographic of participants

Participant ID	**ECR**	**Sex**	**Discipline/field of research**	**Time working with animals (years)**
ECR1	PhD	male	biomedical engineering	4
ECR2	PhD	female	psychology	0.5
ECR3	postdoc	female	behavioural neuroscience	5
ECR4	PhD	male	pharmacology	0.5
ECR5	postdoc	male	nutrition/behavioural science	10
ECR6	PhD	female	behavioural neuroscience	6
ECR7	masters	female	behavioural neuroscience	6
ECR8	masters	female	nutrition/behavioural science	5
ECR9	PhD	female	physiology	3
ECR10	PhD	female	behavioural neuroscience	6
ECR11	postdoc	male	behavioural neuroscience	4
ECR12	postdoc	female	behavioural neuroscience	10
ECR13	postdoc	female	biomedical engineering	6

While initially this study set out to explore research practices, and the interview questions consistently focused on design and reporting, what unveiled itself was the deep emotional impacts and ethical dilemmas researchers experienced when working with animals. These emotions were unsettling and for some, caused contemplation over the validity of their animal

research and future career. Whilst we found some emotional impacts were directly linked to research practices, we could not determine if others influenced experimental conduct; however, we considered the findings important to report.

To make sense of these experiences, both practical and emotional, we briefly summarise the constructed context of our participants situations. Of the thirteen ECRs, four described having an acrimonious relationship with their supervisor and the emotional scars left behind; four spoke more of the positive influence from a nurturing work environment; and five were ambivalent in their approach to their work environment.

As such, two major themes with relevant sub-themes emerged from the coding. These are depicted in the concept map in Figure 1. and further described in this section.

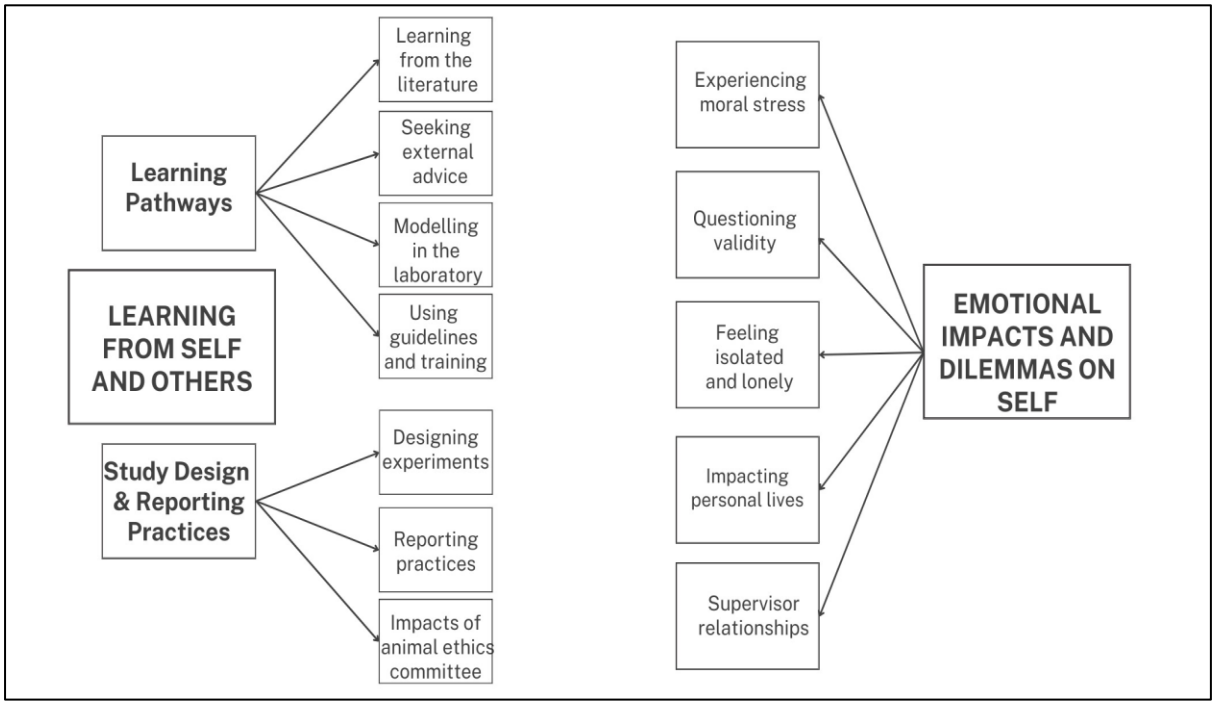

Figure 1. Concept map of influences on ECRs during animal research

4.4.2 Learning from self and others – Learning pathways

Researchers described different learning pathways and influences on design and reporting practices.

4.4.2.1 Learning from the literature

A core part of any ECR's learning experience comes from understanding previous literature. Most talked about needing to stay consistent with what everyone else in the field is using and often copied parts of previous publications for their own protocol: *'we'll go through the literature and see what others have done in the field and you know, pick a couple of tests and see what happens' (ECR10).*

When three ECRs engaged with literature by performing a preclinical SR, they described it had 'opened their eyes' to how poorly papers were reported and designed. This inspired changes to how they conducted future experiments: *'reading those different sections, various bias, you just start knowing which parts people don't do…like randomisation, blinding, housing…I just don't find any studies doing those things, I actually just learnt these for my own study' (ECR2)*.

4.4.2.2 Seeking external advice

Researchers regularly sought advice outside their research team, not only to help perform their animal experiments, but to search for support and connection. However, motivation for seeking that advice differed depending on their situation. Some felt grateful to their supervisors: '*My supervisor was very supportive…if something came up in a faculty meeting that he thought was relevant, he would loop me into training courses' (ECR3)*. Whereas others believed the support and training they received was inadequate. They expressed concern and disappointment, prompting them to actively engage with external mentors: *'there's really no training to*

anything at all which, in my opinion is scary and problematic…so I kept asking my supervisor...and he essentially said no…and that's when I started seeking some outside mentorship from other PIs' (ECR7).

4.4.2.3 Modelling in the laboratory

ECRs recognised modelling of good research practices was important, but the reality was sometimes different. When exemplary modelling occurred, ECRs described feeling fortunate, supported, and proud to disseminate their skills to others. This reinforced a self-belief of competency and confidence in their abilities to design a robust study. However, three ECRs expressed concerns because: *'I don't think relying solely on labs to disseminate best practice resources would be sufficient' (ECR12)* and wanted institutions to play a more active role to prevent a cycling of poor research behaviour. They understood ECRs were trying to do the right thing but '*poor work was mostly because of ignorance and a lack of information…rather than negligence' (ECR3*).

On the contrary, inadequate modelling resulted in feelings of frustration and disappointment. One ECR was dismayed their laboratory *'that claimed more than two decades of experience with animal research'* was not *'aware of, and sensitive to, the wide-ranging benefits of good practice' (ECR1).* Another was overwhelmed by limited mentorship: *'I'm kind of floating or drowning on my own, trying to figure out the ways to ask the questions and carry out the actual physical wet lab experiment',* but acknowledged their experience made them more resourceful and prouder of their work '*given the cards dealt' (ECR7).*

4.4.2.4 Using guidelines and training

For many ECRs, there was limited awareness or implementation of recommended guidelines for improving best practice, such as the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines or protocol pre-registration: *'What is pre-registration? We don't do that' (ECR9).*

Others worried there was inadequate education for ECRs to design robust animal experiments: *'I have not seen…any seminars on how to think about conducting animal research beyond the ethical considerations…there's no one to guide and train people on how to do things properly. They do it from cobbling together from papers, which we know reporting sucks' (ECR3).*

4.4.3 Learning from self and others - Study design and reporting practices

4.4.3.1 Designing experiments

The majority of ECRs did not actively select their animal model, but rather adopted what was already used in their supervisor's laboratory: *'they just always used rats, so there really wasn't much active questioning' (ECR5).* They understood models were chosen based on feasibility, financial constraint, ease of use or perceived translational value.

Choosing sample size was a controversial topic. Methods differed widely and there was no real consensus on the best approach. A few reported using a rule of thumb approach or what their supervisor instructed, but most described performing power calculations because *'we're being called to build in analyses…for ethics' (ECR5)*. A few described analyses as difficult, time-consuming, or counterproductive. For example, ECR3s project involved multiple assays and found it was *'near impossible to find consensus…it gets so lost in the weeds you end up losing a week running analyses, that in the end are very hard to interpret'.* Yet for ECR9, they described power calculations as a helpful tool to moderate moral stress: *'I spent a lot of time*

doing calculations…I mean I love working with animals, but I don't like euthanising them…I tried to co-ordinate how could I use the same animals for at least two projects.'

Randomisation was routine practice, but most described blinding as impractical for their studies, particularly if they were able to discern intervention responses or had resource constraints, such as working independently.

4.4.3.2 Reporting practices

Researchers offered different perspectives on whether their experiments should be published. Three researchers said their data needed to be high quality or impactful to be published: *'the cohort size was too low to be published with an adequate impact' (ECR1).* ECR5 conferred: *'you don't want to publish something that just didn't work'* but then described a need to publish due to significant pressures from funding bodies, moral responsibilities, and career advancement.

Many feared negative results as they believed to be more difficult to publish: *'I don't know if I could convince my co-authors to write a manuscript with null results…the reviewers might tell me, okay, you're not showing anything; what's the point of the paper?' (ECR13).* For PhD and masters' students, there was an added burden of trying to produce something positive for their thesis, and for postdocs, the stress was even more significant due to funding insecurity: *'you only have a contract for one or two years…there is that time pressure to get something out of it…people are more concerned about showing that something works, it's a very precarious time' (ECR13).*

4.4.3.3 Impacts of animal ethics committees

Most researchers were in consensus that ethics applications prompted them to think more deeply about justifying experimental components, such as sample size or endpoints, and that this impacted their study design. Despite finding the process frustrating and time-consuming, it was generally viewed positively because ECRs reasoned it was for the betterment of animal welfare: *'it's frustrating…but I totally understand why it's a lengthy process, why they are so critical of what you're doing…so when I got frustrated, I was like, no this is for a good reason' (ECR8).*

On the other hand, some ECRs believed ethics were picky, focussed on inconsequential issues, lacked ability to determine study worthiness, and requested impractical amendments. One researcher found themselves trading between best and feasible practice: *'yes it would be better if we had done these things…they had certain methods they wanted us to do, but it was going to double the time and require an additional person. It just couldn't be done' (ECR10).*

4.4.4 Emotional impacts and dilemmas on self

Personal reflections, facilitated by a safe space to talk, uncovered ethical dilemmas and emotional stressors faced by ECRS.

4.4.4.1 Experiencing moral stress

ECRs were caring people. They expressed an overwhelming and enduring sense of respect for their animals. Knowing they treated them well, provided care and reduced suffering was incredibly important. In this context, nearly half of ECRs grappled with the inherent tension of caring for animals but subjecting them to treatments which caused harm: *'I need to get over my own sort of (stress), because I just love animals. So, I really hate seeing them given a disease*

for research. But I definitely 100% appreciate that we make advances using that sort of model…but I don't like hurting the mice'(ECR4). Two ECRs talked of forming strong bonds with their animals, with one fearing a betrayal of the relationship by *'doing things wrong that would make the mouse extra mad'* and described themselves as a *'full-time mouse mum'(ECR6).*

This moral stress was more significant for some and manifested in different ways. ECR13 firmly asserted *'hating animal experiments!'* and went on to explain how deeply it impacted them: *'I know it sounds dramatic, but it stresses me out so much, I get, I get nightmares.'*

The task of euthanising animals was recognised as a necessary part of their role, however five ECRs found it distressing and emotionally exhausting: *'every time I have to do a harvest day, I have to go home and hug my cats' (ECR4)* and *'I feel it, you know, I'm killing life. But at the same time, we have to kill them because it's for research. I'm doing something for the betterment of human being. But…I feel that killing an animal is bad' (ECR11)*.

4.4.4.2 Questioning validity

An ethical dilemma ECRs faced was questioning whether their research was worth performing. At different times in their career, five ECRs felt unsure if they still believed in their research and wondered if they should continue to justify sacrificing animals' lives, particularly when research topics were more theoretical or no longer worthy. For example, ECR8 wondered: *'if what I was doing was worth the animal's lives…the learning theory about extinction, it wasn't tangible…I couldn't really feel "am I making people's lives better with this?"'.* ECR7 resonated: *'I really think about like, is this worth it...it seems really hard to justify killing animals when the question is no longer important to you'.*

Not all researchers shared this view. Others argued their research made valuable contributions to science. This was particularly evident when researchers believed their work was highly translational: *'my models provided some evidence that the human mechanism is definitely possible…so that is real translation, that is buffering really close to that translational border' (ECR12).* A temporal aspect of understanding the long-term value of animal research became clearer for some. ECR5 described a mindset shift from their early career where: *'I struggled with sometimes, the validity of what we were doing…some PhD students I supervise expressed similar sentiments, that was tough…in a supervisory role you need to help them understand there are long-term benefits that can be realised'*.

4.4.4.3 Feeling isolated and lonely

Isolation and loneliness were part of the journey for many ECRs in this study. These feelings were unexpected for four ECRs when expectations of their supervisors, or their project, were not met. They described feeling betrayed by the lack of transparency and support: *'it felt like it was all sort of a manipulation to, you know, get me into the country and work on things that he just wanted for his grants and his purposes. It's really overwhelming…it's really isolating' (ECR7).* Similarly, when ECR1 discovered their research team not willing to give basic advice on study design, it was *'really a lonely journey…which was the first huge surprise to me because I thought I was essentially stepping into someone else's shoes'*.

