Title: Investigating the impact of conventional laboratory cages on rodent morbidity and mortality: a protocol for a follow-up systematic review

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Author contributions: AB wrote this protocol (reviewed by JC and GJM). GJM is the guarantor. JC will be the review leader. JC will perform all literature screening, data extraction and risk of bias assessments. AB will act as the secondary reviewer and will perform all of the literature screening, data extraction and risk of bias assessments along with JC. JC will write and prepare the manuscript drafts. Content expertise will be provided by GJM when needed. CBW will be consulted as methodological expert and aid in statistical analyses. All authors will review and approve the final version of the manuscript.

Registration: This protocol is archived in the University of Guelph's repository (The Atrium: https://atrium.lib.uoguelph.ca/xmlui/handle/10214/10046). This protocol was developing using the Preferred Reporting Items for Systematic Reviews and Meta-Analyses - Protocol (PRISMA-P) guidelines (1).

Amendments: Protocol deviations made following protocol registration will be documented in the final systematic review accompanied by the date of the change, a description of the change and the rationale.

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INTRODUCTION

Rationale

Laboratory rodents make up an overwhelming 95% of the animal models used in biomedical research studies (2). They are often housed in conventional "shoebox" cages, which are small, typically barren spaces that limit the expression of many motivated natural behaviours (e.g. 3-5). In these cages, rodents show signs of overall poor psychological and physiological welfare (e.g. 5, 6), which can specifically include: elevated anxiety and/or depression-like behaviour (e.g. 3, 7-17); increased abnormal behaviours such as inactive-but-awake (IBA)

behaviour, where rodents exhibit long bouts of waking inactivity (e.g. 4), and stereotypic behaviours, which are repetitive behaviours indicative of frustration, attempts at coping and/or nervous system dysfunction (e.g. 3, 4, 15, 18-23); and attenuated resilience to acute stressors (e.g. 11, 244, 25). In an effort to address these welfare issues, researchers may house lab rodents in larger cages where rodents are provided with additional resources that enable them to express naturally motivated, species-specific behaviours (often called "enriched cages" (26), but hereafter referred to as "well-resourced cages" [see Defining Key Terms below]). This aligns with the principles established by the Canadian Council on Animal Care (CCAC), which stipulate that ethical lab rodent housing should promote animal welfare by enabling the expression of naturally motivated behaviours and reducing abnormal ones (27, 288); the fact that conventional housing violates these recommendations is just one indication that it does not meet animal welfare standards. In 2020, we conducted a systematic review and meta-analysis to investigate the potential long-term impacts of housing systems on laboratory rodent welfare. Our results showed that when compared to well-resourced cages, conventional housing increases rodent morbidity in stress-sensitive disease models and increases mortality rates, which mirrors some of the physiological consequences of stress seen in humans (29).

Despite over three decades of research highlighting the negative consequences of conventional laboratory cages (e.g. 3-25) there are few guidelines that advise how to effectively design well-resourced environments that reduce rodent stress. In our previous systematic review, resources varied greatly between studies: some only contained a running wheel while others provided a variety of resources that enabled additional behaviours, such as exploration, gnawing, climbing, etc. (29). In our previous study, we were interested in determining which specific resources might be most relevant for promoting lab rodent welfare and health, and which ones are less effective at doing so (30). Our results suggested that housing effects were not impacted by the nature and/or extent of the resources provided (29). However, animal welfare-focused research provides evidence to the contrary: rodents have been observed to show preference for certain resources over others (e.g. 31), and different types of resources have been shown to have differential effects on anxiety-like behaviour (7), suggesting that not all resources are equally effective at promoting lab rodent welfare and reducing stress. Furthermore, reporting quality was low: only a third of included studies described their conventional cages, which made it necessary to make assumptions about the cage design (29). As well, no behavioural observations were provided, making it impossible to confirm how or if provided resources were used as researchers intended (e.g. was a nest box truly used for nesting?) (29). As such, we believe our results to be likely due to a type II error due to poor reporting of cage details, rather than a true null result.

