Theor Appl Genet (1994) 88:479–485 

T. A. Armbrust · E. J. Eisen

# Effect of cryoprotectant and genetic selection for body fat content on embryonic cryosurvival in mice

Received: 25 August 1993 / Accepted: 14 September 1993

**Abstract** Lines of mice selected for high (HF) or low (LF) 12-week epididymal fat pad weight as a percentage of body weight were used to investigate the effects of genotype, two cryoprotectants [glycerol (GLY) and propylene glycol (PG)] and genotype × cryoprotectant interaction on cryosurvival of four and eight-cell embryos. Embryos were collected from selection lines and reciprocal crosses of selection lines (HFLF and LFHF) and frozen by established slow-cool methods. Embryos were thawed for 40 s at room temperature and then placed in a 37° C waterbath for 1 min. Cryoprotectant was diluted from embryos with either 0.5 *M* sucrose (GLY-treated) or 1.0 *M* sucrose (PG-treated). Post-thaw survival was measured as the percentage of embryos developing to 36 h (PTS36), 48 h (PTS48) and hatched blastocyst (PTSHB), respectively. Non-frozen controls were cultured concurrently with frozen embryos. No significant genotype or genotype × cryoprotectant interaction effects were found. Results of the embryo freezing study indicated that selection for high or low fat content did not affect the ability of embryos to survive cryopreservation. There was no indication of embryo heterosis for post-thaw survial. Embryos frozen with GLY survived the freeze-thaw stress significantly better than those frozen in PG ($P < 0.05$). In vitro development of non-frozen controls at 36 and 48 h did not vary significantly among lines, but in vitro development was significantly different among lines at the hatched blastocyst stage ($P < 0.05$). Linear contrasts showed that the embryonic genome was responsible for differential in vitro development at the hatched blastocyst stage between these selected lines (HF > LF; $P < 0.05$); asymmetric response also occurred in that both HF and LF exceeded the unselected control line ($P < 0.05$).

**Key words** Correlated responses · Embryos · Cryoprotectant · Mice

## Introduction

Cryopreservation of mammalian embryos has benefitted agricultural and biomedical research since the first live births from frozen-thawed mouse embryos were reported (Whittingham et al. 1972). Embryos of most livestock species have been successfully cryopreserved (Leibo 1985), and more recently attempts have been made to freeze embryos of non-domesticated species (Dresser et al. 1984). Currently, human fertility treatments are using cryopreservation to solve the moral issues concerned with excess embryos produced during treatment (Chen 1988; Testart 1988). Cryopreservation has affected the cattle breeding industry by allowing breeders to maximize the reproductive output of genetically superior dams using superovulation and in vitro fertilization techniques in conjunction with embryo freezing (Leibo 1985). Possibly the most valuable aspect of embryo freezing is the ability to conserve unique genetic material which might otherwise be lost. Embryo banking of mouse embryos has allowed the preservation of mutant, inbred, and special genetic strains that would otherwise have been expensive to maintain (Mobraaten 1986).

While much progress has been made toward improving post-thaw survival (PTS) of mammalian embryos by manipulating freezing and culturing procedures (Mazur 1985, 1988), few studies have examined the influence of genetic variability on PTS (Whittingham and Whitten

The research reported in this publication was funded by the North Carolina Agricultural Research Service (NCARS), Raleigh, NC 27695-7643. Use of trade names in this publication does not imply endorsement by the NCARS of the products named, nor criticism of similar ones not mentioned

Communicated by G. Wenzel

T. A. Armbrust · E. I. Eisen (✉)
Department of Animal Science, Box 7621, North Carolina State University, Raleigh, NC 27695-7621, USA

*Present address:*
Department of Animal Science, University of Tennessee, Knoxville, TN 37901, USA

1974; Whittingham et al. 1977; Schmidt et al. 1985, 1987; Pomp and Eisen 1990). Only the study by Pomp and Eisen (1990) examined PTS of embryos as a correlated response to selection for quantitative traits. Using lines of mice selected for increased litter size (L +) and increased adult body weight (W +) and crosses of selected lines with a randomly selected control line (K), they showed that the L + maternal source was superior to K in PTS but that L + embryo effects were inferior to K, whereas W + showed no correlated responses in PTS.

