High fat feeding unmasks variable insulin responses in male C57BL/6 mouse substrains

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Abstract

Mouse models are widely used for elucidating mechanisms underlying type 2 diabetes. Genetic background profoundly affects metabolic phenotype; therefore, selecting the appropriate model is critical. Although variability in metabolic responses between mouse strains is now well recognized, it also occurs within C57BL/6 mice, of which several substrains exist. This within-strain variability is poorly understood and could emanate from genetic and/or environmental differences. To better define the within-strain variability, we performed the first comprehensive comparison of insulin secretion from C57BL/6 substrains 6J, 6JWehi, 6NJ, 6NHsd, 6NTac and 6NCrl. In vitro, glucose-stimulated insulin secretion correlated with Nnt mutation status, wherein responses were uniformly lower in islets from C57BL/6J vs C57BL/6N mice. In contrast, in vivo insulin responses after 18 weeks of low fat feeding showed no differences among any of the six substrains. When challenged with a high-fat diet for 18 weeks, C57BL/6J substrains responded with a similar increase in insulin release. However, variability was evident among C57BL/6N substrains. Strikingly, 6NJ mice showed no increase in insulin release after high fat feeding, contributing to the ensuing hyperglycemia. The variability in insulin responses among high-fat-fed C57BL/6N mice could not be explained by differences in insulin sensitivity, body weight, food intake or beta-cell area. Rather, as yet unidentified genetic and/or environmental factor(s) are likely contributors. Together, our findings emphasize that caution should be exercised in extrapolating data from in vitro studies to the in vivo situation and inform on selecting the appropriate C57BL/6 substrain for metabolic studies.

Introduction

Type 2 diabetes (T2D) is characterized by insulin secretory dysfunction and insulin resistance and is often associated with obesity (Kahn et al. 2006). Available treatments, although effective at managing the disease, have not been successful in preventing or reversing T2D. Mouse models are informative for elucidating the mechanisms underlying T2D and developing improved therapies; however, selecting the appropriate model for such studies
is critical. It is well accepted that background strain can have profound effects on mouse phenotype and the response to metabolic interventions (Leiter et al. 1981, Leiter 1989, Funkat et al. 2004, Andrikopoulos et al. 2005). What is less well understood is that variation can also occur even within mouse strains (reviewed in Fontaine & Davis 2016).

The diet-induced obese C57BL/6 mouse model is widely used as it recapitulates numerous aspects of the diabetic phenotype typically seen in obese humans (Surwit et al. 1988, Winzell & Ahren 2004). A number of C57BL/6 substrains now exist. The strain was generated from the C57BL mouse colony originally established by C C Little in 1921 and has been maintained at The Jackson Laboratory since 1948. These Jackson mice were designated C57BL/6J and, at generation F32, some were transferred to NIH. From 1951, NIH-bred mice via brother–sister mating, giving rise to a distinct strain designated as C57BL/6N. Thereafter, NIH distributed these mice to a number of vendors including Charles River (1974; C57BL/6NCrl), Harlan Laboratories (1988; C57BL/6NHzd) and Taconic (1991; C57BL/6N Tac). In 2005, The Jackson Laboratory acquired C57BL/6N mouse embryos that were originally cryopreserved at NIH in 1984 and used these to establish the C57BL/6NJ colony.

Today, C57BL/6 mice are available for purchase from all these commercial vendors, meaning that the different colonies are separated by multiple generations and considerable genetic variation may exist among substrains. No genetic differences were noted between C57BL/6NCrl, C57BL/6NHzd and C57BL/6NTac mice in an analysis of 1,449 single nucleotide polymorphisms (SNPs (Zurita et al. 2011)), though a more recent study of just 100 SNPs revealed some genetic heterogeneity among C57BL/6N substrains, including C57BL/6NJ mice (Mekada et al. 2015). Moreover, multiple genetic differences have been found between C57BL/6J and C57BL/6N substrains (Mekada et al. 2009, Pettitt et al. 2009, Zurita et al. 2011, Simon et al. 2013). One that has been well studied exists within the gene encoding nicotinamide nucleotide transhydrogenase (Nnt), a mitochondrial membrane proton pump that catalyzes the transfer of hydrogen between NAD+ and NADP+. Identified in 2005, the spontaneous in-frame deletion of exons 7–11 in the Nnt gene results in the absence of a functional NNT protein in C57BL/6J mice, leading to impaired mitochondrial function (Toye et al. 2005). Studies have shown that the Nnt mutation is associated with reduced insulin secretion and impaired glucose tolerance in C57BL/6J mice (Toye et al. 2005, Freeman et al. 2006a, b, Aston-Mourney et al. 2007, Fergusson et al. 2014). In contrast, C57BL/6N mice do not carry this mutation. Thus, genetic factors with functional consequences can greatly influence the metabolic phenotype observed among C57BL/6 mouse substrains.