For this systematic review, we will update the previous search (conducted May 2020, already containing 165 relevant articles). We will then email all authors included in the review with an article published in 2007 or later to gather more details about cage conditions, allowing us to more accurately assess which resources are best at reducing rodent morbidity and mortality rates in conventional housing. In addition, we will also revisit included studies to extract rodent body weight data, and use it as a proxy for the presence of abnormal behaviours (thus an indicator of stress), since stereotypic behaviours are often highly active (188, 32) and could reduce rodent weight (making them excessively "skinny"), and IBA behaviour can cause weight gain. This effect is similar to the bidirectional body weight change observed in studies of stressed humans (33). We hypothesize that well-resourced environments which promote species-typical

behaviour and reduce abnormal behaviour will be best at reducing stress. We also hypothesize that not all resources are equally effective at promoting lab rodent welfare and reducing stress. The first hypothesis predicts that large differences in weight across differentially housed rodents will correlate with larger effect sizes, where effect size is the severity of stress-sensitive disease morbidity outcomes and all-cause mortality rates. Our second hypothesis predicts that different resources will have differential impacts on effect size. We also predict that the extent of resources provided will have an impact on effect size, where resources that meet more needs will have a greater effect than those meeting less needs (see Meta-regression below for examples of needs addressed by resources).

Defining key terms

The key terms for this protocol remain the same as in the original (30).

Resource (i.e. environmental "enrichment"): A resource will be defined as any structural item added to the cage determined by the publishing authors to be "enrichment" beyond nesting material alone (i.e. if "enriched" cages only contain nesting material, they will be excluded). This will not include other rodents, olfactory, gustatory or auditory "enrichment".

It is important to note that the term "environmental enrichment" will be used for the purposes of extracting relevant literature and asking authors about cage details, as this is the term most commonly used by researchers to describe any additional resources provided to laboratory rodents. However, we chose to refer to environmental enrichment simply as "resources" since describing an environment as being "enriched" has a positive connotation that implies luxury rather than providing a basic need.

Objectives

The objective of this protocol is to expand on our previous protocol (30), and outline methods for an updating systematic review and extracting more data on potential moderators of effect size. The underlying PICO question remains the same:

For experimental rats and mice, do well-resourced environments impact health compared to conventional housing?

To expand on the original protocol, we will investigate the differential impact of specific resources provided in rodent cages on disease morbidity and mortality. We will also assess if lab rodent weight (used as a proxy for the presence of abnormal behaviours) can explain variations in effect size.

i. *Population:* laboratory rats and mice
ii. *Intervention:* well-resourced housing
iii. *Comparator:* conventional laboratory housing
iv. *Outcomes:* rodent health (mortality data or disease specific morbidity data. See outcomes below.)

Outcomes

As outlined in the original protocol, rodent health will be determined by all-cause mortality data as well as disease specific morbidity data. Morbidity will be assessed in seven diseases determined as relevant due to their positive correlation with stress: cardiovascular disease, major depression, cancer, viral infection, asthma, anxiety disorder, and stroke. The same disease-specific outcomes will be extracted from studies as in the original systematic review. The following table taken from the previous protocol presents a summary of outcome measures that will be extracted for each disease. For more details as well as the rationale, refer to (30).

Table 1. Outcome measures to be extracted for each disease.

Disease	Outcome measures	Tests	Measures
cardiovascular disease	intimal thickening	histological assessment	mm^2 or % of lumen filled
major depression	anhedonia	sucrose preference	% sucrose/ total drinking volume
	helplessness	Forced-Swim Tests	total time immobile
		Tail Suspension Tests	total time immobile
	hippocampal volume	volume measurements by MRI or histological sections	% of brain volume or total volume in mm^3
cancer	tumor burden	tumor volume	mm^3
		tumor weight	in milligrams
		metastasis	number of metastasis or cell count
		number of tumors	tumor count
viral infections	viral load	viral load in relevant organ or blood (e.g. RT-PCR)	viral copy number
	cytokine levels	serum or relevant organ levels of IL-1b, IL-6 and TNFα	protein levels (pg/mL, ug/mL) or mRNA expression (ΔΔCT [cycle threshold] values)
	weight loss	rodent weight	% change from baseline or total weight lost
anxiety disorders	behavioural measures of anxiety	Elevated Plus Maze	% time spent in open arm
		Open Field Test	% time spent in center, total freezing time, rearing, defecation
		Light/Dark Box	% time in light side
		Social Interaction Test	% time spent interacting
asthma	total cell number	total cell number in lung tissue or airspaces	cell count
	eosinophils	total cell number in lung tissue or airspaces	cell count

