Journal of the American Association for Laboratory Animal Science
Copyright 2011
by the American Association for Laboratory Animal Science

Vol 50, No 4
July 2011
Pages 471–478

Superovulation Strategies for 6 Commonly Used Mouse Strains

Charlie Luo,[1] Juliana Zuñiga,[4] Earnessa Edison,[2] Shana Palla,[3] Wenli Dong,[3] and Jan Parker-Thornburg[1,*]

We examined different weight ranges and hormone dosages to determine superovulation protocols for 6 mouse strains commonly used in genetic engineering: C57BL/6NHsd, B6(Cg)-Tyr^{c-2J}/J, B6D2F1/Hsd, FVB/NHsd, BALB/cAnNCr, and Crl:CD1(ICR). Mice from each strain were divided into groups based on weight roughly corresponding to those of 3-, 4-, 5-, and 6-wk-old mice. Mice were treated with 5 IU pregnant mare serum gonadotropin (PMSG) and 5 IU human chorionic gonadotropin (HCG). The weights of mice that produced maximal numbers of oocytes in response to these doses were 14.2 g or less for C57BL/6NHsd, 13.7 g or less for B6(Cg)-Tyr^{c-2J}/J, 6.0 to 9.9 g for B6D2F1/Hsd, 14.5 to 16.4 g for FVB/NHsd, 14.8 g or less for BALB/cAnNCr, and 23.5 g or more for Crl:CD1(ICR). We then compared PMSG dosages of 5 and 2.5 IU per mouse and determined whether 2 doses of PMSG (5 or 2.5 IU, depending on prior results) administered 1 wk apart, followed by the standard HCG injection, would produce more oocytes when compared to a single dose of PMSG. FVB, B6D2F1, BALB/c, and CD1 mice responded best to a single dose of 5 IU of each hormone, whereas B6(Cg)-Tyr^{c-2J}/J mice produced more oocytes after 2.5 IU PMSG. Although C57BL/6 mice given the standard dose produced good numbers of oocytes, the number was higher after 2 doses of PMSG at 5 IU per dose. We conclude that response to superovulation can be optimized based on mouse strain, weight, and the dose and timing of hormone injection.

Superovulation has been used in the production of transgenic mice since the late 1980s to artificially induce ovulation of large numbers of oocytes from limited numbers of female mice. Superovulation facilitates the generation of genetically engineered mice and reduces the number of animals used.[6,10] The use of superovulation to increase oocyte yield for experimental purposes was first described in 1956[5] and subsequently expanded on in 1971.[6] The author noted that the strain (BALB/c and various 129 hybrids) and the weight of the mouse affected response to superovulation hormones. However, despite the expansion of mouse models into additional strains, the development of new, strain-specific superovulation protocols has been limited. Use of suboptimal superovulation protocols leads to euthanasia of increased numbers of female mice to generate the requisite number of oocytes—typically 150 to 300—for manipulation. Later studies, which examined additional mouse strains, noted that the response to superovulation protocols varied depending on the strain of the female donor.[1,2,9,10,16,25] Most of these studies treated female mice of the same age with equal amounts of gonadotropins and then examined the differences in oocyte yield among the strains. The responses to superovulation varied from poor (fewer than 5 oocytes per A/J mouse) to excellent (more than 40 oocytes per 129S1 mouse).[2] These varied responses to superovulation result from genetic differences between strains; the response to hormone-induced ovulation depends on 3 to 4 major genetic differences among strains.[13,14] Although these studies noted strain differences, few authors attempted to optimize the superovulation protocol for different strains.[6,9] To achieve the maximal benefit from superovulation, each strain should be tested to determine its optimal weight (or age) and dosage schedule.[6] One of the strains often used for generation of transgenic animals is the FVB strain. FVB mice exhibited the highest proficiency of transgenic production of all strains tested, [1] but their response to superovulation was quite variable. As a result, use of the FVB strain to produce transgenic mice actually may require the use of more donor female mice because of lack of optimization of the superovulation protocol.

On several occasions over the course of 3 y, we tracked the response to superovulation for FVB, B6D2F1, and C57BL/6 female donors from different vendors across 2 different institutions. For C57BL/6 donors, we noted that the optimal weight range for superovulation appeared to be between 10.5 and 14.4 g, whereas B6D2F1 donors responded optimally at 16.5 to 18.4 g. We observed that, regardless of their age or weight, FVB donor mice did not superovulate as well as did C57BL6 or B6D2F1 donors. In addition, our preliminary results indicated that although younger FVB donors would generate more oocytes, a greater proportion of the oocytes could not be used for injection because they had 3 pronuclei. For the current study, we sought to confirm our preliminary observations by performing a detailed analysis of donor mice by weight and hormone dosage and by extending this analysis to 4 additional mouse strains that are often used in generating oocytes for pronuclear and blastocyst injections. We were able to define target weight ranges for most of the strains examined (FVB being the lone exception) that would result in a good response to standard superovulation procedures. We then used these weight ranges to determine the optimal response of each strain to different hormone dosages and timing of administration.