Although Pomp and Eisen (1990) used glycerol (GLY) as a cryoprotectant, recent studies have evaluated the cryoprotective abilities of glycols such as propylene glycol (PG). Cryopreservation with PG is associated with PTS comparable to or superior to GLY for eight-cell mouse embryos (Renard and Babinet 1984; Hernandez-Ledezman et al. 1988), blastocysts in cattle (Suzuki et al. 1990) and sheep (Schiewe et al. 1991). However, other studies (Miyamoto and Ishibashi 1978; Voelkel and Hu 1992) have reported lower survival rates using PG as a cryoprotectant for murine and bovine embryos. These apparent discrepancies could be the result of confounding cryprotectant with thawing rate, dilution procedure or genotype-by-cryoprotectant interaction, i.e., a differential ranking of PTS of embryo genotypes with different cryoprotectants.

The focus of the research presented here was to examine the role of genotype, cryoprotectant (glycerol vs propylene glycol) and genotype-by-cryoprotectant interaction on the post-thaw survival of mammalian embryos in lines selected divergently for body fat content (Eisen 1987). The low fat line exceeds the high fat line in number of pups born, ovulation rate and preimplantation and total embryos survival (Armbrust and Eisen 1994). One species where embryo freezing has not yet been successful is the pig, possibly due to the high lipid content of the embryos (Fahning and Garcia 1992). Although the lipid content of the mouse embryos from these lines is not known, it is possible that divergent selection for body fat content could modify embryo lipid content and consequently cause a correlated response in PTS. Specific objectives were to (1) test if differences in embryos cryosurvival arose as a correlated response to selection for body fat content in mice, (2) determine if differences in correlated responses occurred at the maternal and/or at the embryo level, (3) determine if embryo heterosis is present and (4) test for interactions of cryoprotectant with the genetic effects described in (1) through (3).

## Materials and methods

### Experimental animals

The lines of mice used in this study were selected within full-sib families in two replicates for high (HF) or low (LF) 12-week epididymal fat pad weight as a percentage of body weight, an indicator trait of body fat percentage (Eisen 1987). Randomly selected controls (RC) were maintained along with selection lines. Selection occurred over 12 generations followed by 7 generations of random selection. At generation 19 the two replicates of each selection treatment were reciprocally crossed to produce the final lines, HF, LF and RC, which were randomly mated in subsequent generations (Eisen and Pomp 1990). This study was conducted with mice from generations 32 and 33. Embryos from the following five populations were studied: HF, LF, reciprocal crosses (HFLF and LFHF; male line written first) and RC.

Experimental mice were housed in a controlled environment at approximately 21°C and 55% humidity with lights on at 0700 hours and off at 1900 hours. Mice were reared in litters standardized at birth to ten pups. From weaning at 21 days to mating they were housed no more than four per cage and allowed free access to Purina Lab Chow 5001 (Purina Mills, Richmond, Ind.) and tap water.

### Embryo collection

Before mating, females were randomly assigned to one of two treatments. Females were mated with a male of the same genotype or, for reciprocal crosses, with a male of opposite genotype, between 1500 and 1700 hours each afternoon. The following morning females were checked for the presence of a copulatory plug, weighed and assigned a sequential number.

Approximately 50–54 h after detection of a copulatory plug, females were killed by cervical dislocation and their oviducts excised into phosphate-buffered saline medium (PB1) (Whittingham 1974) supplemented with bovine serum albumin, fraction V (BSA) (Sigma Chemical, St. Louis, Mo.). Embryos were flushed from the oviducts with PB1 using a 30-gauge blunted needle inserted into the infundibulum. After flushing, the embryos were washed through three 50-µl drops of PB1 and graded as either excellent or poor (Linder and Wright 1983). Excellent embryos were at the four-or-eight-cell stage, spherical and symmetrical with uniformaly sized blastomeres. Embryos which had degenerated blastomeres, cracked zona pellucidae or asymmetrical shape were considered poor and not frozen. All embryos from a donor female were collected, evaluated, frozen, thawed and assayed for survival separately from other donor females.