Other factors contributed to the sense of loneliness. Two ECRs described negative impacts on their mental health due to the gruelling hours of working alone in an animal laboratory: *'I don't think I like the solitary thing for like months on end. It's not great for anyone…I hit real lows and I've seen other people who are in animal research also hit those really like, low moods just because it's never ending. It's just exhausting' (ECR10).*

To help overcome these feelings, three researchers discussed finding solace and strength by sharing stories with those who had similar experiences*: 'we kind of all bonded over the same frustrations…I've been able to make it this far because of the camaraderie' (ECR6)*. This sentiment was echoed by ECR7: *'I really would have quit if I didn't have someone else who had the same experience as me'.*

4.4.4.4 Impacting personal lives

Work-life balance was important. Due to the unrelenting nature of animal research, ECRs often worked long hours, as ECR5 eloquently explained: *'it's just a slog you know…animals don't know it's the weekend'.* Many spoke of being deeply invested in their research but viewed time demands as a significant source of stress and limiting factor for achieving a healthy work-life balance: *'it feels like it's never going to end. It's just exhausting and, well, you can't think about anything else because all of your focus is on this one thing for months of your life' (ECR10).* In fact, the demand and intensity made them question their future career and were *'currently undecided…the solitary work and how much like hard labour it is…I've been considering it from a work-life balance perspective'(ECR10).*

4.4.4.5 Supervisor relationships

The connection between supervisor and ECR had a profound effect on the researcher's journey. A positive connection, alongside a transparent work culture, was beneficial to skill development and researcher wellbeing: *'the lab culture, it's awesome…I feel pretty confident with study design… I feel like I'm in the right place…and my opinions valued for sure' (ECR8).*

On the contrary, negative connections led to feelings of distrust, being silenced and lacking agency. The perceived power imbalance made ECR7 hesitant to question authority because

'it's hard to go against the person who pays for research resources, and who ultimately won't fund the extra validation steps that I think are necessary because it costs more time, animals, resources.' This sentiment was echoed by ECR5 who witnessed supervisors weaponizing research costs, when they heard *'some of my mentors saying in not a very healthy way…like, I paid four thousand dollars for these rats, and we're paying thousands a week in housing. We need to get the most out of them.'* Poor communication resulted in further emotional distress. ECR9 described how poor communication placed them in an ethical dilemma of covering up errors in order to complete their studies: *'We just broke the law by having more animals than we were allowed, and that's because my supervisor didn't communicate…I had a complete mental breakdown…it made me feel like I failed at my job in a way…if I were to have recorded that, my whole PhD would have been over'.*

4.4.5 Future Suggestions

We posed a final question to ECRs, asking for suggestions that might improve experiences of future researchers, particularly in areas they felt they struggled. While many recommended dedicated animal research workshops, further suggestions were offered, and these can be found in Supplementary 6.

4.5 Discussion

This study deepens our understanding of ECR experiences during animal experiments and how they shape research practices. The findings also shine a light on the emotional and moral complexity navigated by junior researchers through individual qualitative perspectives. Their experiences were diverse and often mirrored the situation ECRs found themselves in. That is, a supportive environment encouraged greater self-confidence in research skills, whereas

negative experiences gave way to feelings of isolation and introspective reflections on the value of animal research; however, the two situations were not always mutually exclusive. The findings were clear that practical learning experiences were closely entwined with the emotionality of working with animals.

We found ECRs research methods were heavily influenced by previous literature, current laboratory practices, and supervisory relationships, with limited awareness of recommended best practice tools. Yet wide consensus points to considerable design and reporting flaws in animal literature and the need for ongoing education.[10,23] Although, ECRs are ultimately responsible for their research output, greater awareness of best practices to produce high quality research should remain a priority for institutions.[10] However, it could be assumed education workshops designed for ECRs are heavily targeted at human or broad-based research skills, as many described a lack of dedicated animal workshops.

A good example of impactful research was evident in those who performed preclinical SRs. Our findings mirrored experiences of others, where learnt insights from SRs changed the way ECRs planned and conducted future experiments to be more rigorous and transparent.[24] Many only became aware of how poorly conducted many animal studies were through scrutinising literature this way. Given the influence previous publications have on study design, this attests to the need for more targeted education from institutions, particularly when effective supervision is missing, as some ECRs described.

Researchers' emotional health was negatively impacted by work-related stressors. Recent studies show similar findings, where researchers working in animal laboratories experienced mental and moral stress, and subsequently displayed higher levels of anxiety[25] and reduced quality of life.[26] Further, PhD students and junior researchers were more vulnerable to these effects.[25,26] Unsurprisingly, moral and mental stress can manifest itself in ways affecting

psychological well-being and has the potential to negatively impact outcomes of projects.[27,28] Although we did not find a direct link in this study, it is possible work-related stress has the potential to negatively impact research practices and this warrants further exploration.

Adding to the emotional burden, we found when ECRs felt unsupported or isolated, the more they considered leaving their career as an animal researcher. This was further compounded when ECRs questioned whether their research was 'worthy' or 'impactful enough' to continue sacrificing animal's lives. This topic remains largely taboo and sharing of personal experiences has seldom been reported.[29] It appears the frequency of animal researchers challenging rationale beliefs and openly discussing human-animal relationships in science is not fully understood.

However, over the last few years, attention has focussed on the human emotional strain of working with experimental animals and promotion of supportive working environments.[30-32] An emerging concept of a 'culture of care' (CoC) has been suggested as an additional component to enhance scientific quality and act as a solid foundation for responsible science.[30] CoC describes a multifaceted approach to enhance the well-being of animals, staff, scientific quality, and transparency.[31] The findings of this research strongly endorse implementation of CoC strategies, with an urgent priority to provide better support for ECRs.

4.5.1 Limitations

This study contains limitations. Despite rigorous recruitment globally through social media channels, only thirteen ECRs participated in the study. Through iterative processes, it appeared that data saturation was reached (as defined in the Methods); however, acknowledge this small sample consisted mostly of Australian universities researchers. Of these participants, five ECRs were international students who previously completed education in North American and

European institutions. While this allowed insights to be gained across a specific research environment, findings may be related to their cultural context, and we cannot ensure similar findings would emerge in different settings. As such, applicability of the study's findings to different researchers should be assessed with reference to the sample characteristics.[38] The animal ECR community is broad and complex, and we recognise this study more than likely does not capture its diversity. Further research using a comparative analysis across different disciplines or institutions would be of significant interest to elucidate potential additional insights.

As with any study involving voluntary participation, ECRs who agreed to be interviewed may have had more intense experiences with animal research than those who did not. To address this potential bias, we included researcher's situational context in our findings.

4.5.2 Conclusions

ECRs learning pathways were heavily influenced by supervisory relationships, laboratory practices and literature more than institutional training. A lack of awareness of best practice recommendations was evident, reinforcing the need for more targeted education. An unexpected finding was the moral and emotional turmoil ECRs felt when working with animals and highlights the necessity to better understand the personal strain ECRs experience. This study found that more practical and emotional support is needed for junior animal researchers to improve scientific outcomes and psychological well-being.

4.6 Declaration of conflicting interests

The authors declare that there are no conflicts of interest.

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4.9 Data availability

Data were collected from participants who provided consent for its use for research and publication purposes, but not for sharing as open data.

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4.11 Supporting information

Supplementary File 1: Consolidated criteria for reporting qualitative studies (COREQ): 32-item checklist.

Developed from: Tong A, Sainsbury P, Craig J. Consolidated criteria for reporting qualitative research (COREQ): a 32-item checklist for interviews and focus groups. *International Journal for Quality in Health Care* 2007; 19: 349–357.

No. Item	Guide questions/description	Reported on Page #
Domain 1: Research team and reflexivity		
Personal Characteristics		
1. Interviewer/facilitator	Which author/s conducted the interview or focus group?	Methods - Data collection and analysis; Page 5
2. Credentials	What were the researcher's credentials? E.g. PhD, MD	Methods; Page 5 – Supplementary 2
3. Occupation	What was their occupation at the time of the study?	Methods; Page 5 - Supplementary File 2
4. Gender	Was the researcher male or female?	Methods; Page 5 - Supplementary File 2
5. Experience and training	What experience or training did the researcher have?	Methods; Page 5 - Supplementary File 2
Relationship with participants	Was a relationship established prior to study commencement?	Methods; Page 5 - Supplementary File 2
6. Relationship established	Was a relationship established prior to study commencement?	Methods; Page 5 - Supplementary File 2
7. Participant knowledge of the interviewer	What did the participants know about the researcher? e.g. personal goals, reasons for doing the research	Methods; Page 5 - Supplementary File 2
8. Interviewer characteristics	What characteristics were reported about the interviewer/facilitator? e.g. Bias, assumptions, reasons and interests in the research topic	Methods; Page 5 - Supplementary File 2

Domain 2: study design		
Theoretical framework		
9. Methodological orientation and Theory	What methodological orientation was stated to underpin the study? e.g. grounded theory, discourse analysis, ethnography, phenomenology, content analysis	Methods; Page 4 & 5
Participant selection		
10. Sampling	How were participants selected? e.g. purposive, convenience, consecutive, snowball	Methods - Recruitment; Page 5
11. Method of approach	How were participants approached? e.g. face-to-face, telephone, mail, email	Methods - Recruitment; Page 5
12. Sample size	How many participants were in the study?	Results – Participants; Page 6; Table 1
13. Non-participation	How many people refused to participate or dropped out? Reasons?	Methods - Recruitment; Page 5; Data analysis; Page 6
Setting		
14. Setting of data collection	Where was the data collected? e.g. home, clinic, workplace	Methods – Data collection and analysis; Page 5
15. Presence of nonparticipants	Was anyone else present besides the participants and researchers?	N/A
16. Description of sample	What are the important characteristics of the sample? e.g. demographic data, date	Results – Participants; Page 6/7; Table 1
Data collection		
17. Interview guide	Were questions, prompts, guides provided by the authors? Was it pilot tested?	Methods – Data collection and analysis; Page 6 - Supplementary File 3
18. Repeat interviews	Were repeat interviews carried out? If yes, how many?	N/A

19. Audio/visual recording	Did the research use audio or visual recording to collect the data?	Methods – Data collection and analysis; Page 5
20. Field notes	Were field notes made during and/or after the interview or focus group?	Methods – Data collection and analysis; Page 6
21. Duration	What was the duration of the inter views or focus group?	Methods – Data collection and analysis; Page 5
22. Data saturation	Was data saturation discussed?	Methods – Data collection and analysis; Page 6
23. Transcripts returned	Were transcripts returned to participants for comment and/or correction?	Methods – Data collection and analysis; Page 6 – Supplementary File 5
Domain 3: analysis and findings		
Data analysis		
24. Number of data coders	How many data coders coded the data?	Methods – Data collection and analysis; Page 6
25. Description of the coding tree	Did authors provide a description of the coding tree?	Supplementary File 4
26. Derivation of themes	Were themes identified in advance or derived from the data?	Methods – Data collection and analysis; Page 6
27. Software	What software, if applicable, was used to manage the data?	Methods – Data collection and analysis; Page 6
28. Participant checking	Did participants provide feedback on the findings?	Methods – Data collection and analysis; Page 6 – Supplementary File 5
Reporting		
29. Quotations presented	Were participant quotations presented to illustrate the themes/findings? Was each quotation identified? e.g. participant number	Yes; Results – Pages 9-18
30. Data and findings consistent	Was there consistency between the data presented and the findings?	Yes; Discussion – Pages 18-20
31. Clarity of major themes	Were major themes clearly presented in the findings?	Yes; Page 8 – Figure 1

32. Clarity of minor themes	Is there a description of diverse cases or discussion of minor themes?	Yes; Results – Pages 9-18

Supplementary File 2: Research team reflexivity statement

The primary investigator (HM) is a PhD candidate in her final year at the University of Sydney. She has experience in performing experiments in rodent models and has conducted and aided others in performing preclinical systematic reviews. Therefore, conceptualisation of this study was inspired by lived experience. This lived experience has the possibility of enhancing methodological sensitivity and data interpretation (Honey et al. 2020).

She knew three participants (ECR5, ECR8 and ECR10) from her previous experience in animal research, however, was not directly involved in their research projects or scientific field. At the outset of the interviews, it was discussed that the interviewer was there to be open-minded, non-judgemental and to listen to the participant's experiences to gather data for the purpose of the study. It was re-iterated that anything discussed was to remain confidential and data de-identified immediately. Participants were aware of the voluntary nature of their participation, and that they were free to withdraw from the study at any time up to point of data analysis. Open-mindedness and potential bias on interpretation were discussed during reflexive research meetings.

Participants were not aware of her personal goals or motivation to conduct this study; however, were aware of her experience in animal research and involvement in systematic reviews and the general aims of the study. This was articulated at the outset of the interviews to gain rapport and the aims could be found in the Participant Information Statement sent to each participant prior to gaining consent.

The primary researcher was supported by a highly experienced and diverse research team with expertise in animal research (KR, RB, PK), animal ethics and integrity (KR, PK), research bias in health (KR, LB), mental health clinical practice (NH), and qualitative research (NH, LB).

Their experience enabled the study to be designed and analysis to be undertaken utilising different perspectives.

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Supplementary File 3: Flexible Interview Guide

So really this is more a conversation than an interview. But I just want to let you know:

- Of course, anything we talk about today is confidential. This includes issues of non-ethical practice or scientific misconduct. So, if you disclose this type of information no action would be taken. We're just interested in researcher experiences, and we're not concerned at all with the performance of individual researchers.

Have you got any questions before we get going?

1. So just as a way of introduction, can you just start by telling me about how and why you came to be involved in animal research?

   What field of research are you in? How long have you been involved with animals and what animal is your research in? Are you a student or post doc? Right, are you a domestic or international student?