	cytokine levels	serum levels of IL-4, IL-5 and IFN-γ	protein levels (pg/mL, ug/mL) or mRNA expression (ΔΔCT values)
stroke	infarct volume	histological assessment (e.g. 2,3,5-Triphenyltetrazolium chloride [TTC], hematoxylin and eosin [H&E])	mm^3 or % of hemisphere
	motor and sensorimotor tests	composite scores (Benderson scale, Modified Neurological Severity Score, other scoring systems specified by the authors)	ordinal score data
		Accelerated Rotarod	mean fall score (RPM) or score relative to baseline or latency to fall
		Ledge Tapered Beam Test	foot faults or distance traveled
		Pasta Test	number of paw adjustments
		Limb Placement Test	ordinal score data
	cognitive impairment	Morris Water Maze	% time spent in target quadrant or path length

METHODS

Eligibility criteria

The eligibility criteria will not differ from the original protocol (30). See eligibility criteria below:

Intervention: Resources must be a component of the home cage (we will exclude "play-pen" type studies), and a photograph or clear description of the well-resourced cage must be provided.

Comparator: The comparator group must have the same social housing structure as the intervention group (e.g. individually or group housed). Studies will not be excluded for failure to provide an adequate description of conventional housing conditions. Instead, authors will be contacted to gather better descriptions (see Contacting Studies below).

Report Characteristics: The study must be published in English and must report primary data.

Study Designs: Only *in vivo* controlled trials will be included.

Information sources

To update the search, we will perform the same search, using the same databases for studies published after May 2020.

Table 2. Databases and information sources to be searched, taken from the previous protocol (30).

Database/Information Source	Interface/URL
MEDLINE	Ovid
CAB abstracts	CAB interface
Science Citation Index	Web of Science
Proquest Dissertations and Theses A&I (grey literature)	Proquest
Elsevier	SCOPUS

Search strategy

Table 3. Search strategy developed previously using the Medline database via the Ovid interface. This search was also adapted for the other interfaces listed above.

	Search
#1	exp Murinae/
#2	(mice OR mouse OR Mus OR rodent* OR murine OR rat OR rats).ti,kw,ab.
#3	#1 OR #2
#4	Housing, Animal/
#5	((cage OR caging OR caged OR cages OR environment*) adj3 (enrich* OR naturalistic)).ti,kw,ab.
#6	("voluntary wheel running" or "running wheel" or "wheel running" or "running disk" or "physical activity").ti,kw,ab.
#7	#4 OR #5 OR #6
#8	Cardiovascular Diseases/
#9	("cardiovascular disease*" or "coronary artery disease" or "myocardial infarct*" or "coronary heart disease" or "atherosclero*" or "arteriosclero* myocardial ischemia" or "ischemic heart disease" or "coronary heart disease" or "APOE" or "Apolipoprotein E" or "intimal thickening" or "lumen stenosis" or "lumen occlusion*" or "atherogenic diet*" or "coronary lesion*").ti,kw,ab.
#10	#8 OR #9
#11	Depressive Disorder/ or Depressive Disorder, Major/
#12	("model of depression" or "major depression" or "major depressive disorder" or "depressive disorder" or "forced-swim* test" or "forced swim* test" or "anhedonia" or "sucrose preference" or "social defeat stress" or "tail suspension test" or "chronic mild stress" or "learned helplessness" or "olfactory bulbectomy" or "maternal separation" or "chronic restraint stress").ti,ab,kw.
#13	#11 OR #12
#14	exp Neoplasms/
#15	(carcino* or cancer or malignant or tumor or tumour).ti,kw,ab.
#16	#14 OR #15
#17	exp Viruses/ or exp Virus/ or Virus Diseases/
#18	("viral infection" or virus or "immunodeficiency virus" or HIV or "infectious disease" or "respiratory disease" or "upper respiratory disease" or influenza).ti,kw,ab.
#19	#17 OR #18
#20	Asthma/