### Slow-cool freezing and rapid thawing

After washing, all embryos from a single donor female were placed into a 75-µl drop of one of two cryoprotectants, glycerol (Sigma Chemical, St. Louis, Mo.) or propylene glycol (Sigma Chemical, St. Louis, Mo.), in PB1-BSA and allowed to equilibrate 15 min. Embryos were loaded into a 25-cc insemination straw (Veterinary Concepts, Spring Valley, Wis.), which was then heat sealed at both ends and placed in a controlled rate methanol bath freezer (FTS Systems, Stone Ridge, N.Y.) at 0 °C until all of the embryos had been collected for that day. Cooling proceeded at 1°C/min to −7 °C at which time seeding was induced by touching a forceps precooled in liquid nitrogen to the columns of cryoprotectant, which were separated on either side by air bubbles from the 75-µl column of cryoprotectant containing the embryos. Seeding temperature was held for a total of 10 min; then cooling continued at 0.5 °C/min to −35 °C. After holding for 10 min at −35 °C, embryos were plunged directly into liquid nitrogen. They were stored for 1–4 months before thawing.

To rapid thaw (approx. 20–25 °C/min), straws were held in air (20°–25 °C) for 40 s and then plunged into a 37 °C waterbath for 1 min (approx. 360 °C/min). Embryos were immediately expelled into a 10-x 35-mm tissue culture dish (Falcon Becton-Dickinson Labware, Lincoln Park, N.J.). The dilution of glycerol-treated embryos was accomplished by pipetting embryos into a 75-µl drop of 0.5 *M* sucrose in PB1-BSA solution for 5 min. For propylene glycol, embryos were placed in a 75-µl drop of 1.0 *M* sucrose in PB1-BSA for 15 min. Rehydration consisted of removing the embryos from the sucrose to a 50-µl drop of PB1-BSA for 1 min and then washing them through two more 50-µl drops of PB1-BSA. The embryos were again graded by morphological appearance (Linder and Wright 1983), and the num-

bers of good and poor embryos were recorded. Embryos were considered good if they had at least half of their total number of blastomeres intact; poor embryos were those with less than half of their blastomeres intact.

### Culturing and survival assays

Embryos were cultured in 75-μl drops of Whitten's medium (Whitten 1971) supplemented with BSA and antibiotics under light-grade mineral oil (Fisher Scientific, Raleigh, N.C.) in a humidified atmosphere at 37 °C in 5% $CO_2$. Embryos were evaluated morphologically under a 70 × dissecting microscope (Bosch and Lomb Stereozoom Series, Rochester, N.Y.), and post-thaw survival (PTS) was recorded at 36 h (PTS36), 48 h (PTS48) and at 48 h for hatched blastocyst (PTSHB). At 36 h, embryos were considered good if the number of blastomeres had increased or if a blastocoel cavity was forming. All embryos with a blastocoel cavity at 48 h were counted as good. Development to hatched blastocyst was recorded for generation 33 only. Post-thaw survival at each stage was measured as the number of embryos developing to 36 h, to 48 h or to hatched blastocyst, as a percentage of the number of good embryos at initiation of culture, per donor female (Renard and Babinet 1984).

### In vitro controls

Nonfrozen control embryos for in vitro survival assays were recovered, cultured and assayed using the same methods as those used for frozen embryos. Controls were used to test for genetic differences in development without cryostress and to monitor the culture system for any problems. In vitro development was observed three times for each donor female: development to 36 h (IVD36), development to blastocyst at 48 h (IVD48) and development to hatched blastocyst at 48 h (IVDHB). As for the frozen embryos, development to hatched blastocyst was measured for generation 33 only.

### Statistical analysis

Individual donor females were considered as experimental units for PTS to account for donor female variation within lines-by-cryoprotectant-by-generation subclasses (Pomp and Eisen 1991). Due to the heterogeneity of the variances of measurements taken as percentages, a weighted arcsine transformation (Freeman and Tukey 1950) was performed on all post-thaw survival measurements prior to conducting least-squares analysis for unequal subclass numbers (Harvey 1979). Analysis of PTS data was based on a randomized design according to the following linear model:

$$Y_{ijkl} = \mu + S_i + C_j + G_k + (SC)_{ij} + (SG)_{ik} + (CG)_{jk} + (SCG)_{ijk} + e_{ijkl}$$

where $\mu$ was the overall mean: $S_i$, $C_j$ and $G_k$ were the fixed effects of selection line, cryoprotectant, and generation, respectively; $(SC)_{ij}$, $(SG)_{ik}$, $(CG)_{jk}$ and $(SCG)_{ijk}$ were respective interaction effects; and $e_{ijkl}$ was random residual effect. Age and weight at the presence of a copulatory plug were included in the model as covariates when they reached statistical significance.