2. And when you are conducting your animal experiments, what's your involvement? For example, would you consider yourself the main caregiver, do you perform testing/surgery/ procedures/data collection? Are you supervising others?
3. Are you part of a research group and how's that structured? What's your role is within the investigation team?

<u>Decision making in study design</u>

4. So really, I'm interested to hear about how you go about designing your studies. Can your talk me through what was your role in the development of designing your study/s and the ethics application/s?
5. In general, could you tell me about your experience/s on how you design your experiments? *For post doc; can you talk me through your journey from honours to PhD and now into post-doctoral research on how you've changed the way you design experiments?
6. Were there any barriers or issues you can think of that you came across during the design stage? How did you overcome them?
7. What guidance or resources/tools did you use to develop the protocol?
8. Can you tell me where you learnt about these resources/tools?

9. Are there any specific mentors or individuals in either your research team or outside that you have spoken and sought advice from? Who and why did you seek their advice, in particular?
10. To what extent do you feel your training has equipped you with the skills to design and perform your own experiments?
11. What aspects of designing your experiments did you find the most/least challenging?
12. In general, how do you believe other ECRs feel about their training? e.g., similar, or different to you?
13. What aspects of study design do you feel you need more training or support?
14. To what extent do you believe your research team or institution can provide you with this?
15. Have you used resources/tools outside your institution during the design phase of your study and if so, where did you seek them?
16. How concerned are you that early career researchers don't receive enough training to conduct high quality animal studies?
17. How do you approach components such as selection of animal model, sample size, randomisation and blinding used in your experiments?
18. What practical issues do you believe influence these decisions? Do you think your approach is the most appropriate way? Why?
19. How involved are you in the decision-making process and do you believe you have received enough guidance/training in this area?

    - I noticed you haven't mentioned primary outcomes. When do you decide on the primary outcomes for your study? At design stage or after the experiment?

20. Have you performed or considered performing a systematic review that includes animal studies? If not, why's that? If yes, has this affected or changed the way you performed your study? How?
21. To what extent do you think systematic reviews in animal studies could improve the design of your experiments?
22. In human clinical trials, it is standard practice to pre-register studies, where your protocol is publicly available so everyone can see what you plan to do. To what extent do you believe pre-registration in animal studies would influence your design?

Decision-making in reporting of studies

23. I'd like to chat about reporting studies now. So, after you've completed your data collection, it's now time to write up your manuscript. What determines whether this is a paper and how do you start drafting and editing of your manuscript?
24. What are the next steps after completing the experiment in the context of drafting?
25. Which guidelines, if any, do you use to report your studies and where did you learn about these?
    - How do you implement the guidelines checklist? How do think reporting guidelines impact the way you design your study?
26. How do you approach any perceived conflicts within your research team on how to report studies?

Current understandings about translation in animal research

27. How would you define translation in animal research and what does it mean to you?
28. In the context of translation, how important do you believe animal studies are in contributing to the design of human clinical trials?
29. Thinking about your own experiments, can you describe whether you feel they contribute to human research experiments or clinical practice? If so, how? If not, how do you feel about using animals for scientific research?
30. During the design of you own experiments, how much do you think translation or progression to clinical trials was considered?
31. What component/s of your own animal research do you consider important for animal to human translation?
32. What do you think is the difficult part of animal to human translation?

Research team and role

33. What do you see as the role of your supervisor (or chief investigator) when designing and reporting your animal experiments?
34. How well equipped do you believe your chief investigator is to provide you with the most up-to-date training? Do you feel comfortable asking for further training? Why?
35. Given your position as an early career researcher, do you feel your opinion is valued?
36. Can you discuss the culture of your research team? How do you fit in?
37. Did you experience any pressure or stress at any time during the planning or implementation of your experiments? If so, why?
38. Thinking about the ethics process or committee, what role do you think they play in your experiences when designing your protocol?

Suggestions for improving ECRs decision-making

Can you suggest areas for improvement or strategies in designing and reporting animal studies where you believe ECR's might struggle?

Supplementary 4: Focussed codes

ECR Animal Research Study Codes

Name	Description	Files	References
Learning Pathways	Any words, sentences or paragraphs describing different ways that ECRs learnt or made decisions on their own research practices (in study design or reporting) or were influenced by	15	165
Animal to human translation		13	89
Beliefs about problems with translation		9	17
Collaborating between human and animal researchers		9	14
Concerned for limited translation		7	10
Defining animal to human translation		6	6
Having a positive outlook on translation of own research		8	12
Thinking animal contributes to human research experience not occurring		1	1
Translation is key reason for research		3	4
Understanding benefits of animal research for translation		9	16
Understanding components for better translation		5	6
Awareness of Best Practice Tools & Resources		0	0
3Rs		6	8
Reducing animal waste		4	5
ARRIVE Guidelines		12	25
Assessing ARRIVE guidelines impacts on design		3	3

Name	Description	Files	References
Awareness of ARRIVE guidelines		11	14
Difficulty implementing ARRIVE guidelines		2	2
Implementing ARRIVE guidelines		4	6
Keeping animal research transparent; other researchers learn		4	5
Needing to improve animal research		3	3
Pre-registration		13	40
Awareness of pre-registration		11	17
Feeling pre-registration too hard		2	2
Perceived negative implications for pre-registration		8	9
Seeing pre-registration as a positive		9	11
Systematic Reviews		13	48
Awareness of systematic reviews		10	13
Experience with systematic reviews		8	12
Learning from systematic reviews		6	13
Understanding implications of systematic reviews		9	10
Tools used to develop protocol		4	5
ECRs Limitations		0	0
Believing ECRs have limited views		3	5
ECR's lacking experience		6	7

Name	Description	Files	References
ECRs not aware of financial costs		1	1
ECRs not recognising their limitations		2	3
Ethics Committee		13	53
Beliefs of what ethics committees should do		3	4
Ethics asking the wrong questions; not asking questions; not following through		5	5
Ethics committee worried about the wrong things		3	6
Frustrated with ethics		6	13
Role of ethics committee in study design		12	18
Writing what you think ethics want to hear		3	7
How ECRs learn		0	0
Areas not covered in ECRs training		7	13
Building on previous work		3	3
Doing the same as others in the field		3	4
Doing things by yourself		6	7
Experience in compulsory training		8	14
Experiencing different approaches in different labs		4	9
Forcing students to do courses		1	2
Happy with Lab training		4	4
Learning on the job		4	5
Modelling in the lab		7	16
Seeking advice		0	0

Name	Description	Files	References
Collecting information from external sources		6	9
Collecting information from resources for protocol		8	11
Trial and error		3	3
Troubleshooting		1	1
Understanding importance of good practices		6	15
Understanding previous research		3	6
Pathways of Animal Researchers		0	0
Choosing animal research because of interest		8	12
Choosing animal research for career opportunities		3	4
Difficulty with human research		1	1
Enjoying working with animals		2	4
Feeling forced to do animal research		1	3
Moving to human work anyway		1	3
Sense of moving forward needs to be in animals		1	1
Wanting control		2	3
wanting to do human research		2	2
Worried for career prospects		4	7
Reporting Practices		0	0
Delaying publishing		1	1
Determining what makes a paper		9	13

Name	Description	Files	References
Getting results into public domain		6	12
Pressured to publish		3	5
Reasons for not publishing experiments		9	16
Role in reporting		3	4
Worried about getting negative results		3	7
Resources and Tools		0	0
Lack of dedicated animal resources		6	6
lack of training		6	14
Sharing experiences with other ECRs		5	8
Role in animal experiments		10	23
Actively involved in handling of animals		2	3
Heavily involved in all aspects of experiment		1	2
No involvement in caregiving		1	1
Respecting welfare of animals; getting to know animal through handling		3	4
Study Design Practices		0	0
asking for help		4	9
Barriers in ECRs study design		8	13
Choosing the animal model		5	8
Constantly discussing sample size		2	3
Deciding on power calculations		7	12
Deciding sample sizes		10	16

Name	Description	Files	References
Experience with study design		5	8
Having no choice in animal model		4	5
Justifying sample size used		5	8
Justifying choices		4	5
Justifying the animal model used		5	8
Learning from literature		5	12
Performing other researchers experiments		3	5
Recognising consistency in models		1	1
Seeking guidance outside of supervisor		7	19
Seeking help from Animal Lab Services		1	2
Setting high standards for self		1	1
thoughts on randomisation and blinding		9	15
Understanding the reason of animal model		2	3
Using animal model because that's what the lab uses		3	4
Moral Concerns and Ethical Dilemmas	Words, sentences, and paragraphs describing any emotionality or ethical concerns ECRs used when discussing their experiences working with animals for scientific use	13	51
Concerns about Animal Research		0	0
Chasing positive outcomes		3	7
Concern for environmental aspects of animal research		1	1
Concerns about underpowered studies		3	6
Covering up poor research findings to continue the project		1	1

Name	Description	Files	References
Disappointed with prior animal data		2	3
Discovering poor animal research patterns		7	11
Dismayed and shocked by bad research		1	1
Dodgy science; Scientific misconduct		2	6
Fearing competitive groups		2	4
Moral concerns		6	12
Problems with animal research		3	4
Recognising poor animal research practices		7	16
Repeating same experiments		7	8
Thinking prior evidence is a lie		1	1
Worried about lack of power		5	6
Worried about reproducibility		2	3
Worried about tissue waste		3	4
Confidence as a Researcher		0	0
Assessing impact of own research		5	6
Assessing personal skills to design experiments		6	9
Belief in importance of own research		7	10
disappointed with not getting published		2	2
Disappointed with own results		2	2
Doing things properly		6	11
Feeling confident		8	15

Name	Description	Files	References
Feeling positive about study design		3	3
Figuring out things by yourself		3	4
Idealism		0	0
Envisioning outcomes		4	5
Feeling hopeful for future animal research		2	2
Optimistic about future studies		1	1
Trying to make things perfect		2	4
Understanding hard work worth doing		3	3
Justifying decision to continue on with study		2	3
Leading the whole project		2	4
Looking for challenges		2	2
More experienced researchers desensitised		2	2
Needing help of others		4	6
Needing to perform research correctly		3	8
pride in developing novel research		3	4
Proud of work		1	1
Understanding the core research question and designing to answer it		5	6
Understanding your limitations		4	7
Impacts on personal life		5	7
Leaving animal research		4	6
Reflecting on whether to remain as animal researcher		5	10

Name	Description	Files	References
Isolation and Mental Health		0	0
Connecting with others for support		1	1
Feelings of loneliness and isolation		4	9
Impacts of COVID		6	11
Mental health concerns from animal research		5	9
Working alone or independently		9	18
Lab Culture		0	0
Comradery within the lab		2	4
Feeling valued		7	10
Handling of conflict in research group		3	4
Inherent sexism within team		2	2
Lab size		5	7
Laboratory Culture		7	11
Lacking transparency within research team		2	3
Lacking transparency in lab culture		2	2
Research team interacting		2	2
Supervisor Relationships		0	0
Aligned with supervisor		9	14
Building the supervisors career		4	5
Expectations of supervisor		4	10
Feeling supported		4	7
Feeling uncomfortable with		3	5

Name	Description	Files	References
supervisors' approach			
Handling conflict with supervisor		4	8
Happy with supervisors' ability in study design		6	9
Keeping decisions between student and supervisor		3	4
Lacking Agency & Feeling Silenced		0	0
Being told what to do		5	10
Feeling out of control		2	2
Feeling silenced		3	7
Lacking agency		4	7
uncomfortable standing up to authority		1	2
Limited skills of supervisor		5	6
Passing on of information not happening		2	4
Supervisor lacked ability to train student		3	4
Supervisor taking advantage		1	1
Supervisors' involvement with reporting		5	6
supervisors weaponizing costs		2	2
Not feeling believed or supported		0	0
Not feeling believed		2	2
not feeling supported		3	6

Name	Description	Files	References
Perceived Pressures		0	0
Assessing huge workload		3	5
Barriers in Animal Research		0	0
Design limited by equipment availability		2	3
Discovering lack of equipment		2	3
Expecting different outcomes		3	4
Experiencing limited help		2	4
Having a heavy workload		4	5
Limited by financial constraints		8	20
Pressured to do things you don't want to do		2	3
Proving impact for funding bodies		2	2
Proving your value		1	1
proving yourself to others		2	2
Shifting paradigms		2	2
Time Issues		12	48
Having time limits		7	15
Rushing things		1	1
Wasting time and energy		5	8
Working long hours and time intensive		8	24
Trying to get value for money		1	1
Questioning validity		0	0

Name	Description	Files	References
Don't believe in own research		1	1
Feeling disillusioned with animal research		7	9
Having aversion to performing animal research		3	8
Questioning why we perform animal research		6	14
regretting performing animal study		1	3
Struggling with continuing with animal research		1	1
Thinking animal research is unreasonable		1	2
Thinking deeply about animal research		2	2
Worried about animals use for scientific purpose		1	1
Reason for performing animal research		10	25
feeling happy with approach to animal research		8	11
Feels like no choice in research field		1	1
Interested in animal research		3	6
Reflections on Research		0	0
Accepting things, the way they are		2	2
Reflecting on research		8	11
Sympathetic to animal welfare		10	19
Animal Welfare		3	4
Suggestions for future ECRs	ECRs offered suggestions on strategies/tools/resources/education that they felt may help future ECRs, particularly in areas that they struggled	11	24
Making complex thing easier for students		1	1

Name	Description	Files	References
Making things more interesting for students		1	1

Supplementary 6: Member Checking Document

Preliminary Findings – An invitation to engage and comment

We are excited and curious to present you with emerging concepts and themes from the information collected during the interviews in our study.

The reason we are sending you these preliminary findings is so you can provide commentary on whether any of the experiences or perceptions of others also applied to you? This checks that your responses are accurate and resonate with you. Any responses to this document will be further analysed and incorporated into our findings.