Materials and Methods

Animals. C57BL/6NHsd, FVB/NHsd, and B6D2F1/Hsd male mice and B6D2F1/Hsd female mice were purchased from Harlan Laboratories (Houston, TX). C57BL/6NHsd and FVB/

Received: 29 Nov 2010. Revision requested: 04 Jan 2011. Accepted: 14 Feb 2011.
[1]Department of Biochemistry and Molecular Biology, Genetically Engineered Mouse Facility and Departments of [2]Veterinary Medicine and Surgery and [4]Biostatistics, University of Texas MD Anderson Cancer Center and [4]Department of Neuroscience, Baylor College of Medicine, Houston, Texas.
[*]Corresponding author. Email: jpthorn@mdanderson.org

NHsd female mice were purchased from Harlan Laboratories and then either used immediately for the study if they were the appropriate weight or were bred in brother-to-sister matings for no more than 3 generations to obtain sufficient numbers of female mice of the desired weights. B6(Cg)- Tyr^{c-2J}/J male mice were purchased from Jackson Laboratory (Bar Harbor, ME) and then either used immediately for the study if they were the appropriate weight or were bred in brother-to-sister matings for no more than 3 generations to obtain sufficient numbers of female mice of the desired weights. BALB/cAnNCr and Crl:CD1(ICR) male mice were purchased from Charles River Laboratories (Wilmington, MA); BALB/cAnNCr and Crl:CD1(ICR) females were purchased from Charles River Laboratories and used immediately if they were the appropriate weight and were bred for 1 [Crl:CD1(ICR)] or 3 (BALB/cAnNCr) generations to obtain sufficient numbers of female mice of the desired weights. CD1 female mice were bred to unrelated males; BALB/c mice were bred in brother-to-sister matings.

On receipt from the vendor or after weaning, female mice were allowed to acclimate for 2 d prior to weighing. Female mice began superovulation (described in next section) 2 to 3 d after achieving the correct weight. All mice were maintained under SPF conditions under a 12:12-h light:dark cycle (lights on, 0700 to 1900) at temperatures of 21 to 24 °C. Pathogens excluded from the colony include mouse parvovirus, minute virus of mice, mouse hepatitis virus, mouse norovirus, Theiler disease virus, enzootic diarrhea of infant mice, Sendai virus, pneumonia virus of mice, reovirus 3, lymphocytic choriomeningitis virus, mouse adenovirus, ectromelia virus, K virus, and polyoma virus. The animal facilities are negative for *Mycoplasma pulmonis* and rodent viruses except for the presence of murine norovirus in about 10% of the mice (norovirus is not an excluded pathogen in our facilities). Sentinels are screened quarterly by bacterial culture for *Bordetella bronchiseptica*, *Corynebacterium kutscheri*, *Salmonell*a spp., *Pseudomonas* spp., and *Citrobacter rodentium*; *Helicobacter* testing is not performed. Mice were housed in static microisolation caging (Lab Products, Wilmington, DE) with ad libitum access to Teklad Irradiated Diet 2919 (Harlan Laboratories, Madison, WI) and water treated by reverse osmosis. All animal experiments were approved by the Institutional Animal Care and Use Committee at the University of Texas MD Anderson Cancer Center, an AAALAC-accredited institution.

Superovulation and mating. Superovulation is a technique widely used in transgenic facilities to reduce the numbers of animals used, by enabling greater oocyte production from a single female donor. Because we wanted our results to be broadly applicable for transgenic use, we used techniques that are standard for transgenic facilities. Therefore, our hormone injection times and dosages reflected what we use for our everyday procedures. Pregnant mares serum gonadotropin (PMSG) was obtained from the National Hormone and Peptide Program (Harbor–UCLA Medical Center, Torrance, CA). Human chorionic gonadotropin (HCG) was obtained from Sigma (St Louis, MO). We added 1 mL 0.9% bacteriostatic sodium chloride (APP Pharmaceuticals, Schaumburg, IL) to the original vial of PMSG to obtain a stock solution of 2000 U/mL. The stock solution was stored in a −20 °C freezer (Fisher Scientific, Houston, TX) until thawed for further dilution. We added 900 µL of the first diluted solution to 35.1 mL sterile saline to achieve a concentration of 50 U/mL; 1.2 mL of the 50-U/mL solution was aliquoted into each of 30 microfuge tubes (1.5-mL; Fisher Scientific) and stored in a −80 °C freezer (Thermo-Forma, Marietta, OH) until thawed for injection. We injected each donor mouse with 2.5 or 5 IU PMSG (0.05 or 0.1 mL IP). HCG was resuspended in bacteriostatic water (Hospira, Lake Forest, IL) to 2000 U/mL, aliquoted into 1.5-mL microfuge tubes at 25 µL per tube, lyophilized to dryness in a Savant SpeedVac Plus drying system (ThermoFisher Scientific, Houston, TX), and stored at −80 °C. For use, 1 mL of bacteriostatic sterile saline was added to the microfuge tube to make a 1 mL solution of 50 U/mL; 5 IU HCG was injected intraperitoneally into female mice 47 to 49 h after their last PMSG injection. Female mice were manually restrained for the intraperitoneal injections.

Immediately after HCG injection, female mice were mated 1:1 to male mice of proven fertility and the same strain. Male mice for breeding were maintained in individual cages and ranged in age from 3 to 8 mo; male mice were replaced as they exceeded the age maximum. We used natural mating for production of oocytes rather than in vitro fertilization. We attempted to obviate any day-to-day variation by: 1) ensuring that we used enough female donors to account for variation, 2) observing strict time requirements for weighing and treating the donors, 3) using only proven male mice for breeding and ensuring that matings were spaced a minimum of 5 d apart, 4) 'priming' any stud male mouse that had been unused for more than 3 weeks by mating it to a nonstudy female mouse 5 to 10 d prior to being used for mating during the study, 5) mating only one female donor per male mouse in a session, and 6) ensuring that stud male mice were of optimal mating age (3 to 8 mo of age).

We checked female mice for copulation plugs before 1000 each morning during the first part of the study. However, because fertilization often occurred despite the absence of a noticeable plug, we discontinued recording this information.

Weight analysis. Initial experiments for each strain involved determination of the weight range of female donors that would respond best to our superovulation protocols. We compared online vendor weight tables to estimate the weight range that would correspond to 3-, 4-, 5-, and 6-wk-old mice (Table 1).[3,7,17-23] Donor mice were treated with 5 IU of each hormone and then mated 1:1 immediately after the HCG injection to proven stud males of optimal mating age (3 to 8 mo). The following day, all oocyte donors were weighed, oviducts collected, and oocytes pooled by donor weight and strain, except for the BALB/c strain. BALB/c oocytes were collected and scored for each individual female mouse.