However, genetic differences are not the only likely explanation for phenotypic differences. Adding to these is environmental variation. Seemingly insignificant differences in the micronutrient and macronutrient content, as well as the fat content of rodent diets, have been shown to produce markedly diverse metabolic responses in a single substrain (Omar et al. 2012). An important consideration is that breeding and husbandry practices likely differ among vendors. Further, there may be interactions between genetic and environmental factors that complicate comparison of data between C57BL/6 substrains and their applicability to human disease.

These potential differences make interpretation of glucose metabolism more complex, especially when comparing studies using mice from different vendors. We performed a comprehensive analysis of insulin secretory responses in six C57BL/6 substrains obtained from different vendors within the United States and Australia. Rather than establishing colonies of each substrain at our facility, mice were purchased and used directly in experiments, as this mirrors the paradigm used by most researchers studying glucose metabolism in mice. First, in vitro insulin secretion was compared in islets isolated from the six substrains. Then, in vivo assessments of insulin release in response to intravenous glucose were performed following low or high fat feeding to determine whether in vitro findings translate to responses in a whole-body setting where complex interactions among various hormones and tissues influence the metabolic phenotype. Our findings call for caution in extrapolating in vitro insulin secretion data to an in vivo setting and highlight the critical nature of selecting the appropriate substrain and controls for studies of insulin secretion and glucose metabolism.

### Materials and methods

#### Animals and diets

C57BL/6J (stock #000664; designated ‘6J’ hereafter) and C57BL/6N (stock #005304; 6NJ) mice were purchased from The Jackson Laboratory where they were maintained on diets containing 6.2% fat by weight (#5K52, #5K67;
Islet insulin content was measured in triplicate, as previously described (Zraika et al. 2002). Islet insulin content was measured after acid–ethanol extraction.

**Body weight and food intake measures**

Baseline body weight was measured in all mice prior to initiation of the 10% or 60% fat diets and weekly in mice followed for 18 weeks. Food intake was estimated on a per-cage basis, by weighing food weekly throughout the study; these data are expressed as kcal intake/gram body weight/day.

**Insulin and glucose tolerance tests**

Two days prior to conclusion of the 18-week feeding period, intraperitoneal insulin tolerance tests (1 IU/kg, ITTs) were performed in conscious mice fasted for 4 h (Kooptiwut et al. 2002, Hull et al. 2005). Tail vein blood was collected at 0, 15, 30, 45 and 60 min after insulin administration for glucose measurement. Two days later, intravenous glucose tolerance tests (1 g/kg; IVGTTs) were performed in pentobarbital (80 mg/kg) anesthetized mice fasted for 16 h. In line with our previous studies and to capture both first- (0–5 min) and second- (5–45 min) phase insulin responses, plasma was collected prior to and 2, 5, 10, 20, 30 and 45 min after glucose bolus for glucose and insulin measurements (Kooptiwut et al. 2002, Hull et al. 2005).

**Tissue collection and morphometric analysis of pancreatic beta-cell area**

After the IVGTT, mice were killed and body length (nose to anus), epididymal fat pad mass and inguinal fat pad mass were recorded. Pancreas was excised, fixed in 10% neutral-buffered formalin overnight, paraffin-embedded and sectioned at 4 µm thickness. Sections underwent immunohistochemistry (LeicaBond Max; Leica Microsystems, Buffalo Grove, IL, USA) as follows. Sections were deparaffinized and underwent peroxide block followed by incubation in 10% (v/v) normal goat serum for 20 min at room temperature. Sections were then incubated in anti-insulin primary antisera (A0564, Dako; 1:4000), followed by unconjugated rabbit anti-guinea pig IgGs (1:1000). Antibody binding was detected by goat anti-rabbit poly-HRP polymerized secondary detection and Leica Bond Mixed Refine 3,3′-diaminobenzidine 1-arginine, 0.1 mM isobutylmethylxanthine and 5 µM carbachol (cocktail mediated), as previously described (Zraika et al. 2002). Islet insulin content was measured after acid–ethanol extraction.