#21	(OVA or asthma or asthmatic or "house dust mite" or "papain" or "atopic" or "allergic lung inflammation").ti,ab,kw.
#22	#20 OR #21
#23	Anxiety/ or Anxiety Disorders/
#24	("models of anxiety" or "anxiety disorder*" or "anxiety" or "anxious" or "general anxiety" or "material separation" or anxiogenic).ti,kw,ab.
#25	#23 OR #24
#26	Stroke/
#27	("stroke" or "cerebrovascular disease" or "cerebrovascular disorders" or "cerebral infarct" or "ischemic stroke" or "intracranial hemorrhage" or "intracranial artery disease" or "middle cerebral artery occlusion" or "MCAO").ti,kw,ab.
#28	#26 OR #27
#29	exp Aging/
#30	(longevity or mortality or survivorship or "survival rate" or survival).ti,kw,ab.
#31	#29 OR #30
#32	#10 OR #13 OR #16 OR #19 OR #22 OR #25 OR #28 OR #31
#33	#3 AND #7 AND #32

STUDY RECORDS

Data management

As in the original systematic review, EndNote X7™ (Clairvate Analytics, Philadelphia, USA) will be used to generate references from the search results, and de-duplicated references will be uploaded to DistillerSR® (Evidence Partners, Ottawa, Canada). The pilot test will be redone for the first 100 records for title/abstract and for the first 25 records for full text to ensure consistency between new data reviewers (30).

Selection process

Two reviewers will independently select studies from the search as well as perform the risk of bias assessment. The following was taken from the previous protocol (30) and describes the two stages of the selection process:

Stage 1: Screening the title and abstract for eligibility criteria

i. Is the title and/or abstract available in English?
ii. Does the title and/or abstract describe a primary *in vivo* research trial?
iii. Does the title and/or abstract use laboratory mice or rats for their study?
iv. Does the title and/or abstract use resources as an intervention?
v. Does the title and/or abstract report the use of one of the disease models of interest and/or study survival/mortality?

Reviewers will be asked to answer either YES, NO or UNCLEAR to the above questions, and any study that receives an answer of NO will be excluded.

Stage 2: Full-text screening

i. Is the full text available?
ii. Is the study available in English and over 500 words?
iii. Is it a primary *in vivo* research trial?
iv. Do the researchers use laboratory mice or rats in the study?
v. Does the study use appropriate resources (as described in the exclusion criteria) as an intervention?
vi. Does the study use appropriate standard housing (as described in the exclusion criteria) as a comparator?
vii. Does the study include one of the disease models of interest (i.e. cardiovascular disease, major depression, cancer, viral infection, asthma, anxiety disorders, stroke) and/or is it a mortality study)?
viii. Does the study measure any of the outcomes listed in **Table 1** or report survival/mortality data?

All questions will be answered with either YES or NO. If both reviewers answer NO to any of the questions the study will be excluded, and consensus must be reached if there are conflicting opinions. If no consensus can be reached, a third party on the review team will be consulted.

Data extraction process
Two independent reviewers will extract data from selected studies using a form created in DistillerSR®, which will be pilot tested with 10 references by all reviewers.

Contacting studies
For all eligible studies published in 2007 or later, we will contact the authors via email to obtain additional information on both well-resourced and conventional cage conditions. We will contact corresponding authors once initially, with a follow-up four weeks later if no response is received. We will wait an additional two weeks for a response, before moving on with data analysis (assuming no response will be given). We will also request additional author contact information (e.g. for the first author) if the corresponding author is unable to assist us. Authors will be asked the following list of questions in a survey format:

<u>For "enriched" cages:</u>

1. Do you have any photos (or videos or Powerpoint slides) of your standard (control) cages? If yes, would you be willing to share them with us? Just attach image(s) to this email. *If no photos are available or you are unable to share them, please answer questions 3-4.*

2. Do you have any photos (or videos or Powerpoint slides) of your enriched cages? If yes, again would you be willing to share them with us? Just attach image(s) to this email. *If no photos are available or you are unable to share them, please answer questions 5-7.*

If yes, you have completed the survey.