You will see below that we believe two major themes have emerged: (1) Learning Pathways and Research Practices and (2) Emotional Impacts and Ethical Dilemmas.

We invite you to read through and add further comment to the final questions/statements in the box. Your responses will be de-identified and remain confidential, consistent with your signed consent form.

Thank you again for graciously giving up your time to collaborate in this study.

(1) Learning Pathways and Research Practices

We found that ECRs used many pathways to learn skills for designing, conducting, and reporting their animal studies, but research methods were heavily impacted by their laboratory practices and reading previous literature.

- **Staying consistent –** ECRs wanted to remain consistent with what everyone else in the field is using, particularly when choosing different components for study design, such as animal model, tests, and sometimes for sample size. Often ECRs copied parts of previous publications for their own protocol:

  'We'll go through the literature and see what others have done in the field and you know, pick a couple of tests and see what happens.'

- **Opening their eyes –** when ECRs performed a preclinical systematic review, they became aware of how poorly papers were designed and reported. This changed the way they conducted their own experiments:

  'Reading those different sections, various bias, you just start knowing which parts people don't do…particularly for animal research, and I actually just learnt these for my own study'.

- **Modelling of good practice didn't always happen** – there were different feelings between those who did and those who didn't experience modelling of best practices within their own lab. But both recognised it was important and many thought institutions needed to be more involved in disseminating good practice to avert cycling of poor behaviours:

'If there was, actually like I don't know, some sort of lectures or workshops instead of just relying on students to go and find things themselves, because these things aren't recommended to us. From, usually in the lab, we do things how we've always done things, right. So, nothing ever changes unless there's some sort of direct intervention'.

- **Seeking external advice** – All ECRs regularly engaged with outside help and support, but the motivation for seeking advice differed depending on the researcher's situation. Some felt supported by their supervisors, but others were prompted to find other mentors when they worried that the training from their lab or supervisor was not meeting expectations:

  'there's really no training to anything at all which, in my opinion is scary and problematic…so I kept asking...and he essentially said no…and that's when I started seeking some outside mentorship from other PIs.'

- **Lacking awareness of recommended guidelines** – Many ECRs were not aware or did not regularly implement best practice recommendations below:

  Preclinical systematic reviews*: 'I haven't considered it before' … 'I've heard of them more in the context of clinical research but haven't thought about it.'*

  Protocol pre-registration: *'I don't have any knowledge of that!' … 'I've definitely never come across it but would be an interesting idea for people to do that.'*

  ARRIVE guidelines: *'I unfortunately learnt quite late in my PhD about the ARRIVE guidelines'… 'I remember that phrasing from the ITAR module but I'm not aware of it.'*

- **Choosing sample size –** was a controversial topic and there was no real consensus on the best approach. Power calculations were routine practice, but some ECRS found them difficult, time-consuming, or counter-productive. Most performed them because they were required for ethics but believed they frequently confirmed the 'rule of thumb' approach based on previous experience or reading literature:

  'We're being called to build in power analysis for ethics but were kind of stuck and kind of had to retrofit analyses to justify an n of twelve.'

- **Choosing animal model:** ECRs primarily adopted the animal model already used in their supervisors' laboratory but reasoned the choice was based on financial constraints, ease of use or perceived translational value:

  'They just always used rats, so there really wasn't much active questioning.'

- **Reporting practices -** different perspectives were offered on whether their experiments should be published. Some believed their data needed to be high quality and impactful to be published, whereas others thought all data should always be in the public domain. But many postdocs feared the dreaded null result and felt the added burden of financial and time pressure to publish:

'You only have a contract for one or two years…there is that time pressure to get something out of it…people are more concerned about showing that something works, it's a very precarious time.'

- **Impacts of animal ethics committees –** there was consensus that ethics applications prompted them to 'think more deeply' about justifying experimental components and this impacted their study design. Despite finding the process frustrating and time-consuming, it was generally viewed positively because ECRs reasoned it was for the betterment of animal welfare:

  'It's frustrating…but I totally understand why it's a lengthy process, why they are so critical of what you're doing…so when I got frustrated, I was like, no this is for a good reason.'

- **Future Suggestions –** when we asked you for suggestions or strategies that could improve the way future ECRs learn, especially in areas that you struggled, the majority talked about a need for more support through more dedicated and compulsory animal workshops on topics such as study design, statistics, and reporting; and that this should be an institutional responsibility. It seems that current courses are targeted to human or broad-based educational topics.

  'I think literally having something that is called designing and reporting on animal experiments like, you know, a one-day course maybe for first year PhDs.'

Please add any further comments and consider the statements in the box below:

Laboratory practice and previous literature were the main influences on research practices; however, some were negatively impacted by poor supervisory relationships. Despite low-level awareness of best practice recommendations and tools, there was a desire to engage in dedicated workshops on designing and reporting animal research. Do you agree? Do you want to change anything? Do you want to add anything?

(2) Emotional impacts and ethical dilemmas

Although we initially set out to explore design and reporting practices, over the course of the interviews, what unveiled itself was the deep emotional impacts and ethical dilemmas researchers experienced when working with animals. These emotions were unsettling and for some, caused contemplation over the validity of their animal research and future career.

- **Experiencing moral stress -** nearly half of ECRs grappled with the inherent tension of caring for their animals but then subjecting them to treatments which caused harm, this included euthanasia:

'I need to get over my own sort of (stress) because I just love animals. So, I really hate seeing them given a disease for research. But I definitely 100% appreciate that we make advances using that sort of model…but I don't like hurting the mice.'

- **Questioning validity -** At different times in their career, five ECRs felt unsure if they still 'believed' in their research and wondered if they should continue to justify sacrificing animals' lives, particularly when research topics were more theoretical or no longer worthy:

  'I really think about like, is this worth it...it seems really hard to justify killing animals when the question is no longer important to you'.

  But others argued their research made valuable contributions to science, especially when they thought their research was highly translational:

  'My models provided some evidence that the human mechanism is definitely possible…and so that is real translation, that is buffering really close to that translational border.'

- **Feeling isolated and lonely –** this was a common theme and impacted the emotional health of some researchers. A few described feelings of isolation due to issues with their supervisor, whereas others talked about loneliness caused from the gruelling hours of working alone in an animal lab:

  'It felt like it was all sort of a manipulation to, you know, get me into the country and work on things that he just wanted for his grants and his purposes. It's really overwhelming…it's really isolating.'

  'I don't think I like the solitary thing for like months on end. It's not great for anyone…I hit real lows and I've seen other people who are in animal research also hit those really like, low moods just because it's like never ending. It's just exhausting.'

- **Impacting personal lives –** it seemed a challenge for ECRs to find work-life balance when working with animals. Although ECRs were deeply invested in their research, the demands on their personal lives were a significant source of stress:

  'It feels like it's never going to end. It's just exhausting and it's like, well, you can't think about anything else because all of your focus is on this one thing for months of your life. And it's difficult to disengage.'

- **Supervisor relationships –** appears to have a profound impact on the journey of ECRs. A positive connection, alongside a transparent work culture benefited skill development and wellbeing. But a negative connection and/or poor communication led to feelings of distrust, being silenced and lacking agency:

  'The lab culture, it's awesome…I feel pretty confident with study design. I might not have said the same before. I've learnt a lot. I feel like I'm in the right place…and my opinions valued for sure.'

  'I was like extremely upset…by having more animals than we were allowed, and that's because she (supervisor) didn't communicate…I had a complete mental breakdown about it. It made me feel like I failed at my job in a way.'

Please add any further comments and consider the statements in the box below:

ECRs are caring people. They have an overwhelming and enduring sense of respect for their animals and knowing they treated them well, provided care and reduced suffering was incredibly important. Some researchers experienced deep emotional and ethical impacts that affected their psychological well-being, and they sometimes questioned the impact or validity of animal research. More emotional support for animal researchers would be welcome. Do you agree? Do you want to change anything? Do you want to add anything?

If you have any questions or require further information, please contact heidi.morahan@sydney.edu.au

Supplementary 7: Suggestions or strategies for futures ECRs

The final question posed to ECRs asked for suggestions or strategies to improve the experience and skill development for future ECRs.

Many suggested that institutions should offer dedicated animal research courses and workshops covering topics such as study design, reporting, and statistical analysis. They felt this, along with other individual recommendations seen in the below table, would be beneficial, particularly in the early stages of their post-graduate studies.

ECR1	• Compulsory courses on study design; including power calculations. • Stricter enforcement of the regulations, guidelines and best practice on the supervisors and laboratories to lessen the pressure on the ECRs. • Greater awareness for PHD students of the reality of doing animal research; specifically, that we are supposed to be building on something that is well established.
ECR2	• Performing a preclinical systematic review/meta-analysis to better understand internal biases. • Have access to guidelines on how to conduct a study and how to design a study to reduce the risk of bias.
ECR3	• Online seminars or courses on study design and reporting for animal studies. • Institutions employed specialized high-level technicians who you can consult with and get direct help with and they conducted workshops after ITAR where they introduce resources like preregistration, systematic reviews, ARRIVE guidelines.

	• Protocols and SOPs at an institution/ethics level rather than sitting with one individual or small lab group.
ECR4	• Workshops or group meetings to discuss how to design and report animal studies to better understand that outcomes do matter and what the impact of a study does.
ECR5	• Not rushing into large complex studies that are independently led. • Coursework on statistics. • Ensuring students can search the literature well; conduct a preclinical systematic review.
ECR6	• Utilising technical staff to assist with training and handling. • Dedicated workshops to help improve justification for use of research practices / methods. It can be difficult to gauge whether you're really carrying out research within the acceptable 'gold standard' without having a supervisor that is well-versed in the area and has good mentoring skills. The literature can only get you so far. • Having more buffers and flexibility in study design when new to animal work as it's difficult to anticipate and what to account for.
ECR7	• Choosing and vetting for an appropriate supervisor. • Institutional support for technical training. • Institutional support for issues with supervisory team.
ECR8	• School-based mentorship to source information and assist with designing studies.
ECR9	• More field appropriate workshops that aren't based on human or education research.

ECR10	• Performing preclinical systematic reviews to be more aware of risk of bias in animal studies. • Lectures or workshops on research practices so there is direct intervention to change poor practices.
ECR11	• Have a deep appreciation of an animal's life. • Workshops for ECR's and PhDs on their responsibilities, reporting and how to conduct animal work run by the animal laboratories or ethics.
ECR12	• One day course for 1st year PhDs called 'Designing and reporting on animal experiments.' Make it part of the compulsory courses or smaller compulsory online modules.
ECR13	• Having greater support for stressed ECRs and more transparent and open lab culture. • Instilling greater importance on study design rather than it being an administrative hurdle.

Chapter 5: The culture of care to enhance laboratory animal personnel well-being and the quality of animal research: A scoping review

Chapter 5 has been submitted to Laboratory Animals as:

Morahan, H., Cohen, S., Bero, L., & Rooney, K. (2023). The culture of care to enhance laboratory animal personnel well-being and the quality of animal research: A scoping review

Statement from co-authors confirming authorship contribution of the PhD candidate

The co-authors of the paper: *The culture of care to enhance laboratory animal personnel well-being and the quality of animal research: A scoping review* confirm that Heidi Louise Morahan has made the primary contribution to this study in each of the following areas:

- Conception and design of the research
- Collection and analysis of data, interpretation of the findings
- Writing of the manuscript and critical appraisal of content

In addition to the statements above, in cases where I am not the corresponding author of a published item, permission to include the published material has been granted by the corresponding author.

Heidi Louise Morahan Date: 26 September 2023

As supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

Associate Professor Kieron Rooney Date: 26 September 2023

The culture of care to enhance laboratory animal personnel well-being and the quality of animal research: A scoping review

5.1 Abstract

There has been intense focus to improve the quality of animal research in recent times. An emerging concept of a 'culture of care' has been proposed as another important pillar to enhance scientific quality, with staff wellbeing being a critical aspect. Professionals working with research animals can face moral and psychological burdens and are at risk of experiencing work-related stress. However, data on global prevalence of stress in this population is limited. Equally, it is not clear how these stresses manifest, and what impact it has on an individual's workplace performance and research quality. The purpose of this review was to identify work-related stress, its prevalence and map evidence on strategies to mitigate stresses. In addition, we set out to identify studies assessing the association between work-related stress and research quality. A systematic search was conducted across four databases, in addition to hand-searching relevant references. We included peer-reviewed publications describing work-related stress, culture of care and laboratory animal professionals. A total of 49 publications were included for data mapping. Compassion fatigue was the most described work-related stress, and prevalence across Europe and North America was likely widespread. Multiple strategies to mitigate compassion fatigue and work-related stress were put forward, however limited empirical evidence was available to assess success. Moreover, no studies reported empirical data linking work-related stress with research quality, despite many publications stating the case. Further population specific research and measured assessments are urgently needed to deliver culture of care programs to improve human well-being, animal welfare and research quality.

Keywords: culture of care; work-related stress; compassion fatigue; laboratory animal personnel; scoping review

5.2 Introduction

Scientific animal research comes with many challenges, including problems with reproducibility, and translation.[1-3] It is important to address these issues to promote effective use of research resources, and importantly, the animals' lives. Significant focus has been directed to enhance research quality, with initiatives aimed at improving scientific methods, reporting, and implementation of 3R's.[4-7] However, the emerging concept of a culture of care has been suggested as another important component to enhance scientific quality, while also acting as a solid foundation for responsible science.[8-9]

Culture of care in the context of animal research describes a multifaceted approach to enhance the welfare of animals and staff, scientific quality, and openness and transparency.[10-12] The importance of a culture of care has been recognised in health care professions for many years,[13,14] but is a relatively new concept gaining traction within the animal research community. Following the European Union Directive 2010/63/EU introducing the term 'climate of care',[15] and evidence that mental health of those working in animal laboratories can be negatively impacted by their job,[16-18] several animal research and teaching organisations from the United States, United Kingdom and Europe support the need to better understand, assess and establish a culture of care in animal research institutions.[19-21] This has led to a growing body of research on culture of care in animal research over the last decade.

