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Comparing Genotyping Accuracy Using Buccal Swabs versus Tail Biopsies by PCR in B6;C3-Tg(Prnp-SNCA*A53T)83Vle and B6;C3-Tg(Prnp-SNCA*A53T)83Vle *Snca*^{tm1Mjff} Mice

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Genotyping is a common and necessary procedure performed on genetically modified animals to distinguish carriers from noncarriers of the variants of interest. Established methods involve collection of tissues such as tips of tails or notches of ears. Noninvasive methods have been described but not widely adopted for reasons including inertia to change, needs to adjust PCR protocols, and the lack of validation; noninvasive genotyping methods are a refinement on animal welfare, but questions remain regarding how they compare with invasive methods in terms of genotyping accuracy rate and reproducibility. To gain answers to these questions, we compared the detection accuracy of the transgene and determination of zygosity in B6;C3-Tg(Prnp-SNCA*A53T)83Vle and B6;C3-Tg(Prnp-SNCA*A53T)83Vle *Snca*^{tm1Mjff} neonatal mice between tail biopsies and buccal swabs. Moreover, we weighed and observed mice following genotyping to see if any clinical differences can be discerned. Weight data did not support statistically significant differences in mice undergoing different genotyping procedures and control. No statistically significant difference was found between using buccal swabs or tail biopsies for genotyping with PCR or quantitative PCR. None of the pups swabbed was rejected by the dam. Our findings indicate that buccal swabbing is a more humane and feasible alternative to tail biopsies for high-throughput genotyping.

Abbreviation: qPCR, quantitative PCR

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Introduction

Hundreds of millions of animals, mostly mice and rats, are used for research in the United States each year.[3] Many of these are genetically modified, and most of them are mice.[15] Genotyping these animals becomes necessary to distinguish between animals that harbor the inserted, knocked out, or altered genes from noncarriers.[18] PCR is a high-throughput method most frequently used for genotyping.[22] PCR requires sampling the animals for DNA. For mice, routine practice is to collect tissue such as through tail or ear biopsy.[4,17] The tissues of the tail and ear are richly innervated, and thus questions on how much pain and distress the animals may experience during genotyping have been raised.[1]

If ear notches are used for identification, using the notched-off ear tissues for genotyping would serve both purposes. Tail snips involve cutting off the distal tips of the tails. One to 2 mm suffice for quantity of DNA for PCR. Even such a small amount indubitably causes pain as skin, connective tissue, bone, cartilage, nerves, and blood vessels are permanently severed.[9] It is also worth appreciating that any biopsy will elicit an inflammatory response that although may be subclinical, may nonetheless affect the animal systemically.[26] In addition, the procedure can also be distressing to the human operator as mice may vocalize and bleed from the cut.

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Noninvasive procedures have been described but not been widely adopted.[2,12,16,18] These include buccal swabs[4,13,14,25,27] and collection of hair,[5,21] saliva,[11] and fecal pellets.[8] In the present study, we elected to compare genotyping accuracy from buccal swabs exfoliating epithelial cells to our established practice of tail biopsies. Genotyping accuracy is defined as the detection rate of the transgene when present or agreement of zygosity determination. We hypothesized that the genotyping accuracy of the 2 methods would be equal. Buccal swabs were selected due to the availability of commercial kits designed for this purpose identified by our genotyping partner, allowing for elution of genetic material from the swabs optimized for PCR. To our knowledge, this is the first time that buccal swabs using commercially available, medical-grade, designed-for-purpose swabs had been shipped and used to genotype such a large number of neonatal mice successfully. The ability to obtain sufficient genetic material from buccal swabs has also been well documented across species. In theory, mice as young as neonates from birth can be genotyped by buccal swabs. Interestingly, it has been shown that significantly higher quantities of DNA were obtained from 14-d-old mice than from 28-d-old mice.[6] In practice, genotyping by buccal swabs is limited by how young mice can be reliably and permanently identified; felt tip markers are not permanent, ears need to be large enough to notch clearly, and toes also need to be wide enough to tattoo. At our institution, anesthesia is required for tail biopsies in mice at 21 d of age or older. Analgesia is not required. It is unknown how much unmitigated pain may affect the animal and therefore the study. A noninvasive method such as a buccal swab has the

advantage of obviating the need for anesthesia and analgesia. It would also be beneficial in cases where resampling is needed, as no piece of tissue is taken and lost from the animal.