Planned linear contrasts were carried out on least-squares means of PTS traits as follows:

Selection line divergence: $[(HF)-(LF)]$ measures the correlated response to selection as the difference between the upward- and downward-selected lines.

Reciprocal cross (maternal) effects: $[(LFHF)-(HFLF)]$ estimates divergence due to the maternal genome.

Direct (embryo) effects: $[(HF)-(LF)-(LFHF)+(HFLF)]$ estimates divergence due to the embryonic genome.

Asymmetry: $[(HF)+(LF)-(2RC)]$ tests for difference in the magnitude between upward-and downward-correlated response to selection.

Heterosis (embryo): $0.5[(HFLF)+(LFHF)-(HF+LF)]$ estimates nonadditve genetic effects of the embryo.

## Results

Least-square means of PTS at 36 h culture (PTS36), adjusted for generation effects, are presented in Table 1. PTS36 was significantly greater ($P < 0.05$) for GLY then for PG. Analysis of variance detected no significant differences among lines ($P > 0.05$) or line × cryoprotectant interaction ($P > 0.05$). The covariates, age and weight at mating, were not significant for PTS36 ($P > 0.05$). Least-square means of PTS to blastocyst (PTS48) are presented in Table 2. Results for PTS48 were similar to those for PTS36. Again, GLY had higher PTS than PG ($P < 0.05$). Across cryoprotectants, lines did not differ significantly ($P < 0.05$). Selected lines tended to survive better when frozen in PG whereas crosses had superior survival in GLY, but line-by-cryoprotectant interaction was not significant ($P > 0.05$). Age at mating was not significant, but weight

**Table 1** Least-squares means (arcsine ± SE) of post-thaw survival to 36 h (PTS36)[a] of mouse embryos from selected lines and crosses frozen in propylene glycol or glycerol[b]

| Line | Propylene glycol | | | | Glycerol | | | |
|---|---|---|---|---|---|---|---|---|
| | No. Fem.[c] | No. Fro.[c] | % Rec.[c] | PTS36, % (arcsine ± SE) | No. Fem.[c] | No. Fro.[c] | % Rec.[c] | PTS36, % (arcsine ± SE) |
| HF | 29 | 338 | 98.2 | 41.9(40.0 ± 3.4)[e] | 33 | 395 | 99.2 | 58.7(50.5 ± 3.3)[f] |
| LF | 34 | 408 | 97.3 | 34.8(35.3 ± 3.1)[e] | 34 | 399 | 99.0 | 61.9(52.7 ± 3.2)[f] |
| RC | 58 | 584 | 96.2 | 32.5(33.9 ± 3.4)[e] | 51 | 545 | 98.3 | 58.8(50.2 ± 2.9)[f] |
| HFLF[d] | 29 | 350 | 96.0 | 31.6(32.6 ± 3.4)[e] | 30 | 347 | 99.4 | 64.6(53.7 ± 3.4)[f] |
| LFHF[d] | 36 | 418 | 98.6 | 30.3(32.3 ± 3.2)[e] | 31 | 354 | 99.2 | 66.5(55.5 ± 3.4)[f] |

[a] Percentage of embryos developing in vitro to 36 h per donor female; analyzed by least-squares procedures following arcsine transformation, adjusted for generation effects

[b] Analysis of variance: $P > 0.05$ for line; $P < 0.05$ for cryoprotectant; $P > 0.05$ for line by cryoprotectant; $P > 0.05$ for age at mating; $P > 0.05$ for weight at mating

[c] No. Fem., Number of females; No. Fro., total number of embryos frozen per line; % Rec., percentage embryos recovered after thawing

[d] Crosses of selected lines. Female genotype is listed second

[e,f] Within columns and across rows; PTS36 means with no superscript in common are different ($P < 0.05$)