Standard (control) cages

3) Which items were provided in standard/control cages (check all that apply)?
    a. Bedding (over the whole cage floor [e.g. wood shavings])
    b. Nesting material for sleeping within (e.g. shredded paper)
    c. Shelters or nest-boxes
    d. Other (please specify):
4) Were these cages individually ventilated or conventional/open top?

Enriched cages

5) Were enriched cages individually ventilated or open top?
6) If enrichments were "rotated", were they taken out and exchanged for new objects or just relocated within the cage?
7) Which items were provided in the enriched cage (check all that apply)?
    a. Nesting materials (e.g. shredded paper for sleeping within)
    b. Shelters or nest-boxes
    c. Climbing opportunities (e.g. on the cage bars, on other enrichments, ladders or multi-tiered)
    d. Chewing/gnawing opportunities (e.g. they were provided wood materials)
    e. Other (please specify):

8) Did you record body weight data from your standard (control) and enriched rodents not available in your manuscript? If yes, would you be willing to share them with us? Just attach data to this email along with the age in which the animals were weighed.

<u>For wheel-running studies:</u>

General

1. Do you have any photos (or videos or Powerpoint slides) of your standard (control) cages? If yes, would you be willing to share them with us? Just attach image(s) to this email. *If no photos are available or you are unable to share them, please answer questions 3-4.*

2. Do you have any photos (or videos or Powerpoint slides) of your wheel-running cages? If yes, again would you be willing to share them with us? Just attach image(s) to this email. *If no photos are available or you are unable to share them, please answer questions 5-7.*

If yes, you have completed the survey.

Standard (control) cages

1. Which items were provided in standard/control cages (check all that apply)?
    a. Nesting material for sleeping within (e.g. shredded paper)
    b. Shelters or nest-boxes
    c. Bedding (over the whole cage floor [e.g. wood shavings])
    d. A locked wheel

e. Other (please specify):

2. Were cages individually ventilated or open top?

Wheel-running cages

3. Where wheel-running cages identical to standard cages (other than containing a wheel, e.g. nesting materials and shelters or nest boxes were kept consistent)?
4. Were wheels exchanged for new wheels during the experiment (e.g., at cage change)?
5. Did wheels change position within the cage?

6. Did you record body weight data from your standard (control) and wheel-running rodents not available in your manuscript? If yes, would you be willing to share them with us? Just attach data to this email along with the age in which the animals were weighed.

Data items

In addition to the data extracted from selected papers previously, body weight data will also be extracted from studies when available.

All data extracted are listed below:

i. *Study ID:* authors, title, year and journal
ii. *Study design characteristics:* sample size for control and treatment groups
iii. *Animal model characteristics:* species, strain, genotype (if applicable) sex, age, body weight
iv. *Intervention and comparator characteristics:* cage size, whether open top or individually ventilated (IVC), length of time in caging before disease induction (if applicable), age of introduction, objects included in cage, exercise opportunities, number of animals per cage, nesting materials, frequency of object rotation and/or cage cleaning rates.
v. *Disease model characteristics:* name of model and model description, time from disease induction to outcome measure.
vi. *Outcome measures:* described above (**Table 1**).

OUTCOMES AND PRIORITIZATION

Outcome data to be extracted

Raw data will be extracted for all disease-specific outcomes. Adjusted data will only be extracted if they are the only form of data available. See **Table 1** for outcome-specific data.

Raw data will also be extracted for all-cause mortality. These will include median lifespan and hazard ratios (if these are not reported, data will be extracted from Kaplan-Meier curves using Web Plot Digitizer (https://automeris.io/WebPlotDigitizer/).

