Technical Report

MOLECULAR METABOLISM

The long-lasting shadow of litter size in rodents: litter size is an underreported variable that strongly determines adult physiology

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ABSTRACT

Background/Purpose: Litter size is a biological variable that strongly influences adult physiology in rodents. Despite evidence from previous decades and recent studies highlighting its major impact on metabolism, information about litter size is currently underreported in the scientific literature. Here, we urge that this important biological variable should be explicitly stated in research articles.

Results/Conclusion: Below, we briefly describe the scientific evidence supporting the impact of litter size on adult physiology and outline a series of recommendations and guidelines to be implemented by investigators, funding agencies, editors in scientific journals, and animal suppliers to fill this important gap.

Keywords Litter size reduction; Neonatal growth; Childhood obesity; Experimental models of physiology

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"Studying metabolism in mice is easy!
All you must consider is age,
sex,
strain,
breeding strategy,
body composition,
diet,
bedding,
housing temperature,
genotypes mixed or separated,
microbiome,
institution,
time of day,
fasted/fed,
mouse handling,

and now...

[inhales deeply]

litter size."

Tweet posted on August 18th, 2020 (@skinnyfatPhDKevin Qian) by Kevin Qian, MD/PhD Student, Yale University.

However comical the previous Tweet might sound; it acknowledges that litter size is a biological variable that strongly influences adult physiology in rodents [1—3]. Despite evidence from previous decades [4] and recent studies highlighting its major impact on metabolism, information about litter size is currently underreported in the scientific literature [2]. Here, we urge that this important biological variable should be explicitly stated in research articles. Below, we briefly describe the scientific evidence supporting the impact of litter size on adult physiology and outline a series of recommendations and guidelines to be implemented by investigators, funding agencies, editors in scientific journals, and animal suppliers to fill this important gap.

1. INTRODUCTION

Rodents are widely used in the biomedical sciences as models to help understand human disease and other fundamental physiological processes. Researchers have long recognized the existence of biological variables that may bias or mask important phenotypic outcomes. This is particularly critical in the broad areas of physiology and related metabolic diseases, including obesity, type 2 diabetes, and their associated comorbidities. Given this awareness, researchers, in close collaboration with certified animal suppliers and the editorial board of scientific journals, have undertaken a major effort to systematically report these variables (Box 1). Indeed, several journals are currently implementing their publication policies to include mandatory *"Rigor and Reproducibility Checklists"*, where this and other relevant information must be reported prior to publication. Likewise, several initiatives are already making the case for standardized reporting guidelines for animal research [5]. Clearly, including exhaustive information about animal handling and housing and a full description of the experimental design will contribute to an improved understanding of physiological outcomes. Furthermore, these checklists will be instrumental for ensuring research transparency, reproducibility, and interpretation.

It is now recognised that exposures during the suckling period have a long-term impact on health. This is embodied within the

Box 1
List of common variables that influence adult physiology in rodents

- Species (*Mus musculus*/*Rattus norvegicus*)
- Strain
- Type of diet
- Age
- Sex
- Time of the day (circadian rhythm)
- Fasting vs. feeding conditions
- Animal facility temperature/humidity/enriched environment
- Stress (noise, animal handling, cage changes, etc.)
- Housing/number of animals per cage
- Microbiota
- Parental history
- Season
- Breeding strategy (natural, IVF, superovulation)
- **Litter size**

Developmental Origins of Health and Disease hypothesis, a concept that suggests that suboptimal exposures during critical periods of development have a long-term effect om health. Litter size manipulation in rodents is one way of altering the nutritional plane during this period, with large litters competing more for the mothers' milk and therefore growing more slowly. Litter size during the suckling period is therefore now recognized as an important factor that strongly influences adult physiology in mice and rats, eventually affecting longevity [6,7]. As litter size impacts on long term physiology, it can therefore influence on metabolism when the animal reaches reproductive age. Therefore, the effects of litter size may be extended well into the next generation(s) [8], through intergenerational/transgenerational effects of developmental programming [9]. These effects are thought to be mediated through epigenetic mechanisms [10,11]. This issue further strengthens the necessity to control (or at least report) for litter size because it is a confounding factor that can bias the physiology of rats/mice not only on the parental cohort, but also in the next generation offspring. Although, we will specify recommendations later in this article, we advise standardizing litter size at birth when exploring parental effects. This routine can be implemented when colonies are bred in-house [12].