A culture of care including the well-being of staff is important for laboratory animal professionals (LAPs), as they are at risk of experiencing stress from working with research animals.[10,16] There are many types of work-related stresses (WRS) reported in animal research literature,[22] although data on global prevalence and contributing factors appears limited. Early data came from qualitative work exploring the psychological burdens of conducting animal experiments, including euthanasia,[23] which has sometimes been called the 'caring-killing

paradox'.[24] More recent literature investigated other types of stress, including compassion fatigue, ethical fatigue/conflict,[25] moral stress/distress, or vicarious traumatisation.[26] While these stresses can contribute to compassion fatigue, they can also be experienced in isolation. Compassion fatigue refers to "a psychological syndrome, compromised of secondary trauma and burnout which can adversely affect those who work in caring professions".[27-29] For those working in animal research, there are unique responsibilities inherent to their role that may exacerbate these stresses, such as caring for animals then subjecting them to pain, distress, and death; particularly when strong bonds have been formed. The negative stigma associated with animal research can compound the barrier for LAPs to openly discuss their work or search for support, often leaving them to "suffer in silence".[30] Strategies to prevent or mitigate WRS is an important component of a culture of care and aims to improve animal and human welfare, and subsequently the research quality.[58]

What is not yet well understood, is how these different types of stresses manifest in LAPs and the impact this may have on an individual's workplace performance and quality of experimental outcomes. Further, although many strategies have been proposed to moderate factors contributing to WRS,[28,31] it is not clear which ones are effective in this population. With a view to fostering LAPs well-being and improved experimental quality, this study aimed to synthesise the knowledge of reported WRS in animal research settings and culture of care strategies. We aim to scope and map evidence, from all types of peer-reviewed publications, to build a picture of the current landscape and identify promising areas for future interventions.

A preliminary search of Prospero, Open Science Framework and the Cochrane Database of Systematic Reviews was conducted on 4th May 2023 and no current or underway systematic reviews or scoping reviews on the topic were identified.

5.3 Methods

We chose a scoping review study design as it was the most appropriate method for identifying and mapping emerging evidence and gaps in the literature to inform future research.[32] This design addresses our objectives of identifying WRS and prevalence; types and effectiveness of strategies to promote a culture of care; and assess associations between WRS and research quality. The review was conducted in accordance with the Joanna Briggs Institute (JBI) methodology for scoping reviews[33], the Preferred Reporting Items for Systematic Reviews and Meta-analysis Extension for Scoping Reviews checklist (PRISMA-ScR) (See Supplementary 1),[34] and reflects the framework published by Arksey and O'Malley.[35] The protocol was registered on Open Science Framework on 28th May 2023.[36]

5.3.1 Phase 1 – Identifying the research question

The main research questions for this scoping review were:

- What types of WRS do LAPs experience and how prevalent are they?
- Do any studies exist that assess an association between WRS and research quality?
- What specific strategies have been suggested to promote a culture of care to support the well-being of LAPs and how effective are they?

5.3.2 Phase 2 – Identifying relevant literature

To identify relevant literature, a systematic database search was conducted. We employed the PCC mnemonic (population, concept, and context), as described by the JBI, to guide development of terms to be used in the search strategy design.[33] This study considered publications that have been peer-reviewed (internally or externally). The following publication

types that were considered include experimental studies, qualitative studies, analytical or descriptive observational studies, case studies, review publications, opinion or editorial publications, and systematic reviews focussed on:

- Population: LAPs involved in research and teaching of animal experiments
- Concept: WRS and culture of care relating to human well-being
- Context: scientific animal research settings

A preliminary database search of PubMed and Web of Science was performed, and keywords contained in titles and abstracts of relevant publications and index terms were used to develop a full search strategy. On 9th of May 2023, database searches were conducted in Embase, PubMed, MEDLINE, and Web of Science. The search was limited to those published in English, with no date restriction applied. Final search strategies for each database is available in Supplementary 2.

Database search results were exported to Zotero reference management software (Zotero Version 6.0.26, United States) and records were imported into Covidence systematic review management software (Veritas Health Innovation, Australia) for screening. Further relevant publications were sourced by hand searching the identified literature generated from the database search.

5.3.3 Phase 3 – Selecting the studies

Duplicates were automatically removed, and remaining publications were screened by title and abstract, then full text, against inclusion criteria using Covidence. Publications with no available abstract were individually searched using the University of Sydney library Cross-Search tool. The first phase of screening was performed by two independent reviewers (HM

and KR) and the second phase by the primary researcher (HM). To reduce the risk of bias, 10% of the final publications were assessed by KR. Any conflicts captured by Covidence or uncertainty during full text screening were discussed and the decision to include or exclude publications was mutually agreed. To identify potential stresses directly related to working in animal research settings, publications describing veterinary clinics, animal shelters or other non-research related settings were excluded. A full list of eligibility criteria can be found in Table 1.

Table 1. Eligibility criteria

Inclusion Criteria	Exclusion Criteria
• LAPs involved in research and teaching of animal experiments who experience or at risk of experiencing WRS • Concept of culture of care in animal research relating to human well-being • Peer reviewed (internally or externally) publications • Written in English	• Culture of care relating to animal welfare only • LAPs describing stress not related to work experiences • Non-research related settings • Conference abstracts/poster presentations • When full text not available

5.3.4 Phase 4 – Charting the data

To encompass an integrative synthesis of different types of extracted data (for example, quantitative data from surveys and qualitative data from interviews/ethnographic fieldwork) we used two tools for mapping. First, data items were collated in excel using the following categories: a) author/s; b) year of publication; c) publication type; d) methods (for research publications only); e) participants sampled; and f) key measures/findings relating to stress in LAPs and culture of care. Publications were then imported into NVivo software [QSR

International 2020] for data management and coding of characteristics of WRS (definitions, causes, manifestation, prevalence) and culture of care (definitions, strategies, effectiveness, researcher experience, other notable learnings).

5.3.5 Phase 5 – Summarising and reporting data

We present the selection process for included publications using the Systematic Reviews and Meta-analyses extension for scoping review (PRISMA-ScR) flow diagram.[34] A summary of results from data charting is presented in tabular form and discussed in relation to the research questions.

5.4 Results

5.4.1 Study selection

The results of the database search and selection process can be seen in Figure 1. From the 49 included publications: 19 were review publications; 11 were survey studies; 10 were qualitative studies (6 interview/ethnographic fieldwork; 2 focus group and 2 introspective reflection); 2 were systematic reviews; 2 were commentary publications; 3 were editorial/opinion publications; 1 was a published book chapter; and 1 was a mixed methodology study (survey and interview). A full list of included publications with bibliographic information and other charted data can be seen in Supplementary 3.

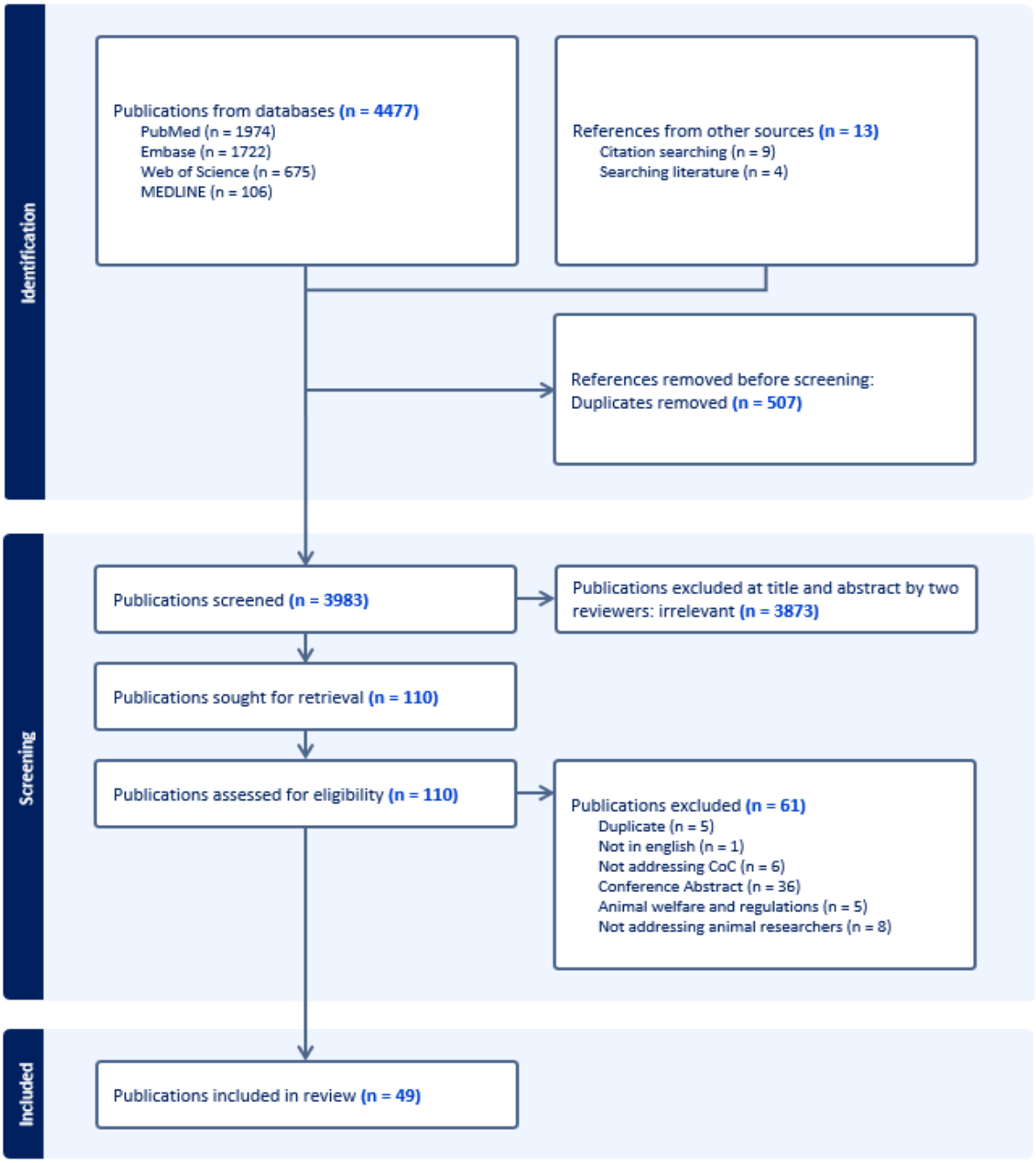

Figure 1. PRISMA-ScR flow diagram displaying the selection process for included publications.

5.4.2 Populations and context

The publication date ranged between 1985 and May 2023, with early publications describing emotional effects of euthanasia on animal researchers.[23,37-39] One of the first included

publications to describe organisational responsibilities for culture of care concerning staff well-being appeared in 2017,[9] and since then, significant interest has been generated on this nascent topic, with a further 38 publications to date. The number of publications included in this scoping review by year can be seen in Figure 2.

References came from a range of countries, however the most addressed population and setting pertained to the United Kingdom (n=10),[12,40-48] North America (United States or Canada) (n=10),[10,16,27,28-30,48-51] and the European Union (n=8).[8,11,17,20,52-54,69] Other references came from settings in Australia (n=2),[39,55] Korea (n=2),[57,70] New Zealand (n=1),[56] Japan (n=1),[20] and China (n=1).[20] A number of review and commentary publications, and systematic reviews addressed the broader animal research community and were therefore more general in nature (n=16).[9,22,23,31,37,38,58-68] In quantitative surveys reporting WRS and related symptoms, women were most heavily represented in populations, with 9 of 11 studies comprising of between 58% to 81% female participation.[16,17,27-29,39,49,58,69] Of note, the two quantitative surveys with more male participants were conducted in Asian settings (Korea, China and Japan). [20,57]

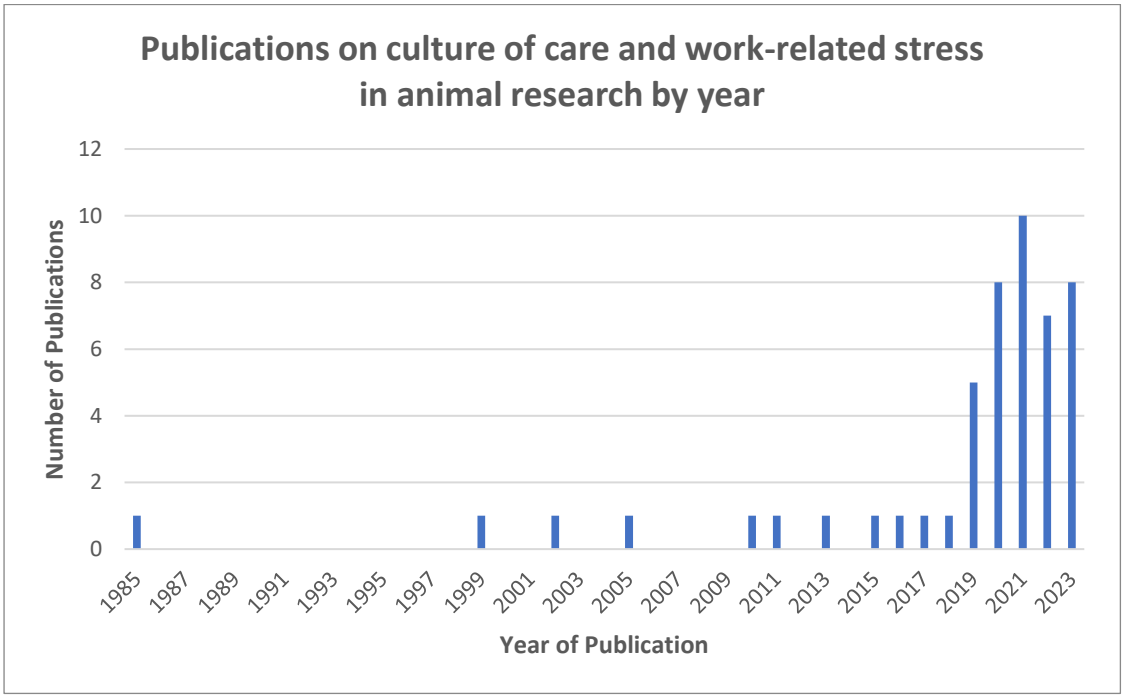

Figure 2. The number of included publications relating to culture of care and work-related stress in laboratory animal professionals by year.

