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Long-term effects of embryo freezing in mice

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ABSTRACT **Embryo cryopreservation does not induce clear-cut anomalies at detectable rates, but several mechanisms exist for nonlethal damage during the freeze–thaw process, and the risk of moderate or delayed consequences has not been extensively investigated. In a long-term study including senescence, we compared cryopreserved and control mice for several quantitative traits. Significant differences were seen in morphophysiological and behavioral features, some of them appearing in elderly subjects. Thus, apart from its immediate toxicity, embryo cryopreservation, without being severely detrimental, may have delayed effects. These results, consistent with other findings, question the neutrality of artificial reproductive technologies and draw attention to the preimplantation stages in developmental toxicology.**

Embryo freezing is common practice in several species including humans. This technique can be lethal to some embryos but is not considered to have any delayed effect. However, several arguments question such a viewpoint. Several targets of major importance to further development and life are present in the early mammalian embryo: the nuclear DNA, of course, and also the mitochondrial genome (1) and early processes such as those related to imprinting (2, 3), which are completed after fertilization. Freezing–thawing involves dramatic cellular and biochemical changes, such as enzyme inactivation (4–6), ionic disturbances, or attack by free radicals (7–9), that through various pathways could damage these critical components or processes. The current opinion—that embryo freezing has no late consequences—relies on the negative results of previous experimental studies (10–15) and on common experience in cattle and humans. More recently, however, damage to the genetic material from freezing has been reported (16–18). Furthermore, in domestic species as in humans, investigations have mainly focused on patent defects at birth or in early life. However, for complex organisms in which many genetic changes, especially mitochondrial, may have only mild or delayed effects, such indicators provide only a limited assessment of the broad spectrum of anomalies that could result from mutagenic or toxic action on the early embryo. More detailed investigation and long-term follow-up are necessary to comprehensively assess embryo-freezing consequences in mammals. We report here the results of a study comparing mice derived from cryopreserved and control embryos in several morphophysiological, sensorimotor, and behavioral traits, from birth to senescence. The experiment was done on two different hybrid genotypes: C57BL6/CBA F_1 (B6CBA) and C3H/DBA2 F_1(C3D2).

MATERIALS AND METHODS

Embryo Collection. After superovulation (pregnant mare serum gonadotropin, 5 international units, and human chorionic gonadotropin, 5 international units, 48 hr later), 8-week-old C57BL/6JIco (B6) and C3H/OuJIco (C3) females were mated with CBA/JIco (CBA) and DBA/2JIco (D2) males, respectively. B6CBA and C3D2 embryos were collected in M2 medium (19), at the two-cell stage (40–42 hr after human chorionic gonadotropin) by oviduct flushing. The morphologically normal embryos of each oviduct were randomly assigned to either the frozen or the control group (60% and 40% of embryos, respectively). The control embryos were cultured for 2 hr in M16 medium (20) before transfer, whereas the others were directly prepared for freezing.

Cryopreservation. The freezing procedure, modified from Renard (21), included a three-step cryoprotection by 1,2-propanediol in M2 medium (0.5 M, 1 M, and 1.5 M, 3 min each) before transferring the embryos in 50 μl of the 1.5 M 1,2-propanediol mixture into 0.5-ml plastic straws. After slow freezing (2°C/min from 2°C to −7°C, manual seeding at −7°C, 0.3°C/min from −7°C to −40°C, 10 min equilibration) in an automated device (Minicool LC 40, Air Liquide, Marne-la-Vallée, France), the embryos were plunged in liquid nitrogen for storage. They were thawed 15 days later, when a new series was collected. Rapid thawing was achieved at room temperature. The cryoprotectant was gradually diluted in M2 medium containing 0.1 M sucrose; then the embryos were cultured for 2 hr in M16 medium before transfer.

Embryo Transfers and Births. Day 1 pseudopregnant females (outbred stock NMRI/I co) received 10 morphologically normal embryos, all from the same group, by oviduct transfer. Age and weight of the females were controlled to be 8 weeks and 28–30 g. In each series, transfers of freeze–thawed and control embryos from the two genotypes were alternated. Eleven experimental series were planned over a 6-mo period to allow a parallel production of control and cryopreserved mice. Birth dates and litter sizes were recorded twice a day from the 19th day after transfer. Litters of less than five pups were excluded. Finally, 193 cryopreserved and 141 control mice were followed until they reached 39 weeks of age. At that time, for practical reasons, only two males and two females randomly selected from each litter were kept (cryopreserved, 90; controls, 75) until they reached 20 mo. To ensure that the experiment was done in a blind manner, the pregnant females and their litters were coded until the young mice were weaned. Then each mouse was coded individually. Males and females were separated at weaning and housed three to five per cage under standard conditions of temperature, diet, and 12 hr–12 hr light–dark photoperiods.