All in all, here we make the case that, in rodent studies, information about birth or preweaning litter size (Box 2) should be implemented in the Editorial Checklists. The effects of suckling-period litter size on adult physiology have been recently reviewed elsewhere [1,3,13,14]. Below, we will summarize the key findings that provide scientific justification for the need to report litter size in studies of adult health and metabolism.

2. THE LONG-LASTING SHADOW OF LITTER SIZE

Litter size reduction during lactation (2–4 pups/female lactating dam) was originally applied as a method to experimentally induce accelerated neonatal growth and early overweight/obesity in rodents [15]. This procedure is now widely used as an experimental model for the study of rapid weight gain in early infancy. Thereafter, rodents suckled in small litters often develop adult obesity and obesity-related metabolic diseases, including insulin resistance, glucose intolerance and diabetes, cardiovascular disease, altered sexual maturation or kidney failure (reviewed in [1,3,14]). In contrast, when rodents are experimentally assigned to large litters during the suckling period (>12 pups/female lactating dam), they experience reduced food intake, reduced neonatal growth, lower adiposity, and improved insulin sensitivity [16–18].

Box 2
Birth and weaning litter size: Similar, but not the same

When described, litter size is usually reported either at birth or at weaning. These two periods will be referred as birth and weaning litter size, respectively. Both birth and weaning litter size have similar impacts on adult physiology. Therefore, for practical purposes, both of them are similarly valid for reporting litter size.

However, the information they convey is slightly different.

- **Birth litter size** reflects gestational litter size and may provide some clue about maternal health and intrauterine progression.
- **Weaning litter size** provides an integrative view of the number of pups that were born *and* survived throughout lactation. Therefore, we favour reporting weaning litter size in strains that exhibit a high degree of cannibalization during lactation. The added value of weaning litter size can be exemplified if for example a female delivers 8 pups but half of them do die during the first week. At the end of lactation, she has suckled four pups only. Consequently, her offspring will behave similarly to a small litter.

Therefore, taking together the small and large litter paradigms, several physiological responses appear to follow a dose–response relationship with suckling-period litter size variation [19]. Specifically, litter size shows a strong negative correlation with several adult physiologic outcomes, including body weight, adiposity, insulinemia and glycaemia (Figure 1). The relationship between litter size and physiologic outcomes is complex and does not necessarily follow simple linear correlation. Unfortunately, the literature is extremely scarce, and careful and exhaustive determination on the association between continuous litter sizes and late-onset physiology is barely reported. To the best of our knowledge, the study by Zhang et al. [19] is one of the very few that carefully dissected the impact of continuous litter sizes (3–10 pups per litter) and body weight at weaning. Interestingly, the negative association between litter size and weaning body weight fitted fairly well into a simple linear correlation. Unfortunately, it is not reported whether these associations persisted throughout life or showed similar correlation with other biological variables. Therefore, additional studies

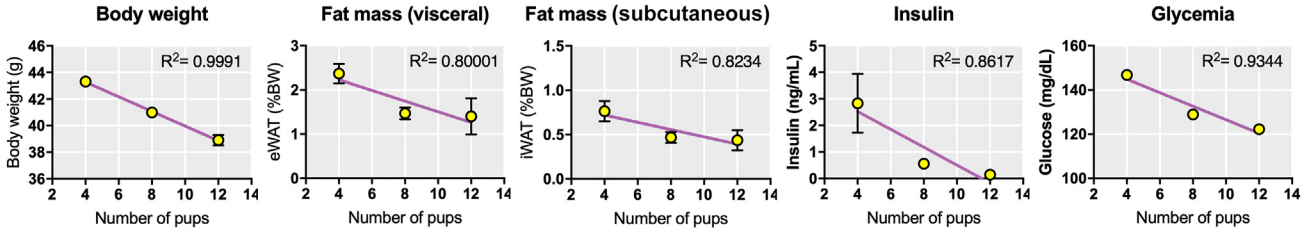

Figure 1: Correlation coefficients between birth litter size and physiological outcomes in 6-month-old ICR-CD1 male mice Supplementary Table 1 Box 3. Litter sizes were adjusted to 4, 8 or 12 pups/dam 24 h *postpartum*. In all cohorts, the female to male ratio was kept at 1:1 when possible. Adult physiological outcomes were recorded in adult mice fed a standard chow, between 8 and 10AM, after a fasting period of 12 h. Visceral and subcutaneous adipose tissues were dissected from the epididymal and inguinal regions, respectively. As discussed in the text, the relationship between litter size and the reported physiological variables is complex and does not necessarily follow a simple linear correlation. Data included in these graphs include unpublished results as well as data partially published in two independent articles [18,44]. Data represent the mean values ± Standard error mean. N ≥ 30 mice in all panels.

including well-controlled large data-sets are warranted to elucidate the actual relationships between these variables [20], which will allow accurate prediction models of disease.