All publications included LAPs involved in experiments in animal research settings; however, not all publications were conducted exclusively in this population or setting. For example, one survey study[39] and one systematic review[63] also included results from veterinary clinic and animal shelter workers. In the case of the survey study,[39] we included data as it was not reported separately, therefore the contribution of LAPs remains unclear.

There was great variability in the description of research settings, with most referencing animal research facilities/institutions/laboratories (n=12), universities (n=8), pharmaceutical animal research facilities (n=7), contract research organisations (n=3), biomedical facilities (n=3), hospitals (n=1), life sciences research institutes (n=1), US army research animal facilities (n=1), and cosmetic animal research facilities (n=1). The remaining publications did not specify the setting but described general animal research settings.

Most publications focused on measuring or describing WRS (n=21) and culture of care (n=15), whereas others focused solely on strategies to moderate WRS or incorporate into a culture of care (n=4). Eight publications focused on a combination of WRS and strategies (empirical data n=4; non-empirical data n=4).

5.4.3 Work-related stress

There was significant ambiguity in terminology used to describe different types of stress, stressors, and symptoms of stress in LAPs. For this review, we have defined and categorised the following terms: WRS (stress), workplace factors contributing to WRS (stressors), and negative effects of WRS (symptoms; personal or workplace).

5.4.3.1 Evidence from quantitative studies (types and prevalence of work-related stress)

The most common WRS investigated in 12 quantitative survey studies was compassion fatigue (n=7).[16,17,20,27-29,49] Although studies described compassion fatigue using a similar definition (as previously described), the parameters differed, with some reporting compassion fatigue as a measure of burnout and secondary traumatic stress (STS),[16,27,29,49] whereas others investigated compassion fatigue as a standalone stress.[17,20,28] The remaining studies investigated perpetrator-induced traumatic stress (n=1),[39] cognitive dissonance (n=1),[68] or stress measured by cortisol levels changes (n=1).[52] One study investigated anxiety levels in researchers[58] which we defined as a WRS symptom. Further details on WRS, associated work-related and demographic factors, and negative effects of WRS reported in quantitative surveys can be found in Table 2.

Table 2. Summary of different types of work-related stress, the risk factors associated with work-related stress and negative effects of work-related stress described across 12 quantitative survey studies

Work-related stress	**Factors associated with work-related stress**	**Negative effects of work-related stress**
Compassion fatigue [16,17,20,27-29,49] Burnout[16,27,29,49] Secondary traumatic stress[16,29,49]	**Work-related factors** Performing euthanasia[29,39,49ab,69] Using physical euthanasia methods[16a] Concern/Perceived stress/pain in animals[16,17,39] Performing tasks in conflict with morals[16,17,20,28] Desire to implement more enrichment[16]	**Personal effects** Reduced quality of professional life [16,17] Anxiety[20,28,57,69] Depression[20,28] Exhaustion or tiredness[20,28] Feeling overwhelmed or sense of hopelessness/helplesness[29,69] Guilt[28]

Perpetrator-induced traumatic stress (PITS)[39] Emotional/Cognitive dissonance[68,69] Stress (measured by salivary cortisol levels)[52*] Mental stress[57]	Lack of animal enrichment[16a] Human-animal interactions[16b,28] Lack of choice or control (euthanasia)[16,17] Working with non-human primates[49ab] Insufficient staffing[20,28] Long hours/workload [16a,20,28] Lack of resources/training for compassion fatigue [20,28] Workplace relationships [20,28,49a] Poor mental and/or physical health[20,28] Good mental health[28] Company image[20,28] Isolation/loneliness [29,49b] Less social support[16,17,39] Communication challenges/inability to talk about work[29] Social distancing[29] Unpredictable work schedule[29] Worrying about health personal/family/animals during COVID-19 pandemic[29] **Demographic Factors** Age[27,57] Female[16a,28,39,57,63b,69] Gender described as other[16a] Education or training role[16b] Working at universities vs contract research institutes[16a] Less experience/time in field [17,27,39] PhD students[17] Lower income[57]	Sadness or apathy[20,28] Frustration[20,28,68] Powerlessness [68] Discomfort/uneasiness [68,69] Invasive/intrusive/recurrent thoughts [39] Disassociation/Avoidance [39,69] Sleep issues, nightmares, or flashbacks [39] **Workplace effects** Decreased ability to perform job[28] Apathy towards job[28]

- contribution of laboratory animal professionals unclear; * – four study participants; a – burnout only; b – secondary traumatic stress only.

In North America, survey data showed that experiencing compassion fatigue at some point during a LAPs career was widespread, with reported prevalence ranging from 52% to 86%. [27-29,49] The highest prevalence was reported in LAPs during the COVID-19 pandemic and the lowest was in US army laboratory animal medicine personnel. These results concur with global data from a study reporting that 52% of respondents from Europe and 56% from China experienced compassion fatigue, but differed with respondents from Japan, where only 35% reported feelings of compassion fatigue.[20]

It appears that the Professional Quality of Life Scale (ProQoL) was often used or adapted to assess the prevalence and/or risk factors associated with compassion fatigue,[16,17,29,49] The ProQoL is a validated tool that involves a questionnaire to measure specific domains, including compassion fatigue and compassion satisfaction, and can also be used to measure burnout and STS. Two significant studies from North America and Spain using ProQoL did not report overall prevalence, however provided empirical data on associated (risk/protective) factors for professional quality of life (i.e., compassion fatigue and compassion satisfaction).[16,17] Three studies developed cross-sectional questionnaires from reviewing literature to identify or characterize the prevalence of compassion fatigue [20,28] or compassion fatigue and burnout.[27] For example, participants were provided with a definition of compassion fatigue and asked if they had personally experienced this. Similarly, another study developed a survey that provided statements to assess associated emotions as an indicator of cognitive dissonance.[68] The prevalence of cognitive dissonance was not reported, however 58% of respondents sometimes, often or always felt discomfort due to animal pain or distress.[68] When investigating PITS, a 2005 study used the IES-R tool to measure current traumatic stress, with 50% of respondents reporting mild to moderate levels of stress.[39] Finally, the State-Trait Anxiety Inventory tool was used to assess anxiety levels in animal and non-animal researchers, with a significant

difference between the groups.[58] This indicated that participation in animal experiments could negatively impact the mental health of animal researchers.

5.4.3.2 Evidence from qualitative studies (types of work-related stress)

We identified seven qualitative studies that directly or indirectly described WRS or factors contributing to WRS mentioned by participants or author (See Table 3).[23,40,45,46,48,55,56] For example, an introspective reflection described the moral stress experienced when involved in pig experiments:

"I found myself doing things to animals that were, in some significant respect, harmful for them… Being called upon to harm animals, even when it is, all things considered, justified, causes moral stress" [56]

The entanglement of human and animal stress within an animal laboratory was identified as something that shaped scientists and technicians' wellbeing, as one participant described:

"*It's hard to see a mouse starting to die, who won't quite make it to an experiment. To have lived in these cages for two or more years, which must be boring for the mice… it is really sad"* [40]

An early study involving ethnographic fieldwork described the discomfort and stress researchers and technicians felt about certain aspects of animal experiments, including performing euthanasia, particularly when strong bonds had been formed:

"This particular dog was always very glad to see us…And of course everybody loved him…then came the day when we finished the study. I actually cry when I think about it (respondent sobs) …we had a real wake and everything. It was terrible" [23]

Table 3. Summary of different types of work-related stress directly or indirectly describing the factors associated with work-related stress and negative effects of work-related stress mentioned by participants across 7 qualitative studies

Work-related stress directly or indirectly described in qualitative studies	Factors associated with work-related stress mentioned by participants	Negative effects of work-related stress mentioned by participants
	Work-related factors	**Personal effects**
Emotional/Cognitive dissonance[23,45,46]	Performing euthanasia[23,45,46,55]	Anxiety[23]
Moral stress[56]	Viewing animals undergoing anaesthesia2[3]	Feeling shame[46]
Euthanasia stress [45,55,47]	Performing or viewing procedures that cause pain, suffering, distress or dying [23,40,45,48,56]	Exhaustion or tiredness [47,55]
Emotional stress [40la]	Conflicting interests of animal welfare and scientific needs [23,40,48]	Guilt[23,46]
	Performing tasks in conflict with morals[55,56]	Sadness or apathy[23,40,46,47]
	Communication challenges/inability to openly talk about work[45]	Desensitisation to animal death[47]
	Making and breaking animal bonds[45,56]	Detachment[47]
	Societal views/social stigma of animal research[45,55]	Frustration[46]
	Developing animal allergies[45]	Anger[23]
	Animal rights activists[45,55]	Powerlessness/Helplessness [23,55]
		Discomfort/uneasiness [23]
		Fear[23,45]
		Feeling isolated[23,46]
		Feeling misunderstood/unappreciated[23]

5.4.3.3 Evidence from non-empirical publications

Publications lacking empirical data, such as reviews, commentary, or editorial publications, did not describe any further WRS other than what was reported in empirical studies. However, we found descriptions of additional factors or triggers associated with WRS that were not reported in empirical studies, such as lack of notification for animal euthanasia (endpoint),[30] making mistakes,[51] aversive work conditions, [51] and negative media. [51] Similarly, an exhaustive list of possible negative personal effects of WRS without empirical evidence was described in these publications: reduced ability to feel empathy,[8,30] hyper-vigilance,[16] fear, [16,27] low morale,

[31] bottled-up emotions,[31,60] difficulty concentrating,[31] voicing excessive complaints about one's job,[31] chronic physical ailments,[30,31] psychosomatic illness,[31] lack of self-care or motivation,[31] problems with personal relationships,[8] substance abuse or other compulsive behaviours,[8,31] suicidal ideations,[18,27,30] and excessive blaming.[60]

5.4.4 Identifying studies assessing work-related stress and research quality

Regarding our research question on assessing how WRS impacts the quality of animal experiments, we found no publications reporting direct empirical evidence. Only one survey study reported that 61% of respondents thought "compassion fatigue often or sometimes affected their ability to do their job";[28] however, they did not elucidate which abilities were impacted and whether this affected the research quality.

Despite this lack of evidence in non-empirical studies, four publications suggested research quality might be reduced due to work-place effects such as lower care-giving for animals,[8,31,51] diminished animal welfare,[31] stress experienced by animals,[31] introduction of scientific variability,[31,51] increased mistakes/safety breaches or occupational health reports,[31,51] decrease in professional decision-making ability,[8] difficulty in effectively performing or completing tasks,[31] difficulty meeting deadlines,[31] increase in absenteeism,[8,31,51] higher employee turnover,[31,51] higher worker compensation costs[31] and greater friction between staff and management.[31,60] One publication extends this theory by describing the importance of positive human-animal relationships to minimise impacts on animal stress, which may reduce abnormal animal behaviour, scientific variability and provides better care and improved animal welfare.[9]

5.4.5 Culture of care strategies

To consider viability for animal research settings to effectively implement culture of care, suggested strategies to mitigate WRS and self-reported coping mechanisms reported in publications were mapped (See Table 4). Across publications, a significant number of suggested strategies were described, however there was considerable overlap. For example, the suggested strategy to implement a compassion fatigue/WRS or well-being/resiliency program was described by many publications; however, these programs often include other strategies reported in Table 4, such as study-end point notifications, memorialising research animals,[51] or incorporating self-care strategies.[10] The most common strategy suggested to mitigate WRS was acknowledgement and validation of LAPs feelings and stress (n=12). It was reported 'recognising compassion fatigue was the first step to addressing it' and this can strengthen a person's coping mechanisms.[51] Other strategies frequently recommended were: providing social support, training and programs, and transparent communication between leadership team, peers, and public.