Parameters Evaluated. *General data.* These included gestation rates and duration, litter size, sex ratio, incidence of external malformations, growth, viability, and morbidity.

Preweaning development. Early development was evaluated during the first 2 weeks after birth, using a set of nine sensorimotor and morphological criteria (22, 23). For each criterion, the individual score was the age (calculated from the

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Table 1. Body weight in adults

Time	Body weight, g							
	B6CBA				C3D2			
	Male		Female		Male		Female	
	C	CP	C	CP	C	CP	C	CP
39 weeks	42.6 ± 0.6	42.3 ± 0.5	32.6 ± 0.7	35.5 ± 0.6	44.0 ± 0.8	44.8 ± 0.7	31.4 ± 0.7	32.6 ± 0.8
67 weeks*	40.9 ± 1.1	47.1 ± 0.8	41.4 ± 1.8	43.2 ± 1.4	41.1 ± 1.3	43.7 ± 0.9	38.5 ± 2.1	41.1 ± 1.7
Variation (%)	(2.4)	(11.1†)	(29.2)	(28.1)	(−3.2)	(0.5)	(25.3)	(25.4)

Observed weights (g ± SEM) with individual variation rates between 39 and 67 weeks. C, controls; CP, cryopreserved. In ANOVA, each observed value was replaced by the difference between this observed value and the value calculated from the regression with litter size. Sample sizes ranged from 25 to 66 at 39 weeks and from 13 to 28 at 67 weeks. Slight discordances between mean values of individual weight variation from 39 to 67 weeks and observed weights at these dates are due to the random sampling made after 39 weeks of age (two males and two females from each litter).

*, Significant overall effect ($P < 0.05$) of cryopreservation. †, Significantly different from corresponding controls ($P < 0.01$, t test).

date of embryo transfer) at which the pup reached the adult pattern. Only the litters of at least six pups were used in this part of the experiment. When the results were analyzed, males and females were pooled, as no difference between sexes has been reported for these parameters (22, 23).

Behavior. Behavioral assessments have proven sensitive enough to provide evidence for subtle teratogenic or mutagenic effects (24, 25). We carried out several behavioral tests on animals between the age of 3 mo and 20 mo. Most tests are standard for which methodology is well known. Some tests evaluated activity and exploratory behavior: running wheel and hole-board tests were done with 3-mo-old mice on a sample of 20 males and 20 females from each group; open-field activity was recorded during 3-min sessions in a computerized device (details in ref. 25) at 7–8 mo in all available subjects ($n = 313$).

Two other tests involved learning processes. The first one was the Krushinsky test (26, 27), which evaluates the ability of an animal to find, without previous experience, a dietary stimulus (milk) that has been presented and then removed from the animal's visual field. The whole test included six sessions of 10 min maximum each over 3 consecutive days. It was done on a sample of 250 mice at 3–6 mo of age. The second test, studying active conditioned avoidance, used the classical shuttle-box, where the animal has to avoid a nociceptive stimulus (an electric shock) preceded by an acoustic and visual signal (for details, see ref. 25). Four tests were performed: (*i*) one test on all available subjects at 7–8 mo; (*ii* and *iii*) tests on mice selected from each litter 7 and 35 days, respectively, after the first test; and (*iv*) a test that occurred 10–12 mo later.

Mandible Morphometry. Mandible shape is a highly heritable mouse quantitative trait, which appears to be under the control of >100 genes (28, 29). Most inbred strains can be characterized by a discriminant analysis of mandible measurements. We studied a sample of 150 subjects between 10 and 12 mo of age. After preparation of the right half of the mandibles, 11 measurements were recorded under a ×10 dissecting microscope. Data were corrected for size by expressing each value as a percentage of the total.