Materials and Methods

Animals. All animals were cared for in compliance with the *Guide for the Care and Use of Laboratory Animals*.[10] All procedures were approved by the University of California, San Francisco's IACUC. The facility housing the animals is accredited by AAALAC International.

Mice used in the study were bred and maintained in our breeding colony. B6;C3-Tg(Prnp-SNCA*A53T)83Vle mice were housed in conventional open-top cages (N10 mouse cage, Ancare, Bellmore, NY) with paper bedding (Yesterday's News, Purina, St. Louis, MO), whereas B6;C3-Tg(Prnp-SNCA*A53T)83Vle *Snca*^{tm1Mjff} mice were housed in individually ventilated cages (NexGen Mouse 900, Allentown, Allentown, NJ). They were fed ad libitum pelleted rodent diet (Teklad 2018 Global 18% protein, Envigo, Indianapolis, IN). They had free access to filtered city and county of San Francisco municipal water. All animals were socially housed. There was no more than one litter at a time with the dam. Weaning was at 21 d of age. Weaned mice were housed in groups of 3, separated by sex. Prior to weaning, the mice had access to an igloo (Bio-Serv, Flemington, NJ) and paper nesting material (Bed'r Nest, Anderson's, Maumee, OH) per cage. Postweaning, the mice had access to a tunnel (Bio-Serv, Flemington, NJ) and paper nesting material, and B6;C3-Tg(Prnp-SNCA*A53T)83Vle mice also had a suspended swing (double mouse swing, Datesand Group, Bredbury, UK). The animal housing room operated under a 12-h light/12-h dark cycle with 10 to 15 air changes per hour. Humidity was maintained between 30% to 70% and temperature was kept between 68 and 79 °F. Open-top cages were changed every week. Individually ventilated cages were changed every 2 wk. The facility in which the mice were housed has known endemic pathogens including mouse hepatitis virus, minute virus of mice, mouse parvovirus, murine norovirus, pinworms, and skin mites.

Mice of the strain B6;C3-Tg(Prnp-SNCA*A53T)83Vle[7] (stock no. 004479, The Jackson Laboratory, Bar Harbor, ME) were used. These transgenic mice express the A53T mutation of human α-synuclein under the murine prion protein promoter and are commonly used to study synucleinopathies such as multiple systems atrophy.[24] We purchased homozygous males to intercross with purchased female B6C3F1/J mice (stock no. 100010, The Jackson Laboratory, Bar Harbor, ME) to produce hemizygous mice. We selected this strain because we have had many years of experience genotyping these mice with a well-established assay. The breeding scheme produces obligate hemizygous mice, allowing for ready comparison between genotyping methods. To demonstrate the ability to discern zygosity, we selected a related strain, B6;C3-Tg(Prnp-SNCA*A53T)83Vle *Snca*^{tm1Mjff}, that our laboratory also regularly breeds for use in experiments. In this way, no mice from either strain were purchased or produced exclusively for this study. B6;C3-Tg(Prnp-SNCA*A53T)83Vle *Snca*^{tm1Mjff} mice were initially developed by intercrossing B6;C3-Tg(Prnp-SNCA*A53T)83Vle with C57BL/6N-*Snca*^{tm1Mjff}/J mice (stock no. 016123, The Jackson Laboratory, Bar Harbor, ME). The *Snca*^{tm1Mjff} allele has been fixed to be homozygous. We bred mice hemizygous at the SNCA*A53T allele to each other, resulting in homozygous, hemizygous, or noncarrier mice.

Sample size calculation. We elected to conduct this study as a noninferiority trial to show that buccal swabs are as good as the standard of tail biopsies. The null hypothesis is that buccal swabs are inferior to tail biopsies for use to genotype mice. Selecting one-tailed α to be 0.025, β to be 0.2, noninferiority margin to be −0.1, genotyping success rate of tail biopsies to be 100%, and that of buccal swabs to be 98%, the total sample size was calculated to be 50 (sample size calculator: two parallel-sample proportions, https://www2.ccrb.cuhk.edu.hk/stat/proportion/tspp_sup.htm).