Table 4. Suggested strategies to mitigate work-related stress and self-reported coping mechanisms in LAPs

Suggested strategies to mitigate work-related stress	Self-reported coping mechanisms
Acknowledgement/Validation9[,10,23,29,30,31,37,38,47,51,59,60,65]	Division of labour/opting out/[45,56]
Social support (staff/personal) 8[,10,23,27,30,31,37,43,47,51,60]	Talking to someone18[,28]
Compassion fatigue/stress support training/programs [8,9,10,29,30,31,51,60,61,64,70]	Getting away from work18[,28]
	Self-care strategies18[,28,29]
Leadership communication and support[8-10,12,27,58,60,61,65]	Social support[52,29]
Open communication/transparency/sharing with peers and public9[,10,12,27,29,38,54,60,66,68]	Physical activity18[,28,52]
	Owning/caring for pets18[,28]
Recognition/positive feedback 9[,10,12,27,29,43,44,51]	Seeking professional help18[,28,29]
Remembrance/tribute/memorialising/ritualisation 9,[10,23,30,38,51]	Turning to religion18[,28]
Self-care strategies (mindfulness/meditation etc) [10,30,40,51,60,65]	Emotional eating18[,28]

Division of labour/opting out /job rotation/better scheduling[8,9,23,27,29,37,61,65]	Releasing emotions18[-28]
Access to counselling services[8-10,29,31,61,65]	Seeking further education18[-28]
Human-animal bonding/naming animals 9[-10,30,38,51]	Use alcohol/drugs/smoking18[-28]
Empowerment of staff [8,9,10,12,58]	Seeking social interaction[52]
Transparency/understanding importance of studies[8,9,30,38,51]	Handling animals in responsible manner[52]
Well-being/Resiliency programs [10,30,31,51,64]	Family support[52]
Increased enrichment/handling/skill training[8-10,54,70]	Cognitive strategy – legitimisation[52]
Strong 3Rs program[10,70]	Shifting responsibility[68]
Considerations for use of higher animals/length of study[70]	Devaluing the animals[68]
Study end-point notification 9[-30,51]	
Rehoming animals 9[-10,54]	
Work environment enhancements promoting healthy lifestyle [27,40,51]	
Ethics committee involvement for LAPs harms[61,65]	
Reporting adverse events/mistakes [44,67]	
Having a voice in ethical decisions[38,51]	
Owning companion/laboratory pet [23,31]	
Physical activity[52,60]	
Fictionalised Story-telling program (Care-full project)[43,66]	
Support for early career staff[8,10]	
Refining euthanasia methods[61]	
Patient involvement[41]	
Personal awareness for increased resources [65]	
Suitable compensation[29]	

5.4.5.1 Effectiveness of strategies

Limited data for the effectiveness of compassion fatigue and WRS programs were available in survey studies, however their primary research aim was not to evaluate strategy success. It appears dedicated programs to support LAPs well-being were not common. Two studies reported very few respondents (<30%) stated their workplace offered compassion fatigue/wellness programs.[18,28] Even when wellness programs/training were accessible, there was limited participation[39] or awareness of their existence.[18,27-29,54] Less than 50% of those working at a pharmaceutical company[54] and a university[27] were aware of the existing culture

of care/wellness program. Furthermore, few felt they would be or were helpful and effective in dealing with compassion fatigue or burnout.[18,27-29] One survey reported 78% of respondents felt current compassion fatigue programs or support measures did not help mitigate compassion fatigue symptoms during the COVID-19 pandemic.[29] In terms of influencing ProQoL scores, counselling/mental health services with or without other forms of support had no effect on compassion fatigue, but institutions offering internal support groups and/or compassion fatigue information did.[29] The other three studies reported between 8-44% of respondents believed compassion fatigue support programs were helpful.[18,27,28]

Five studies investigated self-reported coping mechanisms LAPs used to manage WRS.[18,28,45,52,56] Two survey studies suggested the most effective coping strategies were talking to someone, getting away from work, self-care, social support and physical activity.[18,28] When surveyed about beneficial factors for compassion fatigue programs, respondents reported physical activity/exercise/recreational activities (including monetary reimbursement), work activity groups, quiet place to reflect, self-care training, onsite therapists, peer support, rehoming animals, paid leave and enforcing strict workdays were effective.[18,28] Of note, two qualitative studies described division of labour or opting out of performing difficult procedures, such as euthanising animals, as an effective self-reported coping mechanism for reducing stress.[45,56]

5.5 Discussion

This scoping review highlights how widespread the condition of compassion fatigue and WRS is amongst LAPs, and there is a lack of evidence on the effectiveness of strategies to mitigate stress. Results also showed WRS manifests itself in different ways, with a variety of symptoms and risk factors reported in survey and qualitative studies. However, we could not identify any studies that investigated an association between WRS and research quality. Despite this

finding, understanding, and addressing the personal cost of animal research is likely to have many benefits of improving human well-being, animal welfare and the scientific quality of experiments.

It is not surprising that compassion fatigue has become the most frequently investigated stress in LAPs. The concept has been widely embraced by many professions, as those working in caregiving careers are most vulnerable to its development.[71] For those working in animal research, the dichotomy of caring but causing harm and distress to animals, and other inherent work conditions, places them at significant risk of experiencing compassion fatigue at some point in their career. From the data available, it is clear compassion fatigue and WRS is likely widespread across LAPs working in Europe, North America, and parts of Asia, and we suspect outcomes are likely to be similar across other countries. It is also a real possibility that prevalence could be under-reported as those who experience higher levels of compassion fatigue may face challenges that precluded them from taking part in surveys or have left the industry.[70] In fact, staff retention in animal research is a growing concern,[72] and ensuring a culture of care may contribute to increased job satisfaction and personnel retention.[10]

Many LAPs participating in surveys were female, although this is not surprising given that females are known to dominate animal and veterinary care industries.[73] In two populations where males dominated, compassion fatigue prevalence was lower. Whether this finding is due to cultural context or gender cannot be elucidated, however some studies found females were more likely to experience compassion fatigue[16,28,39,57,69] or STS,[63] yet others found gender did not play a role.[27,16,63] Alternatively, there are known cross-cultural differences in attitudes towards the use of animals in research,[74] which may introduce bias in surveys if compassion fatigue was less reported in certain populations.

The results of this study have important implications for delivery of educational programs to mitigate WRS and potentially reduce negative workplace practices. Unlike health care professions where work-place stress is better understood and culture of care frameworks established, the extracted data suggest animal research and teaching communities are not routinely supporting those who work within their industry. Less than 16% of the general population of LAPs and those working at contract research organisations across Europe and North America indicated their research workplace offered compassion fatigue program.[18,28] Results were not much better for those surveyed in China and Japan, with less than 30% aware.[18]

A striking outcome of this review was the lack of data on the effectiveness of programs and strategies to mitigate WRS in professionals working with research animals. In fact, many strategies described in the included publications have simply been taken from human health care professions. Whether these strategies can be translated into the context of the animal research community is still unknown, but possible, as preliminary evidence for occupational stress intervention programs in other animal care settings, such as veterinary and animal shelters settings, suggest there may be benefits.[75] However, in these settings, staff are not required to intentionally inflict pain, suffering or distress as part of their day to day activities, which may mean LAPs are at higher risk, or strategies applied in other industries may not be as effective. A small but growing number of research facilities in North America, the United Kingdom and other European countries have launched compassion fatigue and culture of care programs[12,30,51,76] and offer the ability for tracking efficacy. We encourage further studies with definable metrics to assess the success of such strategies.

Furthermore, we encourage people across all countries to further explore the concept of a culture of care which is a key example of ‘one welfare’, recognising the interconnectedness of

human and animal welfare and environment.[77] It is well understood positive human-animal relationships are essential for good science, giving rise to positive behavioural and physiological responses.[78] Conversely, negative relationships can have detrimental effects on animal welfare,[77] that can elicit stress responses and subsequently impact research outcomes. We could not identify any empirical research investigating the relationship between WRS and research quality, despite many publications reporting the case. Of course, it is not an unreasonable link, given many described symptoms relate to reduced animal care and work performance. An area of future focus could include questionnaires on negative or positive work impacts, adverse events, and experimental outcomes.

Data revealed some LAPs currently employ their own coping mechanisms, however not all of these were positive tools. For example, some reported using alcohol and drugs, emotional eating, devaluing the animals, and shifting responsibility.[18,28,68] Although many other LAPs employed positive mechanisms, with the most beneficial being talking to others, physical activity, getting away from work, self-care practices and caring for pets.[18,28] Further, division of labour was another coping mechanism used, including having others perform euthanasia.[45,56] The rotation of activities is thought to be an important tool in reducing human wellbeing issues, and this was confirmed by qualitative studies.[45,46]

5.5.1 Limitations

While we endeavoured to retrieve all relevant publications, there is the possibility some were missed, despite systematically searching four large medical literature databases. There were also several publications that reported data from the same studies, potentially over-representing the number of references for stressors and symptoms. Additionally, a small amount of data from non-research settings was included as it was not reported separately, however we believe this has not detracted from findings.[39,63]

Connecting evidence from quantitative, qualitative, and non-empirical publications provided a richer exploration of the data, however there were significant challenges during synthesis where complex perspectives were reported. This was most evident when mapping ethnographic fieldwork and introspective reflections, as they presented data as interconnected thoughts, feelings and experiences related to LAPs, human patients, or authors. While this posed difficulties in drawing meaningful data on WRS prevalence and strategy effectiveness, it did contribute to identifying types of stress and symptoms.

5.5.2 Conclusions

It is clear there is an urgent ethical, and scientific need for the research community to better understand and support those who work with research animals. The limited data has revealed the likely prevalence of compassion fatigue and WRS to be extensive, yet very few facilities appear to explicitly offer compassion fatigue or relevant WRS well-being programs specific to those working with animals in research. Moreover, there is limited awareness and/or uptake of current programs. Unfortunately, many LAPs may not believe these programs can help with dealing with compassion fatigue or WRS and sometimes reported using negative self-coping mechanisms. Numerous strategies have been suggested to mitigate stress and promote a culture of care but to date, there has been no focussed data or peer-reviewed information to assess the metrics of success. Undertaking further research in different geographical locations to identify the well-being of other LAPs populations, along with a strong focus on measuring successful programs, has the potential for even greater improvements for human well-being, animal welfare and research quality.

5.6 Declaration of conflicting interests

The authors declare that there are no conflicts of interest.

5.7 Funding

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5.8 Data availability

Data will be made available on request.

5.9 References

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Preferred Reporting Items for Systematic reviews and Meta-Analyses extension for Scoping Reviews (PRISMA-ScR) Checklist

SECTION	ITEM	PRISMA-ScR CHECKLIST ITEM	REPORTED ON PAGE #
TITLE			
Title	1	Identify the report as a scoping review.	1
ABSTRACT			
Structured summary	2	Provide a structured summary that includes (as applicable): background, objectives, eligibility criteria, sources of evidence, charting methods, results, and conclusions that relate to the review questions and objectives.	2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known. Explain why the review questions/objectives lend themselves to a scoping review approach.	3/4
Objectives	4	Provide an explicit statement of the questions and objectives being addressed with reference to their key elements (e.g., population or participants, concepts, and context) or other relevant key elements used to conceptualize the review questions and/or objectives.	5
METHODS			
Protocol and registration	5	Indicate whether a review protocol exists; state if and where it can be accessed (e.g., a Web address); and if available, provide registration information, including the registration number.	5
Eligibility criteria	6	Specify characteristics of the sources of evidence used as eligibility criteria (e.g., years considered, language, and publication status), and provide a rationale.	7
Information sources*	7	Describe all information sources in the search (e.g., databases with dates of coverage and contact with authors to identify additional sources), as well as the date the most recent search was executed.	6
Search	8	Present the full electronic search strategy for at least 1 database, including any limits used, such that it could be repeated.	Supplementary 2
Selection of sources of evidence†	9	State the process for selecting sources of evidence (i.e., screening and eligibility) included in the scoping review.	6/7 & 9 - Figure 1
Data charting process‡	10	Describe the methods of charting data from the included sources of evidence (e.g., calibrated forms or forms that have been tested by the team before their use, and whether data charting was done independently or in duplicate) and any processes for obtaining and confirming data from investigators.	7/8
Data items	11	List and define all variables for which data were sought and any assumptions and simplifications made.	7/8
Critical appraisal of individual sources of evidence§	12	If done, provide a rationale for conducting a critical appraisal of included sources of evidence; describe the methods used and how this information was used in any data synthesis (if appropriate).	N/A
Synthesis of results	13	Describe the methods of handling and summarizing the data that were charted.	8

SECTION	ITEM	PRISMA-ScR CHECKLIST ITEM	REPORTED ON PAGE #
RESULTS			
Selection of sources of evidence	14	Give numbers of sources of evidence screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally using a flow diagram.	9 - Figure 1
Characteristics of sources of evidence	15	For each source of evidence, present characteristics for which data were charted and provide the citations.	Supplementary 3
Critical appraisal within sources of evidence	16	If done, present data on critical appraisal of included sources of evidence (see item 12).	N/A
Results of individual sources of evidence	17	For each included source of evidence, present the relevant data that were charted that relate to the review questions and objectives.	9-19
Synthesis of results	18	Summarize and/or present the charting results as they relate to the review questions and objectives.	9-19
DISCUSSION			
Summary of evidence	19	Summarize the main results (including an overview of concepts, themes, and types of evidence available), link to the review questions and objectives, and consider the relevance to key groups.	19-21
Limitations	20	Discuss the limitations of the scoping review process.	21
Conclusions	21	Provide a general interpretation of the results with respect to the review questions and objectives, as well as potential implications and/or next steps.	21
FUNDING			
Funding	22	Describe sources of funding for the included sources of evidence, as well as sources of funding for the scoping review. Describe the role of the funders of the scoping review.	22

JBI = Joanna Briggs Institute; PRISMA-ScR = Preferred Reporting Items for Systematic reviews and Meta-Analyses extension for Scoping Reviews.
* Where *sources of evidence* (see second footnote) are compiled from, such as bibliographic databases, social media platforms, and Web sites.
† A more inclusive/heterogeneous term used to account for the different types of evidence or data sources (e.g., quantitative and/or qualitative research, expert opinion, and policy documents) that may be eligible in a scoping review as opposed to only studies. This is not to be confused with *information sources* (see first footnote).
‡ The frameworks by Arksey and O'Malley (6) and Levac and colleagues (7) and the JBI guidance (4, 5) refer to the process of data extraction in a scoping review as data charting.
§ The process of systematically examining research evidence to assess its validity, results, and relevance before using it to inform a decision. This term is used for items 12 and 19 instead of "risk of bias" (which is more applicable to systematic reviews of interventions) to include and acknowledge the various sources of evidence that may be used in a scoping review (e.g., quantitative and/or qualitative research, expert opinion, and policy document).