Statistics. The data were analyzed in a three-way ANOVA to study the effects of treatment (cryopreservation or not), sex, and genotype, except in the preweaning tests for which only genotype and treatment were studied in a two-way ANOVA. A multivariate analysis of variance (MANOVA) and a canonical discriminant analysis were also used for mandible morphometry. Qualitative parameters were compared by χ^2 tests, and viability curves were analyzed by the Cox regression model [procedures from Statistical Analyses System (SAS Institute, Cary, NC), version 6, 1987 and Biomedical Data Package (34)].

RESULTS

General Data. Seventy-nine of the 108 female mice that received embryos became pregnant. Pregnancy rates and gestation durations were remarkably similar in all groups. Litter sizes were lower in C3D2 groups but were not significantly modified by freezing in either genotype. Sex ratios did not differ from expected values. External defects or neonatal deaths were very rare. Postnatal weights showed no significant variations after correction for litter size. Irrespective of cryo-

Table 2. Preweaning development

Criterion	Criterion appearance, days				ANOVA		
	B6CBA		C3D2				
	C	CP	C	CP	Genotype	Treatment	Interaction
R	25.12 ± 0.09	25.35 ± 0.17	25.41 ± 0.11	25.95 ± 0.18	*	*	NS
HP	26.58 ± 0.25	26.54 ± 0.22	27.17 ± 0.25	27.38 ± 0.29	*	NS	NS
DCE	26.52 ± 0.19	25.90 ± 0.17	26.45 ± 0.21	27.17 ± 0.21	*	NS	†
DR	28.06 ± 0.26	27.93 ± 0.25	28.48 ± 0.25	28.69 ± 0.20	*	NS	NS
BH	30.48 ± 0.14	30.12 ± 0.14	30.87 ± 0.17	31.34 ± 0.16	†	NS	*
VP	27.19 ± 0.12	27.22 ± 0.14	27.31 ± 0.12	28.09 ± 0.05	†	*	*
BG	27.09 ± 0.20	27.32 ± 0.09	26.64 ± 0.10	28.00 ± 0.15	NS	†	*
AI	29.87 ± 0.12	30.21 ± 0.17	28.18 ± 0.20	29.71 ± 0.22	†	†	†
EYO	33.21 ± 0.06	33.10 ± 0.13	32.77 ± 0.11	33.97 ± 0.27	NS	†	†

Gestational ages (number of days between embryo transfer and date of recording) of appearance of nine developmental criteria (means ± SEM): R, righting; HP, hindlimb placing; DCE and DR, disappearance of crossed extensor response and rooting response, respectively; BH, bar holding; VP, vibrissae placing; BG, bulging of gum; AI, appearance of the two incisors of lower mandible; EYO, eyelid opening. C, control mice; CP, cryopreserved mice. (*, $P < 0.05$; †, $P < 0.001$). Underlined values indicate significant ($P < 0.05$) differences in partial comparisons between cryopreserved and control mice. Sample sizes were >28 in all groups.

preservation, viability between birth and 67 weeks was lower in C3D2 animals ($P < 0.01$, data not shown). No particular disease was found to occur in cryopreserved mice.

However, in adult and senescent cryopreserved mice we observed increased body weights (Table 1), without any relation to either maternal weight or litter size. The effect of cryopreservation remained significant at 67 weeks, even when data were corrected for litter size. This increase was most obvious in the cryopreserved B6CBA males, which exhibited a 11.1% weight increase between 39 and 67 weeks, whereas the corresponding controls and the C3D2 males showed a 2.4% variation. Patent obesity was observed but not specifically in the cryopreserved mice, and the nature of this abnormal weight gain remained unclear. By contrast, weight evolution was identical in all female groups.

Preweaning Development. Table 2 presents the values recorded for the appearance of the nine criteria studied. Both genotype and cryopreservation induced significant effects (*, $P < 0.05$; †, $P < 0.001$). Development was slower in the C3D2 mice. When significant, the effect of cryopreservation almost exclusively concerned this genotype, in which seven of the nine criteria were delayed in the cryopreserved animals, whereas only one difference between cryopreserved mice and control mice was found in the B6CBA group. Consequently, a significant interaction between genotype and cryoconservation was found in several cases. Two other analyses (ANOVA with data corrected for litter size and a simulation using four subjects per litter without modifying means or variances) confirmed these results, which suggested a differential response of the two genotypes to cryopreservation.