Importantly, this negative association has been reported by many independent laboratories, despite differences in the strains and species utilized (*Mus musculus*, *Rattus norvegicus*), sex, type of diet, and even the actual number of pups included in each group during lactation [2]. For example, offspring of small litters (SL) may include 2–4 pups throughout lactation, medium-sized litters (ML) include 8–12 pups, and large litters (LL) typically contain more than 12 neonates.

Why is suckling litter size critical for adult physiology? An exhaustive review of the potential mechanisms linking litter size and adult physiology is beyond the scope of this *Statement*. Some hints are briefly summarized below. It is suggested that litter size strongly determines food intake during lactation: pups reared in small litters have a higher access to and consumption of maternal milk, which in addition can be higher in triglycerides [18,21,22]. This effect occurs because over a wide range of litter sizes, females do not alter their milk production, which largely depends on maternal heat dissipation capacity [23–25]. In turn, higher food intake in lactation promotes rapid growth (*i.e.,* weight gain), which is primarily accomplished through early fat mass accrual. This early adiposity ignites the signals that lead to the whole spectrum of metabolic disorders, including type 2 diabetes, cardiovascular disease, and adult obesity. These signals may act through *(a)* disrupting the hypothalamic energy balance circuitry, thereby perpetuating hyperphagia [13,26–29], or *(b)* inducing insulin resistance in peripheral tissues [17,18,30–33]. The opposite has been described in rodents raised in large litters: Food intake during lactation is limited, growth rate is reduced, and early fat mass accrual is blunted [19,34–36]. In this setting, large litters are associated with a lean, insulin-sensitive adult phenotype [37–40].

3. AUTHORS' POSITION

Despite extensive evidence showing the association between litter size and adult physiology in rodents, this parameter is rarely reported in the scientific literature unless explicitly manipulated [2]. All signing authors agree that there is now enough scientific evidence to support that litter size in rodents strongly influences adult metabolic health.

Under the previous premise, the signing authors of this article

- *HIGHLIGHT* that litter size has a major influence on adult physiology, and
- *URGE* that this information should be reported, if available, when publishing the data in top-ranking scientific journals, especially those devoted to the fields of obesity/type 2 diabetes and associated metabolic disorders.

In any event, we want to emphasize that lacking this specific information should not necessarily result in rejection of an article. Instead, reviewers should consider, on a single case basis, whether this parameter is central to the paper and if other pieces of data may overcome the lack of information about litter size.

The ideal long-term goal is that future research conducted in rodent models of obesity and metabolic diseases should include information about litter size as part of the published methodology (Box 3). We recognize that this task can be challenging, and requires the concerted contribution of several contributors, including (1) Researchers, (2) the Editorial Board of scientific journals, (3) Funding Bodies and (4) Animal Suppliers. We appreciate that the need to report litter size imposes a great challenge, mainly to researchers and animal suppliers. We believe that researchers will rapidly adapt if reporting litter size is required upon publication and/or funding agencies request these procedures. This effort will be sterile if animal suppliers do not make a major effort to implement systems for tracking their mouse colonies. Ideally, researchers should be able to order small and large litter-derived individuals or, more frequently, animals from litters of near-median size (which, by definition would be the norm in the facility). Although this implementation might be initially envisioned as over cost, in reality, better control of litter size will significantly reduce variability between experiments and the overall cost of the whole experimental setting. We strongly believe that there are currently affordable tracking means for monitoring litter size even in large (academic/private) facilities.

We recognize that there is still a long way to go to make this routine, with many hurdles and barriers to overcome. However, it is worth noting that previous similar efforts have been successfully implemented. For example, it is now beyond question that studies involving animals should report information about both sexes, age, type of diet (and diet composition), or, more recently, the time of the day when the experimental procedure has been conducted. In line, we expect that this statement article will be a ground-breaking step toward including information about litter size (or equivalent variables) in the near future. In the last two sections we will provide guidelines that will require the active contribution from all previous stakeholders to achieve the abovementioned goal. Their potential specific roles and associated activities will be also detailed.