Groups 1 to 4. A total of 217 B6;C3-Tg(Prnp-SNCA*A53T)83Vle mice were divided into 4 groups. We did not select for sex. The mice were from 3 different dates of birth. Rather than attempting 200 timed births at once, we elected to divide 8 litters at a time to each of the 4 groups, until each group had at least 50 mice. This means that the procedures were performed in 3 different sessions. Depending on the group, genotyping procedures performed were either buccal swabs and tail biopsies, swabs only, tail biopsies only, or neither (Figure 1). All procedures were done at postnatal day 7 by the same 3 trained technicians. The entire study was conducted during a period of 3 mo. Aside from directly comparing the agreement of genotype calls from buccal swabs and tail biopsies collected from the same mice, we also wanted to assess whether any differences in weight can be detected depending on the procedures performed on the 4 groups of mice. Mice in all groups were weighed on the day of genotyping, then daily for a week. They were weighed weekly thereafter until 8 wk of age. All mice were identified by toe microtattoos at the same time as the genotyping procedures. For 3 d after, all mice were assessed by a veterinarian daily.

Group 5. An additional cohort of 68 B6;C3-Tg(Prnp-SNCA*A53T)83Vle *Snca*^{tm1Mjff} mice at postnatal day 7 from 10 litters was enrolled into the study at a later date following the conclusion of the collection of data from the procedures described above. Aiming to show the ability for buccal swabs to obtain sufficient DNA for quantitative PCR (qPCR) to compare with tail biopsies for zygosity determination, genotyping was performed by buccal swab and tail biopsy. Mice were identified by toe tattoos. For 3 d after, mice were assessed by a veterinarian daily. Weights were not obtained.

Genotyping. For buccal swabs, pups were restrained by hand at the dorsal scruff and swabs[19] (HydraFlock 6-in. 3318-H, Puritan, Guilford, ME) were inserted into oral cavity, rotated three times on its axis on one cheek. The tips of the swabs were cut into deep 96-well plates.

For tail biopsies, using sharp scissors, the distal 1 to 2 mm of tails were excised. The cut tails were placed into a 96-well plate. Scissors were sterilized by 70% isopropyl alcohol in between each use. The 96-well plates were sealed, then shipped via 2-d shipping to our collaborators, GenoTyping Center of America (Waterville, ME), for PCR.

DNA extraction from buccal swabs was performed by incubation in proprietary extraction buffer at 80 °C while shaking at 1,300 × *g* for a total of 15 min. DNA was used undiluted in PCR reactions. DNA extraction from tail biopsies was performed using a proprietary proteinase K digest at 37 °C overnight and diluted 1:75 for working PCR reactions. The qualitative melt curve Tg(Prnp-SNCA*A53T)83Vle allele assay was detected using internal control primer set M35 and transgene-specific primer

Group	No. of animals	No. of females	No. of males	Procedure at p7
1	55	29	26	swab, tail snip, toe tattoo, weight
2	54	26	28	swab, toe tattoo, weight
3	54	19	35	tail snip, toe tattoo, weight
4	54	23	31	toe tattoo, weight

Figure 1. Numbers of animals and sex distribution in each group. The last column lists the procedures done to mice in each group at postnatal day 7 (p7).

Mix	Forward primer	Forward sequence	Reverse primer	Reverse sequence	Probe	Probe sequence
M35	p10	GGCAAAGGTGGAAATGAAGA	p11	CTCAGACCACACAGGGAATG		
M1964	p2409b	AAAACCAAGGAGGGAGTGGT	p787	CAGTGGCTGCTGCAATG		
M1879	p2330 c	TCAAGTTCAGCCACGATAAAAC	p2330 d	TGATTTGTCAGCGCCTCTC		
M1880	p2330 c	TCAAGTTCAGCCACGATAAAAC	p2331	GCATGACATTTCCCAAGGAC		
M618	p786	TGACGGGTGTGACAGCAGTAG	p787	CAGTGGCTGCTGCAATG	p788	CCCTGCTCCCTCCACTGTCTTCTGG
M5	p13	GCCTTTGAGAGCACCAAGTC	p14	CATTCTGCTCTGAGATGGACA	p15	ACGTCATCCCCAAAGCAGGCTGA

Figure 2. Primer and probe sequences used for PCR.

set M1964 (Figure 2). The qPCR Tg(Prnp-SNCA*A53T)83Vle allele assay was detected using internal control primer set M5 and transgenic specific primer set M618. The quantitative melt curve *Snca*^{tm1Mjff} assay was detected using wild primer set M1879 and mutant primer set M1880. Melt curve PCR reactions were carried out in the presence of Forget-ME-Not™ EvaGreen® qPCR master mix (Biotium, Fremont, CA) products were determined by melt curve analysis on the LightCycler 480 II (Roche, Indianapolis, IN). qPCR reactions were carried out in the presence of Quanta ToughMix (Quantabio, Berverly, MA). Analysis of qPCR zygosity used the ΔCt method.