Behavior. The activity tests, running wheel, hole board, and open-field, showed significant effects of genotype and sex but no overall influence of cryopreservation. However, interactions were found between cryopreservation and sex or genotype (data not shown). In the Krushinsky test, significant overall effects of sex and genotype were also found: for example, the C3D2 mice were slower than the B6CBA mice ($P < 0.001$), and the males were slower than the females ($P < 0.001$) in reaching their best performances. In addition to these natural differences, a significant effect of cryopreservation was seen, which appeared to depend on sex and genotype (Fig. 1): the cryopreserved B6CBA females were significantly less efficient than the control females in reaching their best performance, whereas there were no significant variations due to cryopreservation in either C3D2 sex or in the B6CBA males. Active avoidance conditioning (Fig. 2) also revealed that effects of cryopreservation were present, although less important than those of genotype or sex and in complex relation with them: in the first three tests, where the performances were significantly higher in B6CBA than in C3D2 mice and in males than in females, neither the results nor their improvement was modified by cryopreservation. However, at senescence (at least 10 mo later), the relative decrease of performance diverged among groups, with an interaction ($P < 0.01$) between genotype and cryopreservation: the cryopreserved C3D2 mice, especially the males, showed better results than the control mice ($P < 0.05$), whereas the opposite was observed in the B6CBA mice, especially the females.

Mandible Morphometry. Multivariate analysis demonstrated a significant overall effect of cryopreservation ($P < 0.01$), as well as effects of sex ($P < 0.0001$) and genotype ($P < 0.0001$). There was no interaction between treatment and either sex or genotype. When a three-way ANOVA was done for each of the 11 parameters, three of them showed significant differences due to freezing. The same results were found in a canonical discriminant analysis (Fig. 3), which clearly showed that, here too, the effect of cryopreservation was smaller than that of the sex and genotype.

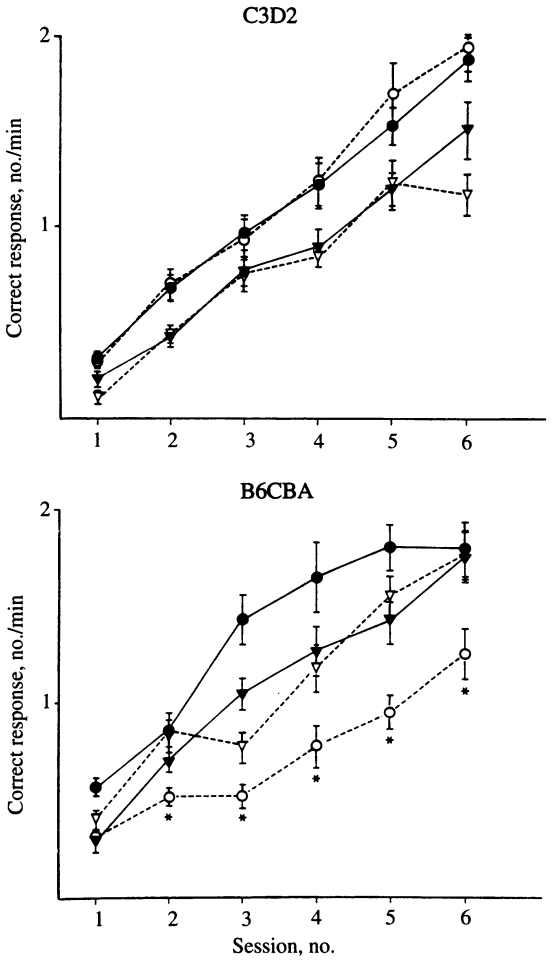

FIG. 1. Krushinsky test. Ratios of number of correct responses over session duration in C3D2 (*Upper*) and B6CBA (*Lower*) mice. ○, Cryopreserved females; ●, control females; ▽, cryopreserved males; ▼, control males. In C3D2 animals, cryopreserved and control subjects obtained very similar results for all parameters, contrasting with the sharp differences between males and females. In B6CBA mice, no effect of cryopreservation was found in males, but cryopreserved females performed less well than controls (*, $P < 0.001$) with increased session durations and fewer correct responses ($P < 0.01$, data not shown). The testing device is a rectangular box with three openings in the front wall where the animal can lap sweetened milk. Six sessions were conducted over 3 days; in each session, the mouse (fasting from the evening before) was given 10 opportunities (trials) to find food. Each trial began when the subject tasted food in the middle opening, at which time the cup was moved left or right. The animal could visually and acoustically follow the movement and (*i*) go to the side where the food had been transferred (correct answer), (*ii*) go to the empty side (incorrect answer), (*iii*) make no choice, in which case food was returned to the middle opening after 1 min. The session was stopped after either 10 trials or 10 min, whatever the number of trials the mouse had then reached. About 30 mice (29–33) between 3 and 6 mo of age were studied in each category.