Dosage and timing analyses. We tested doses of 5 and 2.5 IU PMSG for superovulation of female donors at the weight level that gave the best overall results for each strain. Single PMSG injections were followed 47 to 49 h later by an injection of 5 IU HCG. For all strains, oocytes were collected and scored for each individual donor. After determining the optimal dosage of PMSG, we then used that dosage to determine whether 2 PMSG injections separated by a 1-wk interval, followed 47 to 49 h after the second PMSG dose by an injection of 5 IU HCG increased oocyte number. Oocytes were collected and scored for each individual female donor.

Oocyte collection and analysis. The day after mating, all female donors were euthanized by cervical dislocation by a trained investigator with prior IACUC approval for the technique.

For the weight-group experiments involving C57BL/6, B6(Cg)-Tyr^{c-2J}/J, B6D2F1, FVB, and CD1(ICR) donors, oviducts were collected from all female mice and placed into 2 mL M2 media (Sigma-Aldrich) in a 35-mm culture dish (Fisher Scientific). Each oviduct then was moved to a dish containing 2 mL M2 media (Sigma-Aldrich) and 75 µL hyaluronidase (10 mg/mL; Sigma-Aldrich), where the ampulla was torn open to release the oocytes. After all the oviducts for that group had been

Table 1. Strains and weight groups

Strain	No. of female mice[a]	Weight (g)			
		Group 1	Group 2	Group 3	Group 4
C57BL/6NHsd	20	5.5–10.4	10.5–14.2	14.3–16.2	≥16.2
B6(Cg)-Tyr^{c-2J}/J	20	6–9.9	10–13.7	13.8–15.4	≥15.5
FVB/NHsd	25	8–11.9	12–14.4	14.5–16.4	≥16.5
B6D2F1/Hsd	8	6–9.9	10–13.4	13.5–15.9	≥16
Crl:CD1(ICR)	17	4.5–11.4	11.5–18.4	18.5–23.4	≥23.5
BALB/cAnNCr	16	6–10	10.1–14.8	14.9–16	≥16.1

Weight groups 1 through 4 were chosen to correspond generally to weanling mice at 3, 4, 5 and ≥ 6 wk of age, respectively. Weight ranges were calculated by determining the halfway point between any 2 consecutive weight groups based on an average of vendor age and weight data.
[a]The optimal number of female mice per group needed to yield significant data analyses (see Materials and Methods).

processed, all of the oocytes were collected and placed into a 100-µL drop of KSOM (Millipore, Billerica MA) under embryo-tested mineral oil (Sigma-Aldrich) that had been equilibrated to 37 °C at 5% CO_2.[10,26] Oocytes were allowed to incubate for 24 h, after which the drop was scored for the number of 2-cell, unfertilized, dead, and aberrant oocytes. For BALB/c mice and all of the dosage–timing experiments, oviducts from individual female donors were opened in a 50-µL drop of KSOM (Millipore). These drops were incubated overnight at 37 °C in 5% CO_2 prior to scoring for the number of 2-cell, unfertilized, dead, and aberrant oocytes per female donor. After scoring, C57BL/6NHsd, B6(Cg)-Tyr^{c-2J}/J, and Crl:CD1(ICR) oocytes were allowed to incubate to morula stage before archiving for later use in blastocyst injections. Morula from other strains were archived to obtain straws of wild-type embryos to be shipped with mutant cryopreserved embryos.

Data analysis. Sample size for each weight group was determined by using previously published data for C57BL/6, BALB/c, and FVB/N strains[2] and B6D2F1 and CD1 strains.[15] Because no previous data were available for B6(Cg)-Tyr^{c-2J}/J mice, we presumed that their response would be similar to that of C57BL/6 mice [the original background strain for B6(Cg)-Tyr^{c-2J}/J]. Assuming that data for each dose schedule by weight group within a mouse strain would have the same standard deviation and that a significant difference in the mean number of eggs would be approximately a 100% increase, we set sample sizes to obtain a 2-sided significance (α) level of 0.05 and power of 80% by using the Tukey HSD method[24] for multiple comparisons.

Data collected for each experiment included the total numbers of female donors and oocytes and the numbers of fertilized, unfertilized, dead, and aberrant oocytes. We examined the effects of various treatments by comparing the total number of oocytes and the number of fertilized oocytes per female dose either for a given weight group (for a specific dosage of PMSG) or for multiple doses of PMSG before administration of HCG. The number of female donors and mean and standard error were summarized for data regarding fertilized oocytes and the total number of oocytes. One-way ANOVA (F test) was used to examine the overall difference among the means of the multiple groups. To control the overall type I error rate, the Tukey honestly significant difference test[24] was used for pairwise comparisons to determine which groups differed from each other; all tests were 2-sided. *P* values less than 0.05 were considered statistically significant. All analyses were conducted by using SAS software (version 9.1, SAS Institute, Cary, NC).