Box 3
Editorial Checklist; Animal experiments

Animal experiments- in Methods Section	Yes	No	NA
Ethical Committee approval stated?			
Statement about species and strain/ substrain?			
Statement about sex?			
Age or developmental age status stated?			
Body weight stated?			
Diet information stated?			
Genetic modification status stated?			
- Crossing strategy stated (Het X Het, WT X Het, etc.)?			
Statement about litter size?			
• *Litter size reported?*			
• *Litter size adjusted?*			
• *Fostering procedures included?*			

4. BEYOND LITTER SIZE: OTHER VARIABLES THAT ARE ASSOCIATED TO ADULT PHYSIOLOGY

The Developmental Origins of Health and Disease hypothesis (DOHaD) states that environmental challenges experienced during early life, including the intrauterine and neonatal periods, strongly impact on later biological outcomes [41]. Consequently, both fetal size or intrauterine growth are biological variables that, similar to litter size, strongly influence adult health and disease risk in humans and rodents [42,43]. However, controlling the number of embryos/fetuses or

measuring the intrauterine growth are technically difficult to perform routinely for most research laboratories. And their implementation in large facilities, including animal supplies, is likely unrealistic.

Still, there exist other biological developmental variables, similarly to litter size, that are also strongly associated to adult physiology in rodents, that can be potentially implemented in the laboratory. For example, early growth rate (during the suckling period) or body weight at weaning strongly predict later metabolic outcomes. Both variables are potentially more accurate that litter size for predicting later outcomes because they take into consideration the existing individual variation within the same litter. Importantly, these three variables (litter size, neonatal growth, and weaning body weight) are interrelated biological variables. There is evidence to support that birth litter size modulates early growth that, in turn, influences body weight at weaning. Given this inter-relationship, neonatal growth rate or body weight at weaning would be equally informative than litter size itself in predicting adult metabolic physiology in rodents. However, for practical purposes, here we make the point that reporting litter size is more advantageous than registering other variables and it should be favored for the following reasons:

- It is a great proxy for adult physiology in rodents
- It is easy to measure
- It is scalable to large colonies in big animal facilities, including those from animal suppliers
- It is reproducible
- It is easily comparable across laboratories worldwide.

5. RECOMMENDATIONS

Most mentions of litter size in the literature involve intentional manipulations of this parameter. We posit that the field must also consider the potential impacts of unintentional differences in litter size on metabolic phenotypes in animal models of type 2 diabetes, obesity, and obesity-related comorbidities. Additionally, genetic manipulations

Table 1 — Recommended actions to be taken based on whether litter or growth rate during lactation are known.

	Litter size is known	Litter size is NOT known	
		Is it possible to retrieve some information about litter size and/or neonatal growth rate?	
		YES	NO
Recommended actions	Report litter size (or growth rate) in: • the Material & Methods section AND • the mandatory checklist	**If rodents are bred in-house:** Report the average litter size for the same strain/experimental conditions than reported in the manuscript. **If rodents are not bred in-house:** Request to the animal suppliers the average litter size at birth/weaning for a given species/strain . This data will be based on accumulated historic information.	**No information is available from the animal supplier; it is not possible to reproduce/repeat the procedures in the experimenter's animal facility:** Report this reality as a (potential) limitation that should be carefully evaluated by the editors and the referees.
	Editorial Board Evaluation Editors, assisted with external referees will make appropriate decisions taking into account the available information and the additional scientific merits of the article. Please, note that lacking accurate data about litter size will not be the solely reason for eventual manuscript acceptance or rejection.		

(including transgenesis or gene silencing) can compromise fetal/neonatal viability, resulting in small litters at weaning. These transgenic/knock out (KO) mice might potentially develop a metabolic phenotype independent of the intrinsic genetic manipulation. For this reason, it is extremely discouraged breeding KO mice in homozygosis. If the KO groups are generated through crossing heterozygote mice, now wild type, heterozygotes and KO mice will develop within the same litter. Hence if they exhibit different phenotypes, they can be attributed to the genetic manipulation rather than to differences in litter size. Given the previous considerations, caution should be taken when studying genetic models of disease.