**Table 2** Least-squares means (arcsine ± SE) of post-thaw survival to blastocyst (PTS48)[a] of mouse embryos from selected lines and crosses frozen in propylene glycol or glycerol[b]

| Line | Propylene glycol | | | | Glycerol | | | |
|---|---|---|---|---|---|---|---|---|
| | No. Fem.[c] | No. Fro.[c] | % Rec.[c] | PTS48, % (arcsine ± SE) | No. Fem.[c] | No. Fro.[c] | % Rec.[c] | PTS48, % (arcsine ± SE) |
| HF | 29 | 338 | 98.2 | 40.8(39.8 ± 3.5)[e] | 33 | 395 | 99.2 | 59.4(50.8 ± 3.4)[f] |
| LF | 34 | 408 | 97.3 | 37.0(36.4 ± 3.2)[e] | 34 | 399 | 99.0 | 60.1(51.0 ± 3.3)[f] |
| RC | 58 | 584 | 96.2 | 36.9(37.1 ± 2.8)[e] | 52 | 557 | 98.4 | 65.0(53.8 ± 3.0)[f] |
| HFLF[d] | 30 | 362 | 96.1 | 29.6(31.2 ± 3.6)[e] | 30 | 347 | 99.4 | 68.1(55.7 ± 3.5)[f] |
| LFHF[d] | 36 | 418 | 98.6 | 32.4(33.2 ± 3.3)[e] | 32 | 367 | 99.2 | 67.3(56.0 ± 3.5)[f] |

[a] Percentage of embryos developing in vitro to blastocyst per donor female analyzed by least-squares means procedures following arcsine transformation, adjusted for generation effects. Some line-by-cryopotectant means for PTS48 were slightly larger than those for PTS36 because a few females scored at 48 h were not scored at 36 h and vice versa

[b] Analysis of variance: $P > 0.05$ for line; $P < 0.05$ for cryoprotectant; $P > 0.05$ for line by cryoprotectant; $P > 0.05$ for age at mating; $P > 0.05$ for weight at mating

[c] No. Fem., Number of females; No. Fro., total number of embryos frozen per line; % Rec., percentage embryos recovered after thawing

[d] Crosses of selected lines. Female genotype is listed second

[e,f] Within columns and across rows; PTS48 means with no superscript in common are different ($P < 0.05$)

at mating significantly affected PTS48 ($P < 0.05$). Linear contrasts performed on PTS36 and PTS48 least-squares means for genetic effects of divergence, maternal source, heterosis and asymmetry were not significant (Table 3).

Table 4 summarizes least-square means of PTS to hatched blastocyst (PTSHB). GLY-treated embryos survived better overall than PG-treated embryos ($P < 0.05$). Across cryoprotectants, lines did not differ ($P > 0.05$), nor was there a line × cryoprotectant interaction ($P > 0.05$). However, LFHF PG-treated embryos survived as well as all the other lines in GLY except LFHF. HF and LF embryos frozen in PG had comparable survival rates to RC embryos frozen in GLY. In contrast, RC and HFLF embryos treated with PG had the lowest survival when compared to all lines frozen in GLY and PG, and LFHF had the greatest survival. Age at mating did not affect survival to hatched blastocyst

**Table 3** Linear contrasts[a] (arcsine ± SE) (PTS) for post-thaw survival for three developmental stages

| Linear contrast | PTS36 | PTS48 | PTSHB |
|---|---|---|---|
| Divergence[b] | 1.97(1.28 ± 3.32) | 1.55(1.60 ± 3.45) | −3.09(−2.83 ± 3.62) |
| Maternal[c] | 0.31(0.72 ± 3.38) | 0.98(1.13 ± 3.51) | 8.95(5.87 ± 3.40) |
| Direct[d] | 1.66(0.56 ± 4.63) | 0.57(0.48 ± 4.81) | −12.04(−8.70 ± 5.11) |
| Asymmetry[e] | 7.29(5.20 ± 5.11) | −3.27(−1.85 ± 5.30) | 13.44(8.03 ± 6.11) |
| Heterosis[f] | −1.06(−1.09 ± 2.31) | 0.03(−0.48 ± 2.40) | 4.11(2.34 ± 2.55) |