From: Tricco AC, Lillie E, Zarin W, O'Brien KK, Colquhoun H, Levac D, et al. PRISMA Extension for Scoping Reviews (PRISMAScR): Checklist and Explanation. Ann Intern Med. 2018;169:467–473. doi: 10.7326/M18-0850.

Supplementary 2 – Search Sets

Web of Science – Core Collections

("Animal research*" OR "Laboratory animal*" OR "Preclinical research*" OR "Animal experiment*" OR “Laboratory animal personnel*” OR “Laboratory animal professional*”) AND ("laboratory cultur*" OR "care" OR wellbeing OR "mental health" OR "culture of car*" OR “compassion fatigue” OR “euthanasia stress”)

Pubmed

(("Animal research*"[Title/Abstract] OR "Laboratory animal*"[Title/Abstract] OR "Preclinical research*"[Title/Abstract] OR "Animal experiment*"[Title/Abstract] OR “Laboratory animal personnel*”[Title/Abstract] OR “Laboratory animal professional*”[Title/Abstract])) AND (("laboratory cultur*"[Title/Abstract] OR care[Title/Abstract] OR wellbeing[Title/Abstract] OR "mental health"[Title/Abstract] OR "culture of car*"[Title/Abstract]))

Medline / Embase

((“Animal research*” or “Laboratory animal*” or “Preclinical research*” or “Animal experiment*” or “laboratory animal personnel*” or “laboratory animal professional*”) and (“laboratory cultur*” or “wellbeing” or “mental health” or “culture of car*”)).mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]

Reference	Year	Study ID	Study Type
8	2022	Ferrara 2022	Review article
9	2017	Brown 2017	Book chapter
10	2020	Murray 2020	Review article
11	2021	Bertelsen 2021	Review article/survey tool proposal
12	2020	Robinson 2020	Review article/CoC framework proposal
16	2020	La Follette 2020	Survey research article
17	2021	Goñi-Balentziaga 2021	Survey research article
19	2022	O'Malley 2022	Survey research article
22	2023	Rumpel 2023	Systematic review
23	1999	Arluke 1999	Ethnographic fieldwork
27	2020	Pavan 2020	Survey research article
28	2021	Randall 2021	Survey research article
29	2021	Thurston 2021	Survey research article
30	2023	Grimm 2023	Opinion article
31	2019	Newsome 2019	Review article
37	1985	Wolfe 1985	Review article
38	2002	Herzog 2002	Review article
39	2005	Bennett 2005	Survey research article
40	2019	Friese 2019	Ethnographic fieldwork; qualitative study
41	2020	Gorman 2020	Qualitative study
42	2022	Kirk 2022	Review article
43	2021	Robinson 2021	Commentary article
44	2022	Robinson 2022	Review article
45	2023	Roe 2023	Qualitative study
46	2010	Davies 2010	Qualitative study
47	2023	Williams 2023	Qualitative study
48	2021	Sharp 2021	Ethnographic fieldwork
49	2021	Schlanser 2021	Survey research article
50	2019	Smith 2019	Review article
51	2021	Van Hooser 2021	Review article
52	2020	Glenk 2020	Mixed methodology
53	2019	Hawkins 2019	Review article/survey tool proposal
54	2023	Turkemenian 2023	Survey research article
55	2013	Marks 2013	Introspective reflection paper
56	2016	King 2016	Introspective reflection paper
57	2018	Kang 2018	Survey research article
58	2022	Amarasekara 2022	Review article/survey tool proposal
59	2011	Coleman 2011	Review article
60	2021	Kerton 2021	Review article
61	2021	King 2021	Review article
62	2022	Sanchez-Morgado 2022	Editorial article
63	2015	Scotney 2015	Systematic review
64	2023	Thompson Iritani 2023	Editorial article
65	2020	Tremoleda 2020	Review article
66	2023	Tremoleda 2023	Commentary article
67	2023	Enkelmann 2023	Review article
68	2020	Engel 2020	Survey research article
69	2019	Mamzer 2019	Survey research article
70	2022	Ahn 2022	Review article

Chapter 6: Conclusions and future directions

6.1 Main findings

This thesis established that animal research investigating the effects of highly sweetened maternal diets was conducted to a low standard and was poorly reported, making it difficult to draw robust conclusions from data, and therefore provided limited translational relevance. However, it also established that less experienced animal researchers had low awareness of best practice methodologies, and more often than not, institutions failed to provide practical and emotional support needed, as identified in Chapters 4 and 5. The collective findings of this thesis offer important considerations for fostering improvements to best practice methods, enhancement of research quality, and translational capacity for animal research.

We started this thesis with the aim of addressing the current global epidemic of increased obesity and obesity related diseases by understanding the effects from maternal diets sweetened with sugar, non-nutritive sweeteners, or both. In Chapters 1 and 2, our main findings uncovered that non-nutritive sweetener consumed during pregnancy and/or lactation did not affect the body weight of offspring, however maternal sucrose consumption increased the risk of obesity and poor glucose disposal later in the offspring's life. Subgroup analyses provided further insights into heterogeneity, however given the number of included studies, there was the potential for results to be underpowered and results should be interpreted with caution. Yet overshadowing these outcomes was the fact that most studies had serious concerns with internal validity and reporting. We criticised the way many experiments were designed, analysed, and showed a lack of consideration for clinical relevance.

In Chapter 1, data presented showed rodents were frequently provided with individual non-nutritive sweeteners not commonly consumed by humans, such as saccharin, or at dosages far exceeding human statutory daily intakes. Poor experimental design had real potential to confound results as rodents often find high sweetener concentrations unpalatable[1,2] and as such may lead to reduced food and fluid intake causing lower body weights. Hence, the null result for offspring obesity risk reported in meta-analyses could have been masked by substandard study design. There were other critical design elements specific to maternal studies that were not frequently considered, such as timing of intervention, selecting litter as the experimental unit for analysis (instead of individual offspring) and outcome measures to determine the dynamic relationship between dam and offspring. Subgroup analyses to explore heterogeneity We provided an extensive summary of considerations for future experiments to improve quality and translation.

In Chapter 2, we purposely selected publications that mimicked human consumption of sugar-sweetened beverages, that is sucrose concentrations between 8-20% *w/v*, as an attempt to find outcomes more clinically relevant. Although data included in meta-analyses found effects suggesting the metabolic outcomes of offspring were altered by maternal diet, similar concerns over study design, analysis, and reporting that were reported in Chapter one was again identified. Random allocation, outcome assessment blinding, and allocation concealment were poorly reported, and there was a significant bias towards investigating only male offspring populations. Moreover, the use of individual offspring as the unit of analysis was again highly prevalent, giving rise to inflated sample size and potential false-negative results.[3] Scrutinising the findings of the two preclinical systematic reviews made for disappointing reading, as the reliability of reported outcomes appeared low.

Given the limited evidence for a real-world ecological model for consumption of non-nutritive sweeteners, and to further understand if switching from sugar to non-nutritive sweeteners could ameliorate the deleterious effects of sugar, a preclinical rodent experiment was conducted in Chapter 3. Best efforts were made to address internal and external validity, (ecological validity being a subset of external validity), although we could never be one hundred percent certain of extrapolating animal to human translation due to species differences.[4] Nevertheless, this research was the first of its kind to determine that rats drink commercially available beverages consumed by humans. It informs study protocols of future rodent experiments investigating non-nutritive sweetener diets as a mechanism for obesity control. It also provided evidence that replacing sugar-sweetened beverages with 'diet' beverages showed limited short term metabolic recovery in rodents.

However, as mentioned in the abstract of this thesis, there was a lingering uneasiness about clinical relevance and lack of impact any further animal experiments investigating highly sweetened maternal diets might have on human obesity outcomes. This uneasiness led to the decision to explore other ways for improving scientific quality in animal research more broadly, as this felt significantly more important than using more animals' lives. The overarching theme of fostering best practice methods and translation provided an avenue for advocacy and a distinct change of focus, evident in the final two research chapters.

The substandard level of study design and reporting was well documented in the early chapters of this thesis and led to the exploration in Chapter 4 of how early career researchers, who are heavily involved in conducting animal experiments, made important decisions regarding research practices. The results from this qualitative study showed early career researchers were often not aware of best practice recommendations and were highly influenced by previous

literature and current laboratory practices, which appeared to be suboptimal for some. It seems in this population, negligence was not the driving factor for poor research, but rather a lack of information. They showed a strong desire for better institutional support, specifically requesting workshops that taught the more practical aspects of animal experiments, over and above the introductory ethics courses necessary to perform animal research. Highlighting that knowledge was a powerful tool for improving research quality, data also showed early career researchers who conducted preclinical systematic reviews consistently re-evaluated their research practices and implemented more rigorous methods in future experiments. It is highly likely that providing animal researchers in the early stages of their career with more training and coaching on best practices would contribute to positive research behaviours which will impact the scientific quality of animal studies.

A secondary finding presented in Chapter 4 was the emotionality researchers experienced when working with animals used for scientific use. Despite focussing on study design and reporting behaviours, this unexpected theme was uncovered when early career researchers consistently described emotive and moral challenges they faced. For some, working with research animals negatively impacted their emotional health and displayed symptoms of work-related stress, as described in Chapter 4, and confirmed in Chapter 5. Although we did not find any publications reporting a link between work-related stress and study quality, several review publications described this association and suggested the implementation of a culture of care, including care for staff wellbeing, might contribute to improved research quality.[5-8] This provided us the opportunity to map available evidence in a scoping review investigating the effectiveness of a culture of care to enhance staff-wellbeing and scientific quality, which was presented in Chapter 6.

Results in Chapter 5 confirmed professionals working with animals are at high risk of experiencing work-related stress and limited data showed between 52-86% of laboratory animal professionals in North America and Europe suffered from compassion fatigue at some point during their career. Many early career researchers in our qualitative study described similar experiences, feelings, and thoughts to those who took part in survey studies and other qualitative research included in the scoping review, suggesting that they too may be experiencing compassion fatigue or work-related stress. This included symptoms such as nightmares, guilt, anxiety, exhaustion, uneasiness, or feelings of isolation, which were triggered by the inherent requirements of their job, including performing procedures on animals that caused pain and distress, and euthanasia. Currently, very few animal research institutions offer compassion fatigue or other well-being programs specific to this population and their needs. We also found data was lacking on the effectiveness of suggested strategies to mitigate these stresses and further, there was no empirical data available to link work-related stress with reduced study quality. However, it is not unreasonable to suspect that suffering from work-related stress could have negative implications for research outcomes. Collective data and findings presented throughout this thesis show there is limited practical and emotional support provided by institutions to deliver benefits to drive improvement in scientific quality, animal welfare and staff well-being.

6.2 Future directions

The work presented here identifies three main areas that can be further explored. First, given findings of emotional and moral stress found in early career researchers conducting animal research, correlating with data showing compassion fatigue in laboratory animal professionals is likely widespread across North America and Europe, further research to determine compassion fatigue prevalence in other countries, including Australia, should be prioritised.

Whilst it was not the main aim of Chapter 4, compassion fatigue appeared to be present amongst early career researchers in this study. Unfortunately, we lacked sufficient participant numbers and appropriate study design to assess prevalence. This would be the first step to addressing the psychological challenges when working with research animals and could help identify cultural or contextual differences between different countries. Understanding which strategies in a culture of care are effective for successful program delivery is important, however results presented in Chapter 5 showed a paucity of empirical evidence. It has recently come to our attention that a North American research group is presently conducting a significant study assessing which approaches for mitigating or preventing compassion fatigue are effective. This data will be highly anticipated as currently, most strategies have been taken from human health care professions and we simply don't know if they are transferrable to animal research settings, although it is possible.[9]

At a local level, we believe it would be highly beneficial to identify and assess the current culture at the University of Sydney's animal research facilities, as a measurement that can be reported and followed up. This would be carried out using a comprehensive survey tool developed to understand relevant issues and how to achieve successful implementation for institutional culture of care and compassion fatigue programs. As a committee member of an American university compassion fatigue program stated: "It is better to have a support program and not need it than to need a support program and not have one".[8]

Second, we did not identify any studies in this thesis that established a link between work-related stress and study quality. It has been suggested that laboratory animal professionals experiencing compassion fatigue can exhibit negative work-related performance, including reduced caregiving. It would be of significant interest to further explore how work-related

stress manifests in the workplace and the potential effect this may have on the scientific quality of animal research studies.

The third area for future consideration would be development of workshops focussed on best practice in animal research, to be incorporated into the University of Sydney's training resources and programs. Current practice is that researchers must complete an 'Introduction to Animal Research' course and 'Animal Ethics' training module prior to commencing animal experiments; however, findings in Chapter 4 reported that researchers expected institutions to provide more dedicated animal workshops on designing and reporting preclinical experiments. This is in line with practical strategies recommended by the National Health and Medical Research Council of Australia, where institutions ensure appropriate and ongoing education and training for investigators in current best practice.[10] The goal of providing institutional training, including education on preclinical systematic reviews, would for researchers to be adequately skilled to design, execute, and appropriately report robust studies. In turn, this would address the consequences of poor methodology and enhance robust research outcomes.

6.3 Conclusions

This thesis has explored different ways of fostering best practice methods to enhance study quality and translation in animal research; from evaluating maternal nutrition studies, to understanding what influences research decisions in those less experienced, and finally attempting to identify whether work-related stress has a role to play. The research findings indicate that institutions can and should make significant improvements to better support animal researchers, both practically and emotionally. It was clear that despite promotion of initiatives, recommendations, and guidelines to enhance research quality, other barriers exist. It is important animal researchers, particularly those in the early stages of their career, are

armed with up-to-date research strategies, and are supported through the emotional challenges of the role. This will enable researchers a genuine chance of producing high quality scientific research, fostering translation for better clinical outcomes.

6.4 References

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