Statistical analysis. One-way ANOVA (Excel, Microsoft, Redmond, WA) with statistical significance defined as a P value <0.05 was performed on the accumulated weight dataset separated by sex and then group. It was also performed separated by sex, age, and then group.

McNemar tests were performed on the paired data resultant from buccal swabs and tail biopsies used to genotype the same mice on a 2 × 2 contingency table with statistical significance defined as a P value <0.025 to be consistent with noninferiority study design. (McNemar's χ^2, https://www2.ccrb.cuhk.edu.hk/stat/confidence%20interval/McNemar%20Test.htm#Formula). It was not calculated for the data from the *Snca*^{tm1Mjff} assay as there was no discordance between the 2 genotyping methods.

Results

For groups 1 to 4, only mice in group 1 had both genotyping procedures performed to allow for comparison. The other groups were to assess whether any clinical sequalae were to be observed or weight differences detected resulting from the procedures performed. Fifty-five mice were genotyped in group 1. Each mouse was genotyped twice, once with a swab sample and another with a tail sample. Based on the breeding scheme, all mice carried the transgene. All but one out of 55 swab samples were able to be amplified by PCR, an accuracy rate of 98.18%. All 55 tail samples were able to be amplified by PCR, an accuracy rate of 100%. The McNemar test resulted in a P value of 0.5, not showing a statistically significant difference between the 2 genotyping methods. The single swab sample that did not amplify was contaminated with blood, being visibly red-tinged. Blood is a known inhibitor of PCR.[22]

For group 5, all 68 tail samples were able to be amplified by qPCR. Consistent with the breeding scheme, all mice were determined to be homozygous for *Snca*^{tm1Mjff}. The expected Mendelian ratios for the inheritance of SNCA*A53T would have been 17 homozygous, 34 hemizygous, and 17 noncarriers. Tail samples determined there to be 15 homozygous, 36 hemizygous, and 17 noncarriers. While all swab samples were able to be amplified by qPCR, and all mice were determined to be homozygous for *Snca*^{tm1Mjff}, there was one instance where zygosity could not be confidently determined for SNCA*A53T. Thus, swab samples determined there to be 14 homozygous, 36 hemizygous, 17 noncarriers, and a no call. Comparing the results from the 2 genotyping methods, tail biopsies had an accuracy rate of 100% for the 136 qPCR assays run across both alleles, whereas buccal swabs yielded an accuracy rate of 99.26%. Aside from the one no call from the buccal swabs, the other 135 zygosity determinations agreed with those from tail biopsies. The McNemar test resulted in a P value of 0.5, not showing a statistically significant difference between the 2 genotyping methods.

All mice were noted to appear normal and healthy on all 4 d of veterinary observation, from day 0 when the procedures were performed until day 3 following. No cannibalism, mortality, or failure to thrive was seen. Other than the one mouse that bled during buccal swabbing, no adverse effects were noted immediately after the procedure. In that one mouse, no hemostasis was necessary, as the bleeding stopped on its own. Very minor bleeding was frequently noted following tail biopsy, on the surface where the biopsy took place or later inside the cage. No hemostasis was necessary, however, as it always stopped on their own.

A large amount of weight data was collected. Mice were weighed at 7 d of age, daily until 14 d of age, and then weekly until 8 wk of age (Figures 3 and 4). To our knowledge, this is the first time that growth curves were published for this transgenic strain. No statistically significant difference was found in the weights of females between the entire age range compared across groups 1 to 4. The P value was 0.31 using one-way ANOVA with the dataset of weights of all females in group 1, group 2, and so forth. The P value was 0.15 using one-way ANOVA for the male dataset, also showing no statistically significant difference.

Discussion

This study is a proof of concept that buccal swabbing is a viable, comparable, and noninvasive alternative to established genotyping methods such as tail biopsy. It can safely be done in pups as young as postnatal day 7 in a high-throughput manner. The quantity and quality of DNA collected can support qPCR for zygosity determination. No adverse events, morbidity, or mortality were noted. We have demonstrated that genetic material collected within a swab can survive the rigors of shipping without immediate processing or special transport media. Although our results technically show that buccal swabs have a lower genotyping accuracy rate than tail snips, a 1.82% difference for qualitative PCR, and 0.74% for qPCR, these were not found to be statistically significant. The arithmetic percentage difference would drop to 1.05% if the 3 PCR assays were considered together. One swab that failed was visibly contaminated with blood, a cited possibility in this procedure.[2,4] It is likely the bleeding stemmed from irritation to the buccal mucosa, which can be minimized by gentler swabbing. This was the only swab that was contaminated with blood. We did not submit for genotyping, but swabbing was also performed in animals of group 2. One out of 177 calculates to a 0.56% rate of bleeding from swabbing. It is likely that more skill in avoiding bleeding