Results

Determination of weight ranges for analysis. Our first study was designed to determine a weight range at which each of the 6 strains tested would respond the best to our standard superovulation protocols. Because transgenic facilities often use female donors of a specific age range (rather than weight range), we set up our analysis to reflect weight ranges that generally corresponded to 3, 4, 5, and 6 wk of age across different vendors, by using available vendor data[3,7,17-23] as a guide to predict these weight intervals (Table 1). We assessed the overall number of oocytes (as a measure of the overall capability of a donor to produce) and the number of fertilized oocytes, which would reflect both the receptivity of the female donor to mating and the response of the stud male mouse. As expected, the most responsive weight range varied for each strain (Table 2). C57BL/6NHsd mice showed little difference in superovulation potential at weights as high as 14.2 g, with average numbers of total oocytes at approximately 37 per female and fertilized oocytes at approximately 23.5 per female donor (Table 2). The yield fell as the mice became heavier. B6(Cg)-Tyr^{c-2J}/J females responded best in the weight range of 10 to 13.7 g, with average total oocytes of 32 per donor and average number of fertilized oocytes at approximately 14 per mouse (Table 2). This response was less than that of C57BL/6NHsd, although the albino strain initially was identified as due to a point mutation arising in the C57BL/6/J strain.[8] FVB/NHsd female donors gave more total oocytes (average,14.4 per mouse) when larger than 16.5 g, although they gave greater numbers of fertilized oocytes (average,10.8 per mouse) at a range of 14.5 to 16.4 g (Table 2). The hybrid line B6D2F1/Hsd gave greater numbers of both overall and fertilized oocytes (averages, 30.6 and 25.4 per female donor, respectively) when superovulated at a weight range of 6 to 9.9 g (Table 2). Conversely, our outbred line, Crl:CD1(ICR), showed the opposite results, producing many more oocytes in both categories (averages, 23.3 and 19.3 per donor, respectively) at weights greater than or equal to 23.5 g (Table 2). Finally, groups 1 and 2 BALB/cAnNCr female mice produced significantly ($P < 0.001$) more total and fertilized oocytes than did groups 3 and 4; differences between groups 1 and 2 were not significantly different (Table 2).

Dosage and timing assessments. We used the most responsive weight range for each strain to examine response to different doses of PMSG and to determine whether a second dose of PMSG given 1 wk after the first dose would increase the numbers of total and fertilized oocytes produced. The most responsive weight range was chosen based initially on the range that gave the highest number of fertilized oocytes, using the highest number of total oocytes as the second criterion. For this reason, group 2 was chosen as most responsive for B6(Cg)-Tyr^{c-2J}/J, group 3 for FVB/NHsd, group 1 for B6D2F1Hsd, and group 4 for Crl:CD1(ICR). The numbers for fertilized and total oocytes for both C57BL/6NHsd and BALB/cAnNCr did not vary greatly

Table 2. Weight group analysis

Strain	Group	*n*	Mean no. of oocytes / female mouse (± SE)	Mean no. of fertilized oocytes/ female mouse (± SE)	% of oocytes fertilized
C57BL/6NHsd	1	20	37.7	24.3	65
	2	20	37.0	23.5	63
	3	20	31.4	21.0	67
	4	20	24.6	16.6	67
B6(Cg)-Tyr^{c-2J}/J	1	27	12.3	7.2	58
	2	20	32.0	14.0	44
	3	17	22.2	8.5	38
	4	19	19.8	8.9	45
FVB/NHsd	1	32	10.1	7.3	53
	2	25	10.5	8.4	80
	3	25	12.5	10.8	86
	4	25	14.4	10.3	71
B6D2F1/Hsd	1	8	30.6	25.4	83
	2	8	21.0	16.1	77
	3	15	16.8	13.7	82
	4	8	24.0	16.5	69
Crl:CD1(ICR)	1	28	16.1	12.8	80
	2	19	13.6	11.7	86
	3	20	10.8	7.8	73
	4	19	23.3	19.3	83
BALB/cAnNCr	1	18	39.0 (6.9)	20.9 (3.1)	59
	2	25	47.8 (3.0)	19.0 (2.9)	41
	3	16	7.9 (1.3)	3.1 (0.7)	36
	4	16	10.5 (1.4)	3.9 (0.8)	32

Female mice were weighed and placed into weight groups shown in Table 1 and given 5 IU PMSG followed 47 to 49 h later by 5 IU HCG. For all strains except BALB/cAnNCr, oocytes for a given weight group in a strain were pooled for incubation and counting. Therefore, whereas we could assess which weight groups gave favorable numbers of oocytes, significance could not be established. For BALB/cAnNCr mice, weight groups 1 and 2 yielded significantly ($P < 0.001$) more oocytes than did groups 3 and 4, of which significantly ($P < 0.001$) more were fertilized.

between groups 1 and 2 (no significant difference seen for fertilized oocytes for BALB/c). For these 2 strains, we chose to use group 2 mice due to the difficulty of obtaining group 1 animals. To establish significance, oocyte clusters were collected individually from each donor and incubated separately for analysis. We first compared doses of 2.5 and 5 IU PMSG. With the exception of B6(Cg)-Tyr^{c-2J}/J, all strains showed greater numbers of total and fertilized oocytes after 5 IU PMSG. Total numbers of oocytes improved significantly for FVB/NHsd ($P = 0.005$), B6D2F1Hsd ($P < 0.001$), and BALB/CAnNCr ($P < 0.001$; Table 3). Numbers of fertilized oocytes improved significantly for FVB/NHsd ($P = 0.012$) and B6D2F1 ($P < 0.001$; Table 4). B6(Cg)-Tyr^{c-2J}/J donors had a better response to 2.5 IU PMSG, giving fairly equivalent overall numbers of oocytes to both 2.5 IU and 5 IU, but significantly ($P = 0.003$) greater numbers of fertilized oocytes (13.3 ± 2.1 per female donor; Table 4) at the lower dose.