**In vitro insulin secretion and content**

Islets were isolated from 10-week-old male mice as previously described (Zraika et al. 2002). After overnight recovery, islets were pre-incubated for 90 min in Krebs–Ringer bicarbonate buffer (KRBB) containing 2.8 mM glucose. Thereafter, size-matched islets (n=5 in triplicate per strain) were incubated for 60 min in KRBB (300 µL per tube with the following additions), to determine insulin secretion in response to 2.8 mM glucose (basal), 20 mM glucose (stimulated) or 20 mM glucose containing 10 mM carbachol, 0.1 mM isobutylmethylxanthine and 5 µM carbachol (cocktail mediated), as previously described (Zraika et al. 2002). Islet insulin content was measured after acid–ethanol extraction.
detection reagents (both DS9800, Leica) followed by hematoxylin counterstaining and coverslipping. Whole pancreas sections were then digitized (Nanozoomer Digital Pathology system, Hamamatsu Corporation, Bridgewater, NJ, USA). For each section, total pancreas tissue and insulin-positive areas were determined automatically based on pixel value and density (Visiopharm Software, Hoersholm, Denmark) and verified by manual examination of segmented images, as we have done previously (Rountree et al. 2013). Beta-cell area was expressed as insulin-positive area/total tissue area (%).

Glucose and insulin assays

Plasma glucose was determined using the glucose oxidase method. For ITTs, blood glucose was measured using an AlphaTRAK2 glucometer (Abbott Laboratories). Insulin levels in plasma and in vitro secretion and content samples were determined using the Mouse Ultrasensitive Insulin ELISA (Alpco, Salem, NH, USA).

Data and statistical analyses

Data are presented as mean ± S.E.M. for the number of mice or experiments indicated. Insulin sensitivity was expressed as the inverse incremental area under the glucose curve from 0 to 60 min. Insulin responses during the IVGTT were computed as the ratio of incremental areas under the curve (iAUC) for insulin over glucose for 0–5 min (first phase) and 5–45 min (second phase). Time-course data (body weight, food intake, IVGTTs and ITTs) were analyzed via repeated-measures general linear model, whereas mean data were compared among study groups by ANOVA. Bonferroni correction for multiple comparisons was used for post hoc analyses of both statistical tests. A P < 0.05 was considered statistically significant.

Results

Glucose-stimulated insulin secretion in vitro is uniformly lower in islets from C57BL/6J vs C57BL/6N mice

We first assessed insulin release in islets isolated from all six C57BL/6 substrains at 10 weeks of age. Basal insulin release was similar among substrains, except for 6NTac islets that exhibited increased basal insulin release (Fig. 1A; P < 0.05 vs all substrains except 6NHsd where P = 0.06). Glucose-stimulated insulin secretion was identical between 6J and 6JWehi islets. Islets from all four C57BL/6N substrains (6NJ, 6NTac, 6NHsd and 6NCrl) also had indistinguishable insulin responses. However, when comparing C57BL/6J vs C57BL/6N substrains, islets from C57BL/6J substrains had uniformly lower glucose-stimulated insulin secretion. No differences in insulin secretion in response to high glucose+arginine, IBMX and carbachol (Fig. 1B) or in insulin content (Fig. 1C) were observed among any of the substrains.

Glucose-stimulated insulin secretion in vivo does not differ among C57BL/6 substrains after a low-fat diet

To determine whether our in vitro findings translated to an in vivo setting, we assessed glucose and insulin...
levels during an IVGTT in mice after 18 weeks of low fat feeding. Fasted plasma glucose levels were similar among all substrains after 18 weeks of low fat feeding (Table 1). Glucose levels during the IVGTT were comparable between 6J and 6JWehi mice (Fig. 2A), and among 6NJ, 6NTac, 6NHsd and 6NCrl mice (Fig. 2B). When comparing C57BL/6J and C57BL/6N substrains, only 6JWehi mice exhibited significantly lower glucose levels than 6NHsd mice.