[a] Linear contrasts were performed on least-squares means following arcsine transformation

[b] Divergence: $[(HF) - (LF)]$

[c] Maternal (reciprocal cross) effects: $[(LFHF) - (HFLF)]$

[d] Direct (embryo) effects: $[(HF) - (LF) - (LFHF) + (HFLF)]$

[e] Asymmetry: $[(HF) + (LF) - (2RC)]$

[f] Heterosis: $0.5[(HFLF) + (LFHF) - (HF + LF)]$

**Table 4** Least-squares means (arcsine ± SE) of post-thaw survival to hatched blastocyst (PTSHB)[a] of mouse embryos from selected lines and crosses frozen in propylene glycol and glycerol[b]

| Line | Propylene glycol | | | | Glycerol | | | |
|---|---|---|---|---|---|---|---|---|
| | No. Fem.[c] | No. Fro.[c] | % Rec.[c] | PTSHB, % (arcsine ± SE) | No. Fem.[c] | No. Fro.[c] | % Rec.[c] | PTSHB, % (arcsine ± SE) |
| HF | 13 | 135 | 99.3 | 6.1(13.8 ± 3.7)[e] | 13 | 166 | 100.0 | 21.1(26.6 ± 3.8)[f] |
| LF | 17 | 204 | 98.5 | 7.5(15.8 ± 3.2)[e] | 18 | 217 | 99.5 | 26.0(30.2 ± 3.2)[f] |
| RC | 16 | 170 | 94.7 | 3.2(11.9 ± 3.4)[e] | 14 | 158 | 99.4 | 13.8(23.3 ± 3.6)[f] |
| HFLF[d] | 13 | 161 | 95.0 | 3.2(12.1 ± 3.8)[e] | 12 | 152 | 100.0 | 26.5(30.0 ± 3.9)[f] |
| LFHF[d] | 13 | 151 | 98.7 | 15.1(21.4 ± 3.8)[e] | 13 | 134 | 100.0 | 32.5(32.5 ± 3.8)[f] |

[a] Percentage developing in vitro to hatched blastocyst per donor female analyzed by least-squares procedures following arcsine transformation

[b] Analysis of variance: $P < 0.05$ for treatment; $P > 0.05$ for line; $P > 0.05$ for line by treatment

[c] No. Fem., Number of females; No. Fro., total number of embryos frozen per line; % Rec., percentage embryos recovered after thawing

[d] Crosses of selected lines. Female genotype is listed second

[e,f] Within columns and across rows: PTSHB means with no superscript in common are different ($P < 0.05$)

**Table 5** Least-squares means of in vitro development[a] to 36 h (IVD36), 48 h (IVD48) and hatched blastocyst (IVDHB) of non-frozen control embryos from selected lines and crosses[b]

| Line | Number of females | Number of embryos | IVD36, % (arcsine ± SE) | IVD48, % (arcsine ± SE) | IVDHB, % (arcsine ± SE) |
|---|---|---|---|---|---|
| HF | 16 (6)[d] | 203 (77)[c] | 88.2(69.4 ± 4.4)[e] | 88.0(70.7 ± 4.0)[e] | 73.6(58.9 ± 6.5)[e,g] |
| LF | 20 (11) | 286 (157) | 81.2(66.7 ± 3.9)[e] | 80.8(66.6 ± 3.5)[e] | 52.8(46.5 ± 4.8)[e,f] |
| RC | 37 (13) | 462 (161) | 73.8(60.7 ± 2.9)[e] | 80.3(65.4 ± 2.6)[e] | 43.7(40.5 ± 4.2)[f] |
| HFLF[c] | 17 (8) | 243 (104) | 83.6(67.5 ± 4.3)[e] | 86.8(69.9 ± 3.9)[e] | 82.9(66.4 ± 5.6)[g] |
| LFHF[c] | 16 (6) | 237 (83) | 91.6(73.4 ± 4.8)[e] | 92.7(73.7 ± 4.4)[e] | 62.8(52.2 ± 7.0)[e,f,g] |