We then used the optimal PMSG dosage to test whether 2 doses of PMSG given at a 1-wk interval prior to HCG would induce even greater numbers of oocytes. Donors were given PMSG on day 1 and again on day 8, followed 47 to 49 h later by HCG injection and mating. Crl:CD1(ICR) did not differ significantly in the number of overall or fertilized oocytes produced, although the totals were lower for this strain (Table 3, 5+5). B6(Cg)-Tyr^{c-2J}/J, FVB/NHsd, B6D2F1/Hsd, and BALB/cAnNCr strains responded poorly to the double dose of PMSG, giving significantly lower numbers of oocytes when compared with their response to a single dose of PMSG (Table 3, 2.5+2.5 or 5+5). Although the C57BL/6NHsd strain responded positively to the double dose of PMSG, the numbers of total oocytes were not statistically significantly different (30.4 ± 2.1 oocytes per female with the double dose compared with 26.3 ± 2.6 per female for the single dose, $P = 0.466$)

Discussion

We examined whether the superovulation procedure used in generating genetically engineered mice could be improved for strains commonly used in the procedure. By doing so, we hoped to reduce the number of female donors required to produce oocytes for subsequent manipulations. As noted in early literature on the procedure, superovulation is a hormonal treatment delivered by intraperitoneal injection and is sensitive to both weight and age of the female donor, both of which combine to achieve follicle maturation.[5,6] For BALB/c mice, optimal follicle maturation occurs between 3 and 4 wk of age, after which the follicles become increasingly nonresponsive to hormone treatment.[6] We sought to expand the age and weight analysis

Table 3. Effect of PMSG dosage on total number of oocytes

Strain	PMSG dose(s)	*n*	No. of oocytes/female mouse (mean ± SE)	Overall *P*	Pairwise PMSG treatment comparison	Adjusted *P*
C57BL/6	2.5	20	21.5 ± 2.5	0.056	5: 2.5	0.347
	5	25	26.3 ± 2.6		5+5: 5	0.466
	5+5	20	30.4 ± 2.1		5+5: 2.5	0.044
B6(Cg)Tyr $^{c-2J}$/J	2.5	20	24.8± 2.5	<0.001	5: 2.5	0.824
	5	20	26.8 ± 3.0		5: 2.5+2.5	<0.001
	2.5+2.5	20	13.1 ± 1.6		2.5: 2.5+2.5	0.004
FVB	2.5	25	9.5 ± 1.0	0.004	5: 2.5	0.005
	5	25	15.1 ± 1.3		5: 5+5	0.034
	5+5	24	10.7 ± 1.4		5+5: 2.5	0.776
B6D2F1	2.5	8	13.6 ± 3.3	<0.001	5: 2.5	<0.001
	5	8	45.3 ± 3.9		5: 5+5	<0.001
	5+5	7	17.9 ± 3.2		5+5: 2.5	0.683
CD1	2.5	17	12.3 ± 1.8	0.348	5: 2.5	0.382
	5	17	16.3 ± 2.3		5+5: 5	0.987
	5+5	18	15.8 ± 2.2		5+5: 2.5	0.459
BALB/c	2.5	16	21.6 ± 2.9	<0.001	5: 2.5	<0.001
	5	25	47.8 ± 3.0		5: 5+5	<0.001
	5+5	16	16.3 ± 2.5		5+5: 2.5	0.474

Female mice of the weight group that gave the best overall results for the strain were given 2.5 or 5 IU PMSG followed 47 to 49 h later by 5 IU HCG. For the third group of mice in each strain, we gave 2.5 or 5 IU PMSG followed by a second dose of 2.5 or 5 IU PMSG 1 wk later and then 5 IU HCG 47 to 49 h after the second dose of PMSG. The overall *P* value demonstrates whether differences were observed among treatments. The pairwise comparison of dosages and the adjusted *P* value demonstrate differences between treatments, with the more effective dosage shown first in the comparison.

to additional strains now used for production of genetically engineered mice. We specifically chose to use weight because superovulation is based on a physiologic response to a chemical dosage, and weight is generally a better predictor of this response than age. We also chose to use a standard dosage of 5 IU of each hormone for our first analysis, as it would allow us to determine baseline data for each strain and compare it to our results based on years of hormone use at this dosage. The target number of females to analyze in each group was determined statistically, based on published data[2,15] as well as previous data collected in pilot experiments.

For our initial analysis, we determined the weight range that would result in the greatest oocyte numbers. We and others[6] have noted that mice of a given age range can often have quite different sizes, which was reflected in the size ranges of our weight groups. Our weight ranges were determined by consideration of vendor data and correlated with commonly used age ranges for each strain. Because we gave a standard dose of 5 IU for all mice in the given range, size differences could affect the ability of the female donor to respond to hormone treatment. In general, female mice that vary by weight within a range will experience slightly different amounts of hormone per gram of body weight, although it was unclear from our results whether differences in weight within a range led to differences in superovulation response. However, given that outbred mice gain weight extremely rapidly over the first 2 wk after weaning, female donors of those strains would experience pronounced differences in hormone dose by body weight. Our results suggest that by determining a responsive weight range for superovulation, one can easily screen for which mice are likely to give the best results and rule out those that are over- or underweight.

For our analyses, 5 of the 6 strains tested were assessed by using oocytes that were pooled from the donors in that weight range. This technique is the most common used by transgenic facilities when isolating oocytes.[10] Using pooled oocytes, we were able to determine which weight range group(s) produced greater numbers of total and fertilized oocytes in 4 of the strains tested. However, although use of pooled oocytes resulted in data that we could use to predict the weight range for subsequent experiments, we were not able to establish statistical significance for this portion of the study. As a result of this anomaly, we can only state that our defined 'best' weight range for these 5 strains indicates a trend for animals in our facility, which may or may not be reproducible in other facilities. The lone exception was the BALB/c strain, for which oocytes from each female donor were assessed individually.