DISCUSSION

This study has confirmed that, in mice, embryo cryopreservation does not induce major anomalies, even in senescence. Nevertheless, this technique does not appear to be absolutely free of long-term effects. Indeed, by studying endpoints sensitive enough to show variations due, for example, to sex or genotype, we have also found significant differences between cryopreserved mice and control mice.

The influence of cryoconservation may seem disconcerting because the differences from the controls were of moderate amplitude and could depend on genotype, sex, or age. An

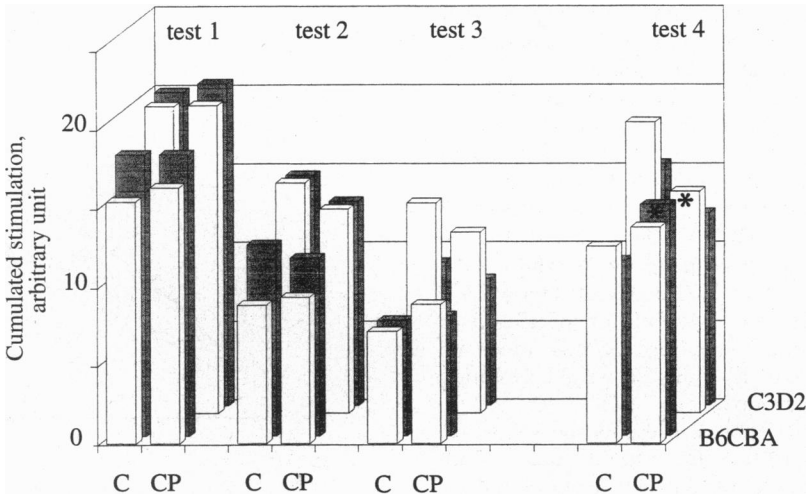

FIG. 2. Conditioned avoidance. Synthetic view of conditioning performances in adulthood and senescence. Bars represent the cumulated durations of conditioned-stimulus delivery over the 50 trials (arbitrary units). Higher durations mean lower performances. C, controls; CP, cryopreserved; light bars, males; dark bars, females. Test 1 was done at 7–8 mo of age on all available mice. Further performances were assessed on two males and two females randomly selected from each litter. Tests 2 and 3 occurred 7 and 35 days after test 1, respectively. Test 4 was performed when mice were between 17- and 20-mo-old. Sample sizes were 30–66 in test 1 and 10–26 in test 4. Each test lasted 25 min (one trial every 30 sec, 50 trials). *, Different from controls ($P < 0.05$, genotype–treatment interaction: $P < 0.01$).

immediate, essential concern is whether such differences reflect real biological changes. Gradual variations are frequent in multifactorial traits. Variable responses to a given agent in relation to the genetic background are commonly observed as well, even within one species and, with respect to cryopreservation, strain or individual differences have been demonstrated concerning embryo postthaw viability in mice (30) or cytogenetic damage in human fibroblasts (18). Furthermore, if one considers the possible explanations for initial alterations due to cryopreservation (whether these be nuclear mutations, mitochondrial mutations, or epigenetic changes), it would in fact, be surprising to find clear-cut and constant phenotypes because the number, as well as the precise localization, of these events could differ from one mouse to another. Thus, here as in other experiments where low teratogenic or mutagenic risks were assessed (24, 25), subtle and complex effects might actually represent an appreciable part of the induced phenotypical changes. Although such effects are more difficult to assess and explain, they must retain our attention. In this experiment, the number of statistically significant differences and the fact that several of them were evidenced as main effects in the analysis make it very unlikely that they are all due to sampling variations. One should also note that when a significant effect of cryopreservation was found, its size [|difference|/SD, as defined by Cohen (31)] was often similar to the values found for either sex or genotype. However, only the demonstration of the involved cellular events will unequivocally confirm that late effects of embryo freezing can actually exist.