Table 1  Body weight, fasted plasma glucose and insulin in C57BL/6 substrains.

<table>
<thead>
<tr>
<th>Baseline body weight (g)</th>
<th>18 weeks</th>
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<tbody>
<tr>
<td></td>
<td>Body weight (g)</td>
</tr>
<tr>
<td>6J LF</td>
<td>25.3 ± 0.4</td>
</tr>
<tr>
<td>6J LF</td>
<td>25.4 ± 0.41</td>
</tr>
<tr>
<td>6JWehi HF</td>
<td>28.2 ± 0.81</td>
</tr>
<tr>
<td>6NJ LF</td>
<td>27.5 ± 1.01</td>
</tr>
<tr>
<td>6NHsd LF</td>
<td>25.0 ± 0.6*</td>
</tr>
<tr>
<td>6NTac HF</td>
<td>24.3 ± 0.7</td>
</tr>
<tr>
<td>6NJ LF</td>
<td>26.5 ± 0.51</td>
</tr>
<tr>
<td>6NJ LF</td>
<td>26.9 ± 0.31</td>
</tr>
<tr>
<td>6NJ LF</td>
<td>27.1 ± 0.81</td>
</tr>
<tr>
<td>6NTac LF</td>
<td>22.0 ± 0.61</td>
</tr>
<tr>
<td>6NJ LF</td>
<td>24.8 ± 0.61</td>
</tr>
<tr>
<td>6NCrl LF</td>
<td>25.2 ± 0.61</td>
</tr>
</tbody>
</table>

Data are mean ± s.e.m. N=7–12 per group, except for body length and fat pad mass where N=3–12.

P<0.05 vs same substrain fed a low-fat diet; †P<0.05 vs 6JWehi fed the same diet; ††P<0.05 vs 6NTac fed the same diet; †††P<0.05 vs 6NHsd fed the same diet.

Fasted plasma insulin levels did not differ among substrains after low fat feeding (Table 1). In keeping with our in vitro findings, insulin responses during the IVGTT were identical between low-fat-fed 6J and 6JWehi mice (Fig. 2C). In addition, IVGTT insulin responses among low-fat-fed 6NJ, 6NTac, 6NHsd and 6NCrl mice were similar (Fig. 2D). However, in contrast to our in vitro findings, when comparing C57BL/6J vs C57BL/6N substrains, IVGTT insulin responses were comparable among...
low-fat-fed mice from all six C57BL/6 substrains (Fig. 2C and D). Similarly, iAUC-insulin/glucose for 0–5 and 5–45 min were comparable among all substrains.

**In vivo insulin responses after a high-fat diet are similar among C57BL/6J substrains but not C57BL/6N substrains**

Given that IVGTT glucose and insulin responses were similar among low-fat-fed C57BL/6 substrains, we next sought to determine whether all substrains also responded similarly to metabolic stress (high fat feeding). Thus, we also performed an IVGTT in mice fed a 60% fat diet for 18 weeks. 6NJ mice developed significantly higher fasted plasma glucose levels compared to all other substrains, except for 6NTac ($P=0.07$) (Table 1). Glucose levels during the IVGTT were significantly higher in 6J vs 6JWehi mice (Fig. 3A). Among C57BL/6N substrains, 6NJ had higher glucose levels throughout the IVGTT than 6NTac, 6NHsd and 6NCrl (Fig. 3B). Additionally, 6NHsd mice had higher glucose levels than 6NCrl. When comparing C57BL/6J vs C57BL/6N substrains, 6NHsd mice exhibited an increased insulin response compared to both 6J and 6JWehi mice, whereas the response in 6NCrl mice was higher than that in 6J mice. In contrast, insulin responses in 6NJ and 6NTac mice did not differ from those in 6J or 6JWehi mice. iAUC analyses showed that high-fat-fed

![Figure 3](https://example.com/figure3)