By looking at total numbers of oocytes, we could assess overall response to the hormone. We anticipated that numbers of fertilized oocytes would be dependent both upon the male performance and female receptivity. Because the male mice we used were optimized for performance, we generally regarded the number of fertilized oocytes as a marker of female receptivity, and that their receptivity would be influenced by weight range and hormone administration. Of note, we had several situations in which total number of ooytes was lower but the number of fertilized oocytes was increased in one weight range compared with another, indicating that the female mice were more receptive to the male mouse. We also saw the opposite result, with number

Table 4. Effect of PMSG dosage on number of fertilized oocytes

Strain	PMSG dose(s)	*n*	No. of fertilized oocytes/ female mouse (mean ± SE)[a]	% fertilized oocytes/ female mouse	Overall *P*	Pairwise PMSG treatment comparison	Adjusted *P*
C57BL/6	2.5	20	10.8 ± 1.9	51	0.177	5: 2.5	0.617
	5	25	13.4 ± 1.7	53		5+5: 5	0.550
	5+5	20	16.3 ± 2.4	52		5+5: 2.5	0.151
B6(Cg)Tyr $^{c\text{-}2J}$/J	2.5	20	13.3 ± 2.1	59	0.003	2.5: 5	0.009
	5	20	6.2 ± 1.4	25		2.5+2.5: 5	0.988
	2.5+2.5	20	5.8 ± 1.4	43		2.5: 2.5+2.5	0.006
FVB	2.5	25	7.0 ± 1.1	68	0.011	5: 2.5	0.012
	5	25	12.0 ± 1.3	79		5: 5+5	0.070
	5+5	24	8.1 ± 1.3	73		5+5: 2.5	0.778
B6D2F1	2.5	8	9.3 ± 3.8	51	<0.001	5: 2.5	<0.001
	5	8	38.0 ± 4.7	84		5: 5+5	0.001
	5+5	7	13.7 ± 2.8	75		5+5: 2.5	0.712
CD1	2.5	17	7.5 ± 1.8	53	0.504	5: 2.5	0.473
	5	17	11.4 ± 2.9	54		5+5: 5	0.795
	5+5	18	9.3 ± 2.1	51		5+5: 2.5	0.849
BALB/c	2.5	16	14.4 ± 2.4	69	0.131	5: 2.5	0.434
	5	25	19.0 ± 2.9	41		5+5: 5	0.124
	5+5	16	11.6 ± 2.0	73		5+5: 2.5	0.777

Female mice of the weight group that gave the best overall results for the strain were given 2.5 or 5 IU PMSG followed 47 to 49 h later by 5 IU HCG. For the third group of mice in each strain, we gave 2.5 or 5 IU PMSG followed by a second dose of 2.5 or 5 IU PMSG 1 wk later and then 5 IU HCG 47 to 49 h after the second dose of PMSG. The overall *P* value demonstrates whether differences were observed among treatments. The pairwise comparison of dosages and the adjusted *P* value demonstrate differences between treatments, with the more effective dosage shown first in the comparison.

[a]Percentage of total oocytes that developed to the 2-cell stage.

of total oocytes higher but that of fertilized oocytes lower in one weight range compared with another. This result was particularly obvious for the BALB/cAnNCr strain, thereby verifying data suggesting that BALB/c male mice are only semifertile.[11]

This study produced several interesting observations. The hybrid strain, B6D2F1/Hsd, responded best to superovulation hormones when treated at 6 to 9.9 g, a weight range that occurs immediately after weaning. The total number of oocytes and the number of fertilized oocytes obtained were greater for this group than for any of the other strains tested. This finding correlates well with previous data obtained on F1 strains of 129 × C57BL/6,[6] which showed that these donors superovulated best immediately after weaning (21 d of age) in response to 4 to 5 IU PMSG. Conversely, the weight range that was optimal for our outbred strain, Crl:CD1(ICR), was the highest weight range tested, corresponding to 6 wk of age or older. Inbred strains generally performed best at intermediate weight ranges, and the optimal ranges occasionally spread across 2 weight groups (for example, C57BL/6 groups 1 and 2). The C57BL/6NHsd strain responded better to superovulation (that is, gave higher oocyte yields) than did the B6(Cg)-Tyr$^{c\text{-}2J}$/J strain for all weight ranges. The C57BL/6J albino strain differs from the C57BL/6J strain due to a spontaneous point mutation in the tyrosinase gene[8] as well as at 2 single-nucleotide polymorphisms.[27] Because we used C57BL/6N in our analysis, we suggest that the differences we observed could be due to the differences between J and N substrains of C57BL/6, given that these sublines differ at approximately 103 polymorphisms, with mutations in the *Nnt* and *Cdh23* genes in the J substrain.[27] Direct comparisons of albino with black and N with J would be instructive in future work.

Our second study examined the response of our 6 mouse strains to different dosages and timing of PMSG administration. We initially assessed each strain's response to 5 IU of hormone. This dose was chosen in light of results from the literature (Table 5).[1,2,6,10,14,16,25] The results we obtained were comparable to those reported previously for C57BL/6, F1 hybrid lines, the ICR outbred strains, FVB substrains, and BALB/c substrains. We extended the analysis to include a B6-albino strain, B6(Cg)-Tyr$^{c\text{-}2J}$/J, which was the one strain that responded better to a lower dose of PMSG.

We also performed these experiments by using 2.5 IU for comparison. We chose this lower dose given our previous observations that C57BL/6NHsd mice appeared to have a better response to 2.5 IU PMSG than to 5 IU, the standard dose. In fact, whereas C57BL/6NHsd mice produced fairly similar numbers of both total and fertilized oocytes to both 5 IU and 2.5 IU, the B6(Cg)-Tyr$^{c\text{-}2J}$/J strain responded significantly better (in terms of producing fertilized oocytes) to 2.5 IU PMSG; all other tested strains gave greater numbers of oocytes when injected with 5 IU PMSG. We did not test higher levels of PMSG in our analysis because we previously have not had good results with higher concentrations.