What would the eventuality of such delayed consequences after embryo freezing imply? This can be discussed at different levels. One level is the individual one, where the main problem is the extent to which these effects would disturb or alter the subject's capacities and health. From this point of view, changes such as those we have observed might be considered as a negligible risk, when compared with others. However, depending on the mechanisms involved, other anomalies might be identified in the future. Furthermore, there is no certainty that the intensity of a given effect would be the same in every species, and effects evaluated as being similar by objective methods might not have an identical meaning in all cases. For instance, the risk of a body-weight increase at senescence may not be considered in the same way in cattle or laboratory mice as in the long-living human. Finally, beyond the consequences for the cryopreserved subject comes the question of risks for the next generations, a major problem that we could not address here. Obviously, a much more comprehensive understanding of the effects of embryo cryopreservation in different species is needed to answer these questions.

Another more general level would concern some aspects of the relation between environment and reproduction. Our

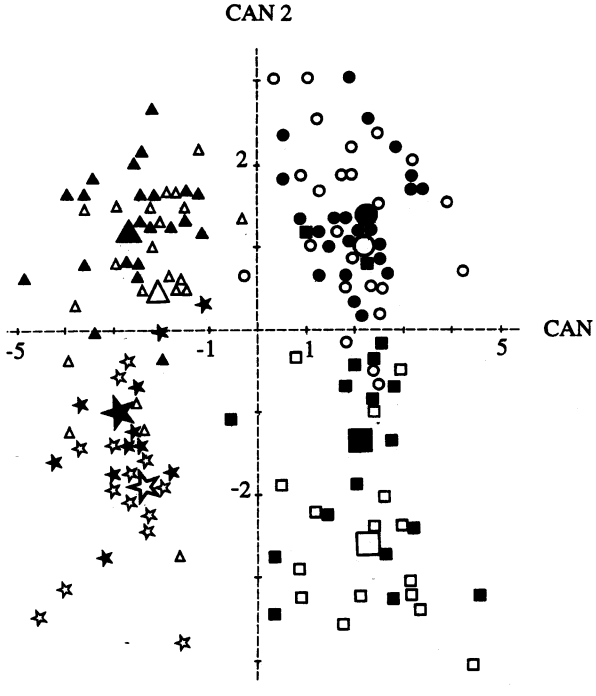

FIG. 3. Mandible morphometry. Individual and mean values of the two main canonical variables (CAN 1 and CAN 2, accounting for 96% of total variance), calculated from the 11 mandible shape parameters (canonical discriminant analysis, SAS procedure DISCRIM). Large symbols, mean values; ● ○, B6CBA males; ▲ △, B6CBA females; ■ □, C3D2 males; ★ ☆, C3D2 females; light symbols, cryopreserved subjects; dark symbols, controls. Sex and genotype are clearly separated. Differences between cryopreserved and control subjects are less important but are significant ($P < 0.05$, t test) in three of the four sex–genotype combinations for canonical variable 2.

results suggest that, beyond its immediate damage, embryo freezing, without being highly detrimental, may not be completely neutral. Similar doubts are emerging about other types of embryo manipulations, such as cloning, and support the idea that events occurring at early preimplantation stages might affect later processes (32). As recently outlined by Reik *et al.* (33), this raises concerns about possible unexpected consequences of modern reproductive technologies. More generally, it would also give more importance to the preimplantation embryo as a potential target to environmental hazards.

We conclude that substantial arguments support the hypothesis that embryo freezing can have delayed consequences. A large variability of these effects according to genotype is to be expected, which commands the greatest caution in extrapolating from one species to another. However, the possibility of delayed consequences could perhaps justify a more limited use of this technique in clinical practice until clearer conclusions about its effects in human embryos can be drawn. We suggest that progress in this field might come from investigating freezing damage to mitochondrial DNA, nuclear DNA, and to the early nucleo–cytoplasmic interactions.