**Figure 3** Plasma glucose (A and B) and insulin levels (C and D) during an IVGTT in C57BL6/J (A and C) and C57BL6/N (B and D) substrains fed a high-fat diet for 16 weeks. Substrains are denoted as follows: Panel A: 6J, triangles; 6JWehi, hexagons; and panel B: 6NJ, circles; 6NHsd, squares; 6NTac, inverted triangles and 6NCrl, diamonds. N=5–12 per group. *$P<0.05$ 6J vs 6JWehi; †$P<0.05$ 6NJ vs 6NHsd, 6NTac and 6NCrl; ‡$P<0.05$ 6NHsd vs 6NCrl; ‡$P<0.05$ 6NCrl vs 6NJ; 6NHsd vs 6NJ and 6NTac.

![Figure 4](https://example.com/figure4)

**Figure 4** First- (A) and second- (B) phase insulin responses during the IVGTT, computed as the ratio of incremental areas under the curve for insulin/glucose from 0 to 5 and 5 to 45 min, respectively, in C57BL6/J substrains following 18 weeks of low fat (open bars) or high fat feeding (closed bars). N=5–12 per group. *$P<0.05$ vs high-fat-fed 6NCrl.
6NCrl mice had increased first-phase secretion compared to all other substrains and increased second phase secretion relative to 6NJ mice (Fig. 4).

High fat feeding results in an increased insulin response in all C57BL/6 substrains except 6NJ

Within substrains fed a high- vs low-fat diet, only 6NJ mice developed fasting hyperglycemia, with no significant differences occurring for the other substrains (Table 1). High-fat-fed 6J, 6JWehi, 6NHsd and 6NCrl mice exhibited significantly elevated fasted insulin levels (Table 1). During the IVGTT, glucose levels were higher in 6J and 6NJ high-fat-fed mice compared to those in low-fat-fed mice of the same strain (Figs 2 and 3). Insulin responses increased with high fat feeding in all substrains (Figs 2 and 3), except 6NJ (Bonferroni post hoc P = 1.0). Analysis of iAUC data showed increased first-phase insulin release in 6NCrl high- vs low-fat-fed mice (Fig. 4).

High fat feeding in 6Hsd and 6NJ mice results in decreased insulin sensitivity

Insulin sensitivity, assessed by ITT, following 18 weeks on a low-fat diet was comparable among all six substrains (Fig. 5). Further, no differences in insulin sensitivity were observed among substrains after 18 weeks of high fat feeding. When comparing substrains fed a low- vs high-fat diet, 6NJ and 6NHsd were the only substrains that showed a significant decrease in insulin sensitivity (Fig. 5B and C).

Differences in body weight, body length, fat pad mass, food intake or beta-cell area do not explain the variable insulin secretory responses among high-fat-fed C57BL/6N substrains

At baseline (10 weeks of age), body weight was lower in 6NTac mice than all other substrains, whereas 6JWehi mice were heavier than 6NJ, 6NTac and 6NCrl mice (Table 1). Body weight during 18 weeks on a low-fat diet was similar between 6J and 6JWehi mice (Fig. 6A), whereas 6NHsd mice were heavier than 6NTac mice (Fig. 6B). Given the lower baseline body weight in 6NTac mice, percent weight gain was also computed (Fig. 6C). When comparing C57BL/6J and C57BL/6N substrains on a low-fat diet, weight gain was significantly greater in 6NHsd, 6NTac and 6NCrl than both 6J and 6JWehi mice (Fig. 5C; and P = 0.08 for 6NCrl vs 6J), and final body weight was higher in 6NHsd mice vs 6NJ, 6NTac, 6J and 6JWehi mice (Table 1). In contrast to the findings on a low-fat diet, 18 weeks of high fat feeding resulted in similar weight gain in all six substrains (Fig. 6A, B and C), with the exception of 6NTac mice, which exhibited greater weight gain compared to 6J, 6JWehi and 6NHsd high-fat-fed mice (Fig. 6C). No differences were observed in body weight at
the end of the study among any of the six substrains fed a high-fat diet (Table 1).

Body length did not differ between 6J and 6JWehi, 6NHsd, 6NTac or 6NCrl mice after 18 weeks on a low-fat diet, whereas 6NJ mice were shorter than 6NHsd and 6NCrl mice on a low-fat diet (Table 1). No differences were observed in body length among substrains after 18 weeks of high fat feeding. When comparing low- and high-fat-fed mice within substrains, only 6J and 6NJ high-fat-fed mice were longer (Table 1).