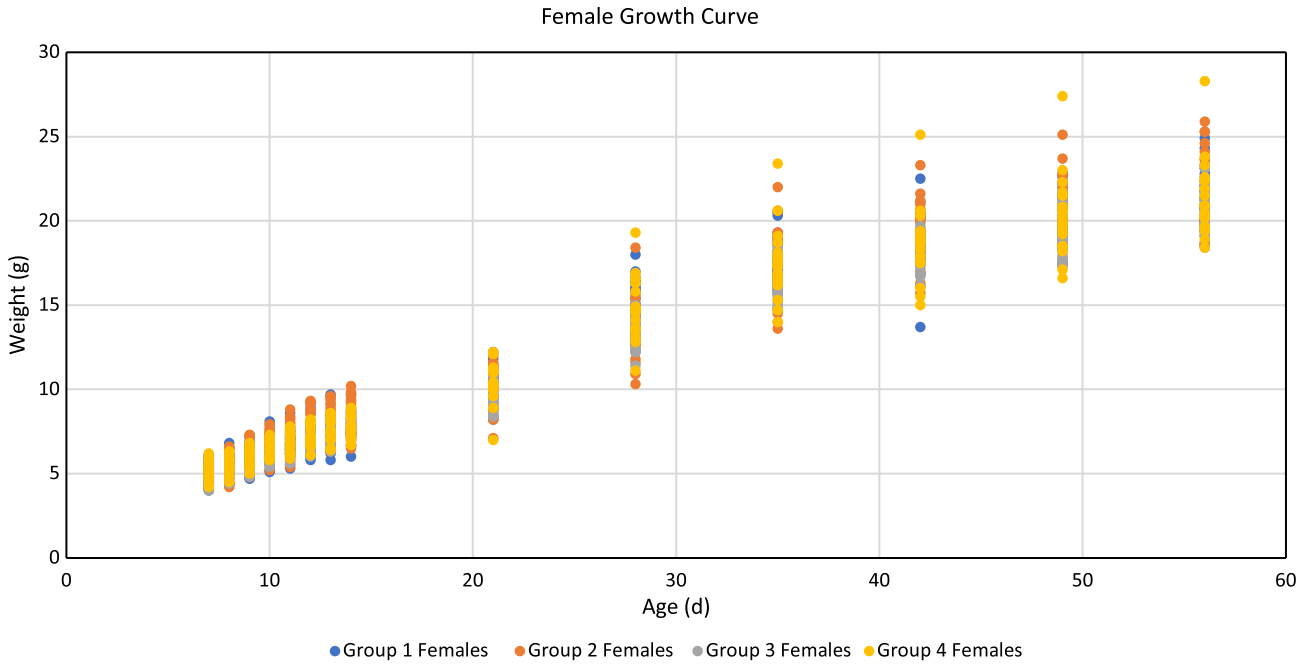

Figure 3. Scatterplot of all weights obtained from female mice of all 4 groups from 14 timepoints ($n = 97$). The groups are denoted by different colors, overlaid on each other. The general impression is that mice from all groups appear to gain weight and the weights remain similar to each other.

during the swabbing procedure would come with more practice. Swabs with visible blood should also not be used.

We aimed to investigate whether any clinical effects can be discerned following the different combinations of genotyping procedures performed in groups 1 to 4. Using weight as an objective clinical variable, mice having undergone procedures with presumably more pain and distress may exhibit weight loss or lower weight gain. The extensive weight data collected did not support this. No clinically relevant sequalae in the mice due to genotyping procedures done compared with controls were seen. This is consistent with previous studies finding no links between tail biopsies in mice and resultant differences in weight or biometric data such as heart rate and temperature.[1,4,23] Weight may be too insensitive to clinical effects of one-time momentary pain or distress for it to be a useful variable for comparison. Although animals in the control group were not subjected to genotyping procedures, they were nonetheless handled to be weighed and toe tattooed. Handling alone has been shown to