After establishing the best dose of PMSG, we then determined whether performing 2 PMSG injections separated by a 1-wk interval would result in greater numbers of oocytes. We chose this regimen because of our previous results in which FVB

Table 5. Comparison of responses of various mouse strains to superovulation hormones

Strain		PMSG dosage	Mean total no. of oocytes	Mean no. of fertilized oocytes	Mating scheme	Reference no.
Hybrid strains						
	B6AF1	5 IU	45.4	ND	IVF	14
	B6C3F1	5 IU	31.9	25.6[d]	IVF	16
	B6CBAF1	5 IU	19.8[c]	17.5[c]	IVF	4
	B6D2F1/Hsd	5 IU	45.3	38	Natural	Current study
	B6D2F1/Tac	5 IU	NR	11.19[e]	Natural	1
	B6SJLF1	5 IU	19.3[b]	14.2[b]	IVF	25
	CLGF1 x 129 B6AF1	4 IU	67.4	ND	Natural	6
Inbred strains						
	BALB/cAnNCr	5 IU	47.8	19	Natural	Current study
	BALB/cCrYok	5 IU	20.9	15.3[d]	IVF	16
	BALB/cJ	5 IU	15.08[f]	ND	IVF	2
	B6(Cg)Tyr^{c-2J}/J[a]	2.5 IU	24.8	13.3	Natural	Current study
	C57BL/6J	5 IU	25.68[f]	ND	IVF	2
	C57BL/6J	5 IU	46.92	ND	IVF	14
	C57BL/6J	5 IU	33.7	26.7[d]	IVF	16
	C57BL/6J	5 IU	26.5[b]	17.1[b]	IVF	25
	C57BL/6NHsd	5 IU	30.4	16.3	Natural	Current study
	C57BL/6NTac	5 IU	NR	10.4[e]	Natural	1
	FVB/NHsd	5 IU	15.1	12	Natural	Current study
	FVB/NTac	5 IU	NR	9.99[e]	Natural	1
	FVB/NTac	5 IU	19.49[f]	ND	IVF	2
Outbred strains						
	Crl:Cd1(ICR)	5 IU	16.3	11.4	Natural	Current study
	CrjICR	5 IU	17.4	11.6[d]	IVF	16

ND, not determined; NR, not reported

[a]B6-albino results are reported for a dosage of 2.5 IU PMSG, as that was the optimal dosage for that strain. All other strains examined in the current study are reported for a dosage of 5 IU PMSG.

[b]Results recorded as a total per 10 female donors; this number was obtained by dividing the total given by 10.

[c]Results recorded as a total for 6 female donors; this number was obtained by dividing the total given by 6.

[d]Results recorded as % of total number of oocytes per female donor; this number was obtained by multiplying the total by the percentage.

[e]Results recorded as the average number of fertilized eggs per experiment (n = 15 female donors per experiment); this number was obtained by dividing the average number of eggs by 15.

[f]Results recorded as total number of oocytes produced for a given number of female donors; this number was obtained by dividing the total number of oocytes by the number of female donors.

female mice dosed in this manner with PMSG unexpectedly had a greater yield of oocytes. This experience is in direct contrast to experimental observations that 2 or 3 PMSG injections (separated by 24-h intervals) reduced the number and quality of oocytes.[4] We sought to replicate our preliminary observations and determine whether they were due to prolonged priming of the donors or to increased weight of the mice (after a 1-wk hiatus). However, we were unable to generate the expected increase in oocyte number in FVB/NHsd female mice. Two doses of PMSG decreased the overall number of oocytes in this strain, although the number of fertilized oocytes was not affected significantly. In fact, the majority of lines tested showed only slight differences in numbers of fertilized oocytes, in contrast to results from the previous study, which used a shorter dosage interval,[4] with several notable exceptions. For B6D2F1/Hsd donors, 2 doses of PMSG decreased both the total number of oocytes and number of fertilized oocytes. This effect appears to be due both to a negative effect of the PMSG and to the increasing weight of the females, as oocyte numbers were equivalent to group 3 results rather than the expected group 2 results. For BALB/cAnNCr female mice, weight appears to be the major factor in the decrease of oocytes, in that fertility drops off sharply from group 2 to group 3, and the 2-dose PMSG regimen would result in donors progressing from weight group 2 to group 3 due to the 1-wk delay in mating. This result correlates well with previous observations.[6] C57BL/6NHsd strains produced overall greater numbers of fertilized oocytes after receiving 2 doses of PMSG. This result was unexpected but fortuitous, because many projects use C57BL/6N strains. If a project is stalled unexpectedly after PMSG injection, female C57BL/6N donors can be reinjected the following week without compromising their fertility. In fact, several strains showed only slight decreases in fertilized oocyte numbers when given 2 doses of PMSG (Table 4). Thus, with the exception of projects using B6D2F1 and

BALB/c strains, any project in which PMSG has been given but the mice are not used immediately is still viable—the female mice can be placed on the shelf for a week and then reinjected, with only a small decrease in oocyte numbers. A comparison of ovarian histology over time after single and double doses of PMSG would indicate whether hormone-induced follicles from the first injection are stalled and then later released after HCG administration or whether they are actively degrading, with additional follicles maturing after the second dose of PMSG.

This study has shown that the weight of donor female mice can be used as an optimizing factor for the response to superovulation and that the dosage and timing of PMSG injections that will produce the greatest numbers of oocytes will differ depending on the strain used. However, we caution that these results are dependent on the source of PMSG, as well as on close attention to the preparation and storage of the hormone. Our results suggest that the source of mice (particularly C57BL/6) could affect superovulation results and should be considered when assessing project needs. Various other factors can affect results also, including light times, housing, room location and noise or vibrations in the animal room. Our analysis demonstrates that for our facility and for several commonly used strains, the weight of the female mice and dosage of PMSG are key factors in maximizing superovulation rates, providing other variables are controlled.