Epididymal fat pad mass was similar among substrains after 18 weeks on a low-fat diet, with the exception of 6NHsd mice, which had higher fat pad mass compared to low-fat-fed 6J and 6JWehi mice (Table 1). No differences were observed among substrains fed a high-fat diet, and no change in epididymal fat pad mass was observed within substrains fed a low- or high-fat diet (Table 1). In contrast, although inguinal fat pad mass did not differ among substrains fed either a low-fat diet for 18 weeks or a high-fat diet for the same period, a significant increase in inguinal fat pad mass was observed when comparing mice receiving low- vs high-fat diet for all substrains (Table 1).

Food intake did not differ among C57BL/6 substrains during 18 weeks on a low-fat diet (Fig. 7A and B), except that 6NHsd low-fat-fed mice had lower food intake than 6NTac low fat-fed mice (Fig. 7B). No differences were observed in food intake for substrains during 18 weeks of high fat feeding (Fig. 7C and D).

Finally, beta-cell area was similar among C57BL/6 substrains following 18 weeks of low fat feeding, and the same duration of high fat feeding (Fig. 8). When comparing mice that received low- vs high-fat diet within substrains, only 6JWehi mice exhibited a significant increase in beta-cell area on a high-fat diet (Fig. 8).

Discussion

In this study, we performed a comparison of insulin secretory responses, *in vitro* and *in vivo*, among two C57BL/6J substrains and four commonly used C57BL/6N substrains. *In vitro*, we found that islets from C57BL/6N substrains secrete significantly more insulin in response to glucose than islets from C57BL/6J substrains. Further, minimal variability was seen among either the four C57BL/6N substrains or the two C57BL/6J substrains. In contrast, *in vivo* insulin responses to glucose following 18 weeks of low fat feeding showed no differences among any of the six substrains. When mice were challenged with a high-fat diet for 18 weeks, insulin responses increased to a similar extent among C57BL/6J substrains. However, variability was evident among C57BL/6N substrains. In particular, 6NJ mice showed no increase in their insulin response after high fat feeding, resulting in the development of hyperglycemia. Conversely, 6NHsd and 6NCrl mice had a substantially higher insulin response than other substrains (C57BL/6J or C57BL/6N). Collectively, these data demonstrate that within-substrain variability can occur under certain conditions, in this case, among C57BL/6N mice upon adaptation to high fat diet.
feeding. Thus, our findings emphasize the importance of selection and use of appropriate control (sub)strains for in vitro and in vivo studies.

Genetic differences between the C57BL/6J and C57BL/6N substrains have been well documented, with numerous SNPs having been reported to date (Mekada et al. 2009, Pettitt et al. 2009, Zurita et al. 2011, Simon et al. 2013). Additionally, it is well known that C57BL/6J mice harbor a five-exon deletion in the Nnt gene, whereas C57BL/6N mice do not (Toye et al. 2005, Mekada et al. 2009). This Nnt deletion mutation has been shown to associate with reduced glucose-stimulated insulin secretion (Toye et al. 2005, Freeman et al. 2006b). Also, increased expression of full-length Nnt can enhance insulin secretion (Aston-Mourney et al. 2007, Wong et al. 2010). In our study, the presence of the Nnt mutation was an important determinant of glucose-stimulated insulin secretion in vitro. Specifically, islets from C57BL/6J substrains (harboring the Nnt mutation) secreted significantly less insulin in response to glucose than those from the C57BL/6N substrains (expressing wild type Nnt). Further, islets from all four C57BL/6N substrains secreted similar amounts of insulin in response to glucose. Notably, insulin secretion in response to a cocktail of secretagogues, which act via multiple pathways to elicit insulin exocytosis, was similar among all six substrains. The latter suggests the Nnt mutation affects only glucose-stimulated insulin secretion in vitro. In contrast to our in vitro findings, presence of the Nnt mutation did not result in lower glucose-stimulated insulin secretion in vivo, following either a low- or high-fat diet. These divergent findings using in vitro vs in vivo measurements likely occur due to the complex regulation of insulin release in vivo, of which Nnt mutation status is only one component. Although the latter is determined by the specific substrain, other factors such as interactions between various organs (e.g. pancreas, liver, muscle, fat, brain) and hormones (e.g. insulin, glucagon, incretins, catecholamines) also influence insulin release in vivo and are independent of substrain.