Potential data variation will be reduced if litter size is either known or adjusted between groups. This is a critical, non-trivial, aspect: Controlling litter size would result in reduced variability and therefore will have a positive impact on one of the 3 R's (Reduction), because the number of replicates can be dramatically cut down. Likewise, reduction will impact on the cost of the experimental procedures because, again, the number of rodents to be used for a specific procedure can be significantly reduced.

Below, we summarize several scenarios that researchers might encounter and offer possible actions and guidelines aimed at providing as much detailed information as possible for targeting journal editorial requirements.

5.1. Reporting litter size

The simplest situation to handle is when litter size is known (Table 1; green box). Typically, this occurs when rodents are bred in house and researchers record and/or adjust the number of pups in each litter. Hence, the data can be readily referred to in the *Methods* section and *Mandatory Checklists* provided by the journals (Box 3). Importantly, if differences in litter size between experimental groups are detected, then the final conclusions and metabolic implications should be carefully re-evaluated. For the sake of transparency, we argue that this is the type of information that external reviewers and editors should seek to fully evaluate metabolic phenotypes in mice and rats.

The problem arises when litter size is unknown (Table 1; orange box). Then, the question is whether some information (albeit *ad hoc*) might be retrieved. If the mouse/rat colony is bred and maintained in the investigator's animal facility, we strongly recommend implementing processes for recording litter size. We recognize that this might be tedious and add extra workload to the laboratory. Indeed, recording the number of pups at birth/weaning is performed in some animal facilities as a means for tracking their population. Hence, reporting litter size at weaning would be a good practice because it correlates with the development of obesity and its comorbidities [2]. In fact, weaning integrates information regarding the number of pups that *(a)* were born and *(b)* survived throughout lactation. Noticeably, in this scenario, the exact litter size will be unknown. However, if the average litter size is reproducible and variability is not exceedingly high, it would be considered a good proxy for the experimental litter size. This type of information should be clearly stated in the material and methods section.

The red flag might be raised when no information regarding litter size, either at birth or at weaning, is available (Table 1; red box). This might happen when using novel KO or transgenic models, a rodent obtained from the wild, or an uncommon mouse/rat strain for which no data have been previously reported. Researchers should report this situation in the checklists and methods section during the submission process. **We want to emphasize that, although lacking information about litter size might be an important limitation, this would not be sufficient justification for manuscript rejection.** Referees and editors must evaluate whether lacking litter size information is key for the final interpretation of the data, and there are other merits that outweigh this limitation.

To conclude, achieving the previous goal will require close cooperation between researchers and animal suppliers. Despite the potential economic cost, maintaining active records of litter size at birth and/or at weaning for on-going colonies will be extremely helpful for the scientific community. We consider that this information can be implemented in the future, especially for strains that are not widely