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1. Wallace, D. C. (1992) *Science* **256,** 628–632.
2. Monk, M. & Grant, M. (1990) *Development (Cambridge, U.K.),* Suppl. 55–62.
3. Latham, K. E. & Solter, D. (1991) *Development (Cambridge, U.K.)* **113,** 561–568.
4. Rammler, D. H. (1967) *Ann. N.Y. Acad. Sci.* **141,** 291–299.
5. Fahy, G. M. (1986) *Cryobiology* **23,** 1–13.
6. Carpenter, J. F. & Crowe, J. H. (1988) *Cryobiology* **25,** 244–255.
7. Alvarez, J. G. & Storey, B. T. (1992) *J. Androl.* **13,** 232–241.
8. Petrenko, A. Y. (1992) *Cryobiology* **29,** 144–152.
9. Whiteley, G. S. W., Fuller, B. J. & Hobbs, K. E. F. (1992) *Cryobiology* **29,** 668–673.
10. Ashwood-Smith, M. J. (1985) *Cryobiology* **22,** 423–433.
11. Sidyakina, T. M. & Golimbet, V. E. (1991) *Cryobiology* **28,** 251–254.
12. Glenister, P. H., Whittingham, D. G. & Lyon, M. F. (1984) *J. Reprod. Fertil.* **70,** 229–234.
13. Glenister, P. H. & Lyon, M. F. (1986) *J. In Vitro Fertil. Embryo Transfer.* **3,** 20–27.
14. Mobraaten, L. E. (1986) *J. In Vitro Fertil. Embryo Transfer* **3,** 28–32.
15. Mobraaten, L. E. (1987) *Cryobiology* **24,** 586 (abstr.).
16. Shaw, J. M., Kola, I., MacFarlane, D. R. & Trounson, A. O. (1991) *J. Reprod. Fertil.* **91,** 9–18.
17. Bouquet, M., Selva, J. & Auroux, M. (1993) *Biol. Reprod.* **49,** 764–769.
18. Rudd, N. L., Hoar, D. I., Williams, S. E. & Hennig, U. G. G. (1989) *Genome* **32,** 196–202.
19. Quinn, P., Barros, C. & Whittingham, D. G. (1982) *J. Reprod. Fertil.* **66,** 161–168.
20. Whittingham, D. G. (1971) *J. Reprod. Fertil. Suppl.* **14,** 7–21.
21. Renard, J. P. & Babinet, C. (1984) *J. Exp. Zool.* **230,** 443–448.
22. Roubertoux, P. L., Ragueneau, S., Baumann, L. & Semal, S. (1987) *Behav. Genet.* **17,** 453–454.
23. Roubertoux, P. L., Nosten-Bertrand, M., Cohen-Salmon, C. & Lhotellier, L. (1992) in *Techniques for the Genetic Analysis of Brain and Behavior: Focus on the Mouse,* eds. Goldowitz, D., Wimer, D. & Wahlsten, D. (Elsevier, Amsterdam), pp. 423–444.
24. Vorhees, C. V. (1974) *Teratology* **10,** 269–274.
25. Auroux, M., Dulioust, E., Selva, J. & Rince, P. (1990) *Mutat. Res.* **229,** 189–200.
26. Krushinsky, L. V., Astaurova, N. B., Kouznetzova, L. M., Otchinskaya, E. I., Poletaeva, I. I., Romanova, L. G. & Sotskaya, M. N. (1975) in *Current Problems in Behavioral Genetics,* eds. Fedorov, V. K. & Ponomarenko, V. V. (Nauka, St. Petersburg, Russia), pp. 98–110.
27. Poletaeva, I. I., Popova, N. V. & Romanova, L. G. (1993) *Behav. Genet.* **23,** 467–481.
28. Bailey, D. W. (1985) *J. Hered.* **76,** 107–114.
29. Festing, M. F. W. (1972) *Nature (London)* **238,** 351–352.
30. Schmidt, P. M., Schieve, M. C. & Wildt, D. E. (1987) *Biol. Reprod.* **37,** 1121–1128.
31. Cohen, J. (1977) *Statistical Power for the Behavioral Sciences* (Academic, New York).
32. Ozil, J. P. (1990) *Development (Cambridge, U.K.)* **109,** 117–127.
33. Reik, W., Römer, I., Barton, S. C., Surani, M. A., Howlett, S. K. & Klose, J. (1993) *Development (Cambridge, U.K.)* **119,** 933–942.
34. Dixon, W. J., Brown, M. B., Engelman, L. & Jennrich, R. I. (1990) *BMDP Statistical Software Manual* (Univ. of Calif. Press, Berkeley).