Notably, the literature is mixed with respect to metabolic phenotypes among C57BL/6J and C57BL/6N substrains on either a low- or high-fat diet. Some studies have found no differences between C57BL/6J and C57BL/6N substrains; for example, insulin release was shown to be similar in lean 6NTac mice vs either...
lean 6JWehi (Wong et al. 2010) or 6J mice (Alonso et al. 2012), in line with our findings. Also, high-fat-fed 6J mice were observed to have similar glucose tolerance to 6NTac mice (Harley et al. 2013). However, other studies have demonstrated significant differences. These include lower insulin release in low-fat-fed 6J vs 6NCrl mice (Fergusson et al. 2014) or 6NJ mice (Fisher-Wellman et al. 2016) and in high-fat-fed 6J vs 6NJ mice (Fisher-Wellman et al. 2016), poorer glucose tolerance in high-fat-fed 6J vs either 6NJ (Nicholson et al. 2010, Fisher-Wellman et al. 2016) or 6NTac (Simon et al. 2013) mice, and differential weight gain between high-fat-fed 6J and either 6NTac (Harley et al. 2013) or 6NJ (Nicholson et al. 2010) mice. Although these studies provide important information about metabolic responses to low or high fat feeding, it is difficult to generalize their conclusions. One reason is that the majority of studies comparing C57BL/6J vs C57BL/6N mice have included only one of each substrain, with little explanation as to why the particular substrains were chosen. A gap in our knowledge exists regarding variability in metabolic phenotypes within C57BL/6J or C57BL/6N substrains obtained from different vendors. A recent review by Fontaine and Davis discusses this issue and emphasizes the importance of considering substrain variability for development of diabetes mouse models (Fontaine & Davis 2016). Recommendations are made regarding the need to understand and document the background strain of genetically modified mice, as well as sourcing mice for an entire study from a single, approved vendor.

Our study design enabled a direct comparison of insulin secretory profiles within substrains from different vendors. Comparison within C57BL/6J substrains (6J vs 6JWehi) demonstrated that insulin release in vivo following low fat feeding was indistinguishable. Further, these mice had an identical increase in insulin release in response to high fat feeding and did not differ in any of the other parameters measured except glucose levels during the IVGTT. Although the latter cannot be explained by changes in insulin sensitivity or beta-cell area, other factors such as glucose-dependent glucose disposal and glucose metabolism by the liver may have contributed to elevated glycemia in high-fat-fed 6J vs 6JWehi mice. Among C57BL/6N substrains (6NJ, 6NHsd, 6NTac and 6NCrl), insulin release in vivo after low fat feeding was comparable. In contrast, significant variation was seen among the C57BL/6N substrains following high fat feeding, particularly with respect to insulin release. 6NHsd, 6NTac and 6NCrl mice demonstrated increased insulin release, with 6NHsd and 6NCrl mice exhibiting significantly greater insulin responses than 6NJ mice. Strikingly, 6NJ was the only substrain that did not exhibit an increase in insulin release following high fat feeding, and as a result, these mice displayed hyperglycemia. The latter is inconsistent with a recent study where high-fat-fed 6NJ mice displayed marked increases in insulin secretion during an i.p. glucose tolerance test (Fisher-Wellman et al. 2016). A potential explanation for the discrepant findings is that the high-fat diets differed in both fat and sucrose contents, which could influence metabolic responses.