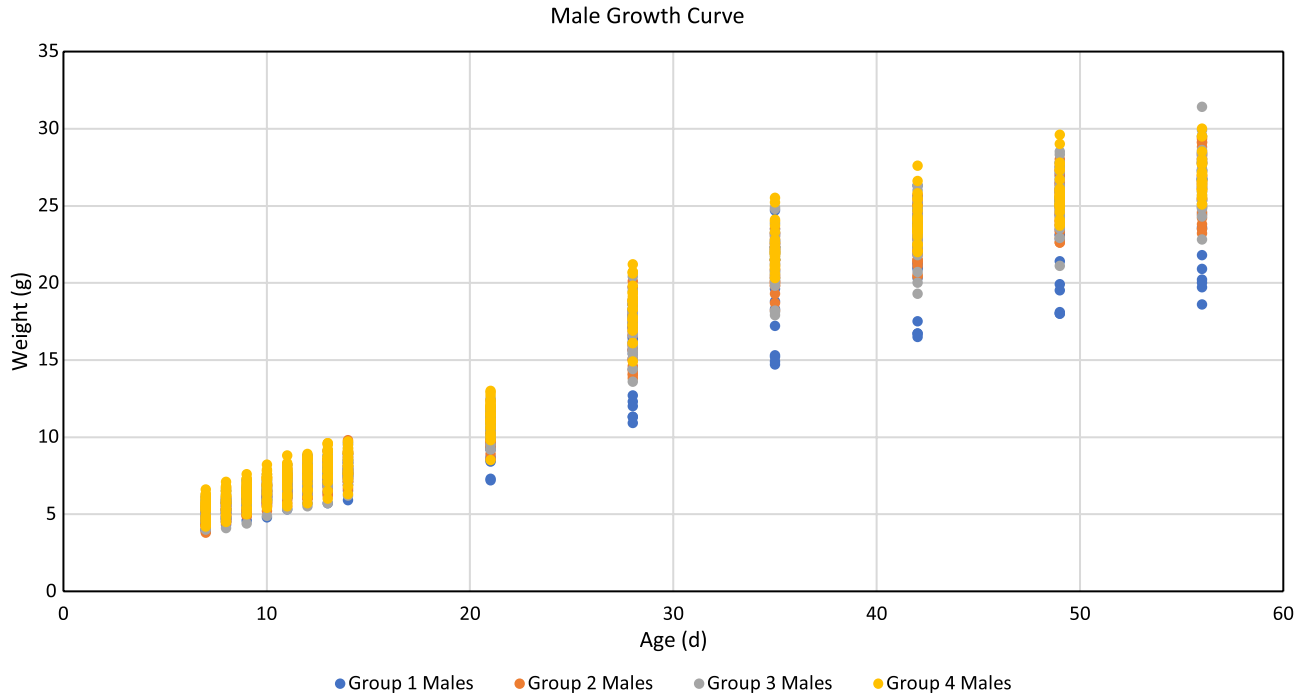

Figure 4. Scatterplot of all weights obtained from male mice of all 4 groups from 14 timepoints ($n = 120$). The groups are denoted by different colors, overlaid on each other. The general impression is that mice from all groups appear to gain weight and the weights remain similar to each other.

be the most stressful intervention to mice.[4] Because weighing requires handling, the stress that all animals had to experience may have made resolving minute differences in weight even more difficult.

The 3Rs (replacement, reduction, and refinement)[20] dictate that we should minimize pain, distress, or the lasting harm that research animals may experience. Adopting a noninvasive method for genotyping presents an opportunity for refinement for this common procedure. It may also help lessen the emotional toll exerted on the persons genotyping. Swabbing the buccal cavity of a mouse does not cause permanent mutilation, obvious pain, assured bleeding, or chance of infection. Because of this, no anesthesia is necessary even in postwean mice, which would reduce exposure risk to personnel. Buccal swabs allow for repeat genotyping, which may be necessary in multiple experimental situations to confirm that animals with the correct genotypes were mated and enrolled into studies, or to confirm recombination in cre-lox systems, when the cre driver is inducible and ubiquitously expressed. All of these reasons make buccal swabs a more humane and superior procedure for genotyping than tail biopsies. For broad adoption, financial analysis with a time study may be warranted to more fully evaluate feasibility for your specific institution. The swabs are made of single-use, disposable plastic with an environmental impact and recurring cost. In our experience, swabbing takes comparable time to cutting tails in prewean animals; however, without needing anesthesia in postwean animals, swabbing is faster.

Conflict of Interest

The authors have no conflicts of interest to declare.

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