Acknowledgments

We acknowledge the assistance of David Galipp, Chad Smith, and Jennifer Alana. We appreciate the critical reading of our submission by Dr Richard Behringer. This study was supported by the GLAS program of AALAS (to JPT) and by Cancer Center Support Grant NCI no. CA016672 (GEMF and RASF–Houston).

References

1. **Auerbach AB, Norinsky R, Ho W, Losos K, Guo Q, Chatterjee S, Joyner AL.** 2003. Strain-dependent differences in the efficiency of transgenic mouse production. Transgenic Res **12:**59–69.
2. **Byers SL, Payson SJ, Taft RA.** 2006. Performance of 10 inbred mouse strains following assisted reproductive technologies (ARTS). Theriogenology **65:**1716–1726.
3. **Charles River Laboratories.** 2010. Research models and services US. [Cited 29 November 2011]. Available at: http://www.criver.com/SiteCollectionDocuments/rm_mm_c_2010_US.pdf
4. **Edgar DH, Whalley KM, Mills JA.** 1987. Effects of high-dose and multiple-dose gonatotropin stimulation on mouse oocyte quality as assessed by preimplantation development following in vitro fertilization. J In Vitro Fert Embryo Transf **4:**273–276.
5. **Gates AH.** 1956. Viability and developmental capacity of eggs from immature mice treated with gonadotrophins. Nature **177:**754–755.
6. **Gates AH.** 1971. Maximizing yield and developmental uniformity of eggs, p 64–75. In: Daniel JC, editor. Methods in mammalian embryology. San Francisco (CA): WH Freeman.
7. **Harlan Laboratories.** [Internet]. Research models by product type. [Cited 29 November 2011]. Available at: http://www.harlan.com/products_and_services/research_models_and_services/research_models_by_product_type/
8. **LeFur N, Kelsall SR, Mintz B.** 1996. Base substitution at different alternative splice donor sites of the tyrosinase gene in murine albinism. Genomics **37:**245–248.
9. **Legge M, Sellens M.** 1994. Optimization of superovulation in the reproductively mature mouse. J Assist Reprod Genet **11:**312–318.
10. **Nagy A, Gertsenstein M, Vintersten K, Behringer R.** 2003. Manipulating the mouse embryo: a laboratory manual, p 148–149. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press.
11. **Ohta H, Sakaide Y, Wakayama T.** 2009. Age- and substrain-dependent sperm abnormalities in BALB/c mice and functional assessment of abnormal sperm by ICSI. Hum Reprod **24:**775–781.
12. **Ozgunen KT, Erdogan S, Mazmanoglyu N, Pamuk I, Logoglu G, Ozgunen T.** 2001. Effect of gonadotrophin dose on oocyte retrieval in superovulated BALB/c mice. Theriogenology **56:**435–445.
13. **Spearow JL.** 1988. Major genes control hormone-induced ovulation rate in mice. J Reprod Fertil **82:**787–797.
14. **Spearow JL, Barkley M.** 1999. Genetic control of hormone-induced ovulation rate in mice. Biol Reprod **61:**851–856.
15. **Sugiyama F, Kajiwara N, Hayashi S, Sugiyama Y, Yagami K.** 1992. Development of mouse oocytes superovulated at different ages. Lab Anim Sci **42:**297–298.
16. **Suzuki O, Asano T, Yamamoto Y, Takano K, Koura M.** 1996. Development in vitro of preimplantation embryos from 55 mouse strains. Reprod Fertil Dev **8:**975–980.
17. **Taconic.** [Internet]. Animal models: Black 6; traditional inbred mice. [Cited 29 November 2011]. Available at: http://www.taconic.com/user-assets/Images/growth_charts/C57Black6Mice.jpg
18. **Taconic.** [Internet]. Animal models: FVB; traditional inbred mice. [Cited 29 November 2011]. Available at: http://www.taconic.com/user-assets/Images/growth_charts/FVA_Mice.jpg
19. **Taconic.** [Internet]. Animal models: B6D2F1 NIH; traditional hybrid mice. [Cited 29 November 2011]. Available at: http://www.taconic.com/user-assets/Images/growth_charts/B6D2F1.jpg
20. **Taconic.** [Internet]. Animal models: BALB/cA Bom; traditional inbred mice. [Cited 29 November 2011]. Available at: http://www.taconic.com/user-assets/Images/growth_charts/BALB-cABom.jpg
21. **Taconic.** [Internet]. Animal models: ICR; traditional outbred mice. [Cited 29 November 2011]. Available at: http://www.taconic.com/user-assets/Images/growth_charts/ICR_mice.jpg
22. **The Jackson Laboratory.** [Internet]. Body weight information—JAX mice strain BALB/cJ. [Cited 29 November 2011]. Available at: http://jaxmice.jax.org/support/weight/000651.html
23. **The Jackson Laboratory.** [Internet]. Body weight information—JAX mice strain C57BL/6J. [Cited 29 November 2011]. Available at: http://jaxmice.jax.org/support/weight/000664.html
24. **Tukey JW.** 1953. The problem of multiple comparisons. Unpublished manuscript. In: The collected works of John W. Tukey VIII. Multiple comparisons: 1948–1983, p 1–300. New York (NY): Chapman and Hall.
25. **Vergara GJ, Irwin MH, Moffatt RJ, Pinkert CA.** 1997. In vitro fertilization in mice: strain differences in response to superovulation protocols and effect of cumulus cell removal. Theriogenology **47:**1245–1252.
26. **Whittingham DG.** 1971. Culture of mouse ova. J Reprod Fertil Suppl **14:**7–21.
27. **Zurita E, Chagoyen M, Cantero M, Alonso R, Gonzalez-Neira A, Lopez-Jimenez JA, Landel CP, Benitez J, Pazos F, Montoliu L.** 2011. Genetic polymorphisms among C57BL/67 mouse inbred strains. Transgenic Res **20:**481–489.