6NHsd, 6NTac and 6NCrl substrains were shown to be genetically identical in an analysis of 1449 SNPs (Zurita et al. 2011). Of note, the 6NJ substrain was not included in this analysis. However, a more recent study of 100 6NJ-specific SNPs revealed that 89 of these were common to the 6NTac substrain, whereas only 78 or 76 were found in 6NCrl and 6NHsd mice, respectively (Mekada et al. 2015). Thus, genetic differences could underlie the lower insulin responses in 6NJ mice compared to 6NCrl and 6NHsd mice following high fat feeding. Similarly, differences in body weights at baseline and after high fat feeding may be genetically determined. However, other factors also likely play a role, including disparities in intrauterine, microbiotic and/or epigenetic conditions, as well as environmental nuances among housing locations. One example where phenotypic differences between C57BL/6J and C57BL/6N substrains have been attributed to environmental rather than genetic factors is a study demonstrating that 6J, 6NHsd, 6NTac and 6NCrl mice bred/reared for 10 weeks in a uniform environment no longer differed in airway responsiveness, vs when they were studied one week after receipt from vendors (Chang et al. 2012). In our in vivo studies, mice were maintained in a uniform environment for 18 weeks, thus factors such as caging, bedding, water, light/darkness cycle and air quality are unlikely to have contributed to the observed differences among high-fat-fed C57BL/6N mice. In addition, although vendor diet differed among the substrains, these discrepancies did not correlate with the differences in insulin secretory responses observed. For example, in 6J vs 6JWehi mice, where fat content in vendor diets differed the most (i.e. 6J: 6% vs 6JWehi: 9% w/w fat), insulin secretion was identical. Also, 6J and 6NJ mice were fed an identical diet at Jackson Labs; yet, their responses to a high-fat diet were divergent.

To better the understand underlying causes of the variable insulin responses among high-fat-fed C57BL/6N substrains, we performed ITTs to estimate insulin sensitivity...
as the latter is a major regulator of the insulin response to glucose in vivo (Bergman et al. 1981, Kahn et al. 1993). Among all high-fat-fed C57BL/6N mice, glucose responses during the ITT were comparable, even in 6NJ mice, which showed no increase in insulin release following high-fat feeding. Thus, the degree of insulin sensitivity does not predict which C57BL/6 substrains will exhibit high vs low insulin secretion in vivo. One important caveat is that the ITT is a relatively crude measure that does not differentiate between peripheral and hepatic insulin resistance and does not inform on glucose disposal under steady-state conditions. Future work could include hyperinsulinemic–euglycemic clamps to provide more detailed information in this regard.

Another parameter that may have contributed to variable insulin responses following high fat feeding is beta-cell mass. Surprisingly, few studies have compared this measurement among C57BL/6 substrains. Chow-fed 6J and 6NCrl mice had similar beta-cell mass (Fergusson et al. 2014), and chow-fed 6JWehi and 6NTac mice had similar pancreatic insulin content (Wong et al. 2010), in line with our data showing no differences in beta-cell area among substrains on a low-fat diet. To our knowledge, ours is the first reported comparison of beta-cell area among high-fat-fed C57BL/6 mouse substrains. We found beta-cell area to be increased only in high- vs low-fat-fed 6JWehi mice, and observed no differences among C57BL/6 substrains following high fat feeding. Even for 6NJ and 6NCrl mice that exhibited marked differences in insulin responses following a high-fat diet, beta-cell area was comparable. These data suggest that the variability in insulin responses among C57BL/6 substrains does not occur due to differences in beta-cell expansion.

A limitation of our study is that we only examined two C57BL/6J substrains. Thus, although the insulin responses in these mice were identical under all conditions tested, we cannot rule out the possibility that analysis of additional C57BL/6J substrains would have revealed variability similar to that seen among the C57BL/6N substrains. A further limitation is that this study only included male mice. Thus, it remains unknown whether variability occurs in insulin responses among female C57BL/6 mice.

Collectively, our data demonstrate that C57BL/6J and C57BL/6N substrains differ in their insulin response in vitro, but not in vivo after a low-fat diet. Further, variability exists in the in vivo insulin response to high fat feeding among C57BL/6N, but not C57BL/6J substrains. Based on these findings, caution should be exercised in extrapolating findings from in vitro studies to the in vivo situation and care should be taken in selecting the appropriate C57BL/6 substrate for studies in metabolic research.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
R L H and S Z conceived and designed the study, analyzed data and wrote the manuscript. J R W and B M B designed and performed experiments and analyzed data. M D S and G S B performed experiments and analyzed data. S A contributed mice, analyzed data and edited the manuscript. S Z is the guarantor of this work, and as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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