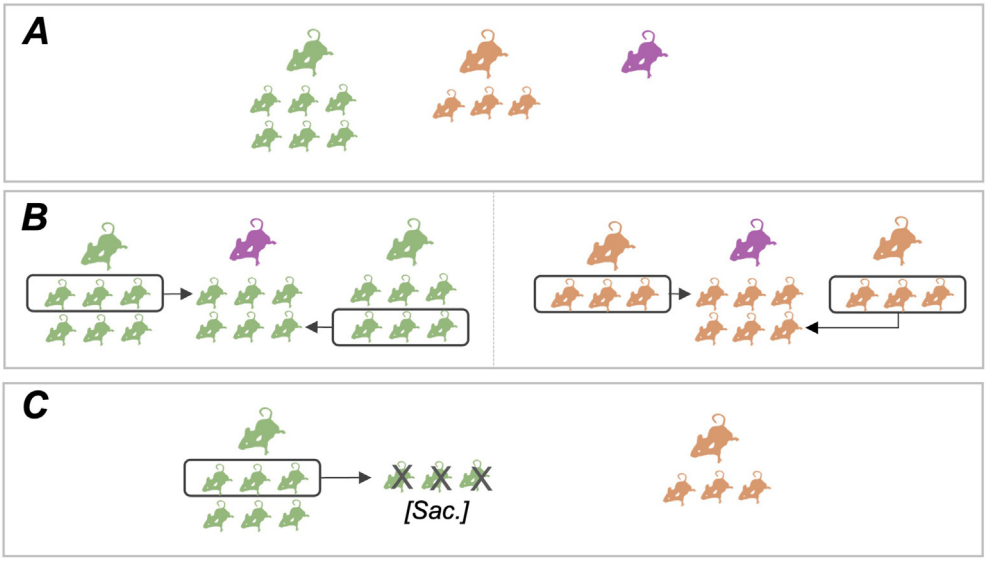

Figure 2: Litter size manipulation. ***A,*** Theoretical example. The control female (green) has 6 pups, whereas the "experimental" one (orange) has 3. The foster female is depicted in purple. ***B,*** fostering option #1. Three pups from two control/"experimental" cohorts were transferred to a foster female. The foster mother will now breed 6 control or 6 "experimental" pups, depending on their origin. ***C,*** litter size manipulation. In this example, the litter is adjusted to match the size of the group that delivers the lower number of babies. In this example, the size of the control group is culled to 3 pups.

used or show huge variations in litter size, which can introduce important phenotypic variation among offspring.

5.2. Adjusting litter size

In addition to **reporting litter size**, **adjusting litter size** between groups might be considered under specific circumstances. For example, when litter size is consistently and significantly different between experimental groups, or when average litter size is exceedingly variable in a specific strain of mice or rats. The goal here would be that all experimental groups will nurse the same number of pups during the suckling period.

Adjusting litter size after birth is by no means a straightforward action and requires specific responses to particular experimental settings. For example, let us imagine a situation in which the wild-type control female mice deliver an average of 6–9 pups per litter, whereas the females in the experimental group deliver only 3 (Figure 2A). As stated in the previous section, at the minimum, this situation should be reported in the mandatory checklists. In addition, it is also possible to manipulate the offspring to maintain the same number of pups/dam during the suckling period. We can accomplish this goal in at least two ways.

Regarding the first method, we could use wild-type foster females. For example, it would be feasible to transfer the offspring of two transgenic females to a foster female that will now care for the same number of pups throughout lactation as the controls (Figure 2B). Likewise, the same procedure should be applied to the offspring of control females: 3 pups from 2 independent cohorts should be transferred to an independent foster female. This will ensure that stress associated with the fostering itself will be similar in both experimental groups. With this manipulation, stepmothers will care for an average of six pups throughout lactation. Therefore, eventual metabolic differences between the two groups would be attributed to the specific experimental manipulation rather than differences in suckling litter size.

We would like to remark that while fostering procedures will help to maintain divergent groups in similar experimental conditions, it is by no means straightforward, and several difficulties should be considered. First, fostering generates a huge stress in both the stepmothers and the pups. Second, preparing the foster females is challenging, as they should deliver their own offspring at the same time as experimental groups. Third, preparing foster females may double the number of animals required for an experiment, which increases the economic cost and space in the animal facility. Hence, given these limitations, in practical terms, we suggest applying this methodology to situations when no other option is feasible.

An alternative to the fostering approach would be adjusting the cohorts to the average of the group producing the lower number of mice (Figure 2C). In the current example, the number of pups in the C group should be reduced to 3, while no further manipulations should be taken in the experimental group. It is arguable that, in this specific example, maintaining 3 neonates/female throughout lactation will induce some degree of overweight. However, this effect will be similar in all experimental groups, and if differences arise between them, they will be mostly attributable to the experimental manipulations rather than birth litter size itself.

To finish, all manipulations described in the previous examples, including litter size adjustments and/or fostering, should be performed around days 2–3 postpartum for at least two reasons. First, manipulating the offspring at this age would reduce the risk of maternal cannibalization. Second, maintaining the original number of neonates would ensure optimal stimulation of lactation performance.

6. CONCLUSION

- Litter size during the suckling period is a highly reproducible variable that strongly influence adult physiology in rodents.
- Currently, information about litter size is underreported in the scientific literature.
- Given its significant impact on adult physiology, the signing authors agree that efforts must be made to explicitly report this information to the scientific community.
- Reporting litter size might be extremely challenging, especially when animals are obtained from commercial sources. Nevertheless, we encourage all stakeholders—researchers, editorial boards in scientific journals, vendors—to strive to achieve this long-term goal.
- Finally, we want to emphasize that missing information regarding litter size would not be the sole reason for rejecting the publication of a scientific article. Referees and editors should carefully evaluate whether this piece of information is key for fully understanding metabolic phenotypes of a given experimental model or whether there are other merits that outweigh the lack of this specific information.

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CONFLICTS OF INTEREST

None declared.

DATA AVAILABILITY

No data was used for the research described in the article.

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