Continuous measures

For continuous outcome measures, mean, standard deviation (or standard error) and sample size will be extracted to calculate and report standardized mean difference in the meta-analysis. For studies which do not report specific sample size (but give a range of sample sizes) the smallest possible sample size will be used to give the most conservative estimate of the 95% confidence interval. For studies that do not report the mean and standard deviation in the text (main text or supplemental), we will extract data from graphical information using Web Plot Digitizer (https://automeris.io/WebPlotDigitizer/). Studies that do not report how error bars were generated (standard deviation or standard error) will be excluded from the meta-analysis. Body weight data will be extracted as a continuous measure and converted to a standardized mean difference to compare between studies.

Extracting outcomes from studies with multiple experimental groups
Since biochemical studies may have multiple experimental groups, we are specifying which groups will be used to extract data *a priori*. The following guidelines will be followed (taken from the previous protocol (30).

a) Exclude any rescue experiments (e.g. loss of function or gain of function).
b) Extract data on all genotypes or strains (other than those mentioned above).
c) Extract data on both sexes if sex is reported separately and include both in the meta-analysis separately.
d) When multiple time points are reported, extract data from the last time point prior to full disease recovery (this includes body weight data).
e) Exclude control groups in which the intended disease model fails to generate disease in control animals.
f) Exclude data on drug treated groups or other intervention groups.

Risk of bias in individual studies
Risk of bias was previously addressed using the Systematic Review Protocols for Animal Intervention Studies (SYRCLE)'s risk of bias tool. The RoB assessment will be updated to include the relevant studies found in our updated search. This tool and checklist can be found here: https://bmcmedresmethodol.biomedcentral.com/articles/10.1186/1471-2288-14-43#Tab3

DATA

Data synthesis
We will use R studio to conduct random effects meta-analyses, as was performed previously (29). A meta-analysis will be conducted for each disease individually and all-cause mortality data separately, and all diseases pooled (for meta-regressions, see below). For each disease, a meta-analysis will only be performed if enough studies report similar outcome data (more than two studies). Heterogeneity will be assessed using I^2 and a value >50% will be considered substantial heterogeneity (30, 34).

Subgroup analysis
Subgroup analyses will be conducted for each disease measure (column two, **Table 1**).

Meta-regressions
All other potential sources of heterogeneity (including species [rat vs. mouse], sex, and resource type) will be assessed using meta-regressions. We will also convert body weight to standardized mean differences and use meta-regressions to assess whether data heterogeneity changes with differences in weight between differentially housed rodents. Cancer and viral infection studies will be excluded from this analysis due to cancer-induced and infection-induced weight loss confounds.

To assess whether specific resources provided correlate with effect size and contribute to data heterogeneity, we will use email responses from researchers (see above). As in the original systematic review, we will then classify resources into subgroups (blind to study authors and results) based on the opportunities they provide:

i. space
ii. foraging opportunities
iii. shelter/hiding opportunities
iv. opportunities for physical exercise (e.g. wheel)
v. novelty/exploration
vi. chewing/gnawing opportunities
vii. climbing/3-D movement opportunities
viii. extra nesting material/sleeping places

We will also assess resources for "red flags" that could potentially reduce how effective resources are at reducing stress. These are:

a) Resources that are supplied for less time than the disease can develop/remit in.
b) Novelty that is rotated very often (e.g. daily) that may induce a neophobic response.
c) Animals that are old (> middle aged) and are likely to be anhedonic or timid when faced with change.
d) Animals that are paired or group housed male mice.

Meta-bias
We will assess the potential for publication bias using funnel plots if ≥10 studies are available for any individual meta-analysis (35).

Confidence in cumulative evidence
We will assess evidence strength in all studies using the Grading of Recommendations Assessment, Development, and Evaluation (GRADE) (36) guidelines.

DISCUSSION
This systematic review will expand on our previous systematic review and address two questions: 1) what resources confer the most benefit to experimental mice and rats? And 2) Can body weight (as a proxy for abnormal behavior) indicate the stress-reducing nature of these resources? The results of this review will allow us to determine which resources may be most relevant for promoting laboratory rodent welfare, thus allowing researchers to refine rodent housing in ways most likely to reduce stress.

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