[a] Percentage of embryos developing in vitro to specified stage per donor female, analyzed by least-squares procedures following arcsine transformation; adjusted for generation and weight at mating effects
[b] Analysis of variance: IVD36 and IVD48: $P > 0.05$ for line; $P > 0.05$ for generation; $P > 0.05$ for generation by line; $P > 0.05$ for weight at mating
[c] Refers to genotype of embryo. Donor female genotype is listed second
[d] Numbers in parentheses are for IVDHB, which was only measured in generation 33
[e,f,g] Within columns; means with no superscript in common are different ($P < 0.05$)

**Table 6** Linear contrasts[a] (arcsine ± SE) for in vitro development (IVD) for all developmental stages

| Linear contrast | IVD36 | IVD48 | IVDHB |
|---|---|---|---|
| Divergence[b] | 5.03(2.69 ± 6.02) | 7.20(4.10 ± 5.48) | 20.82(12.34 ± 8.39) |
| Maternal[c] | 8.03(5.98 ± 6.70) | 5.95(3.86 ± 6.10) | −20.09(−14.18 ± 9.56) |
| Direct[d] | −3.00(−3.28 ± 8.44) | 1.26(0.24 ± 7.69) | 40.91(26.53 ± 11.24)* |
| Asymmetry[e] | 19.84(14.73 ± 8.16) | 8.23(6.44 ± 7.43) | 39.03(24.34 ± 11.34)* |
| Heterosis[f] | 3.88(2.35 ± 4.20) | 5.34(3.20 ± 3.82) | 9.64(6.61 ± 5.59) |

* $P < 0.05$
[a] Linear contrasts were performed on least-squares means following arcsine transformation
[b] Divergence: $[(HF) - (LF)]$
[c] Maternal (reciprocal cross) effects: $[(LFHF) - (HFLF)]$
[d] Direct (embryo) effects: $[(HF) - (LF) - (LFHF) + (HFLF)]$
[e] Asymmetry: $[(HF) + (LF) - (2RC)]$
[f] Heterosis: $0.5[(HFLF) + (LFHF) - (HF + LF)]$

($P > 0.05$), but weight at mating was significant ($P < 0.05$). Linear contrasts showed no significance of specific genetic effects in PTS at the hatched blastocyst stage of development (Table 3).

Line-by-generation and line-by-generation-by-cryoprotectant effects were not significant. Because of significant generation ($P < 0.05$) and generation-by-cryoprotectant interactions ($P < 0.05$) for PTS36 and PTS48 (data not shown), analyses of variance were conducted on a within-generation basis for PTS traits to determine if line or line-by-cryoprotectant-effects were being masked in the analysis across generations; in no case were these effects significant within generations 32 and 33 (data not shown).

Least-squares means of IVD36 and IVD48 of non-frozen control embryos are given in Table 5. In vitro development did not vary significantly between lines at either developmental stage ($P > 0.05$). Analysis of variance detected no significant generation or line-by-generation effects. Percent IVD was highest for LFHF embryos and lowest for RC embryos. As with PTS36 and PTS48, linear contrasts of genetic effects were not significant (Table 6).

There were significant differences between lines in IVDHB for non-frozen controls ($P < 0.05$) (Table 5). RC embryos developed less frequently to hatched blastocyst than HF or HFLF embryos. HFLF embryos had significantly higher IVD than LF and RC embryos. Linear contrasts performed on least-squares means (Table 6) showed a large, positive effect of asymmetry (24.3 ± 11.3) and direct embryo effects (26.5 ± 11.2) ($HF > LF$; $P < 0.05$). Although divergence was also positive ($HF > LF$), it did not reach statistical significance because of a nonsignificant negative ($HF < LF$) maternal effect. Embryo heterosis was not significant for IVDHB.

## Discussion

The results of this experiment indicate that selection for high or low fat content in mice did not result in significant embryo- or maternal-correlated responses in post-thaw embryo survival at three developmental stages. Therefore, the possibility that correlated responses in embryo cryosurvival might somehow have occurred because of a change in lipid content of the selected embryos must be rejected. The only previous report on the effects of selection on post-thaw embryo survival showed a positive maternal and a negative embryo effect on PTS following selection for high litter size (Pomp and Eisen 1990); in contrast, the same study found no correlated response in PTS in a line selected for large body weight. In the high litter size line, the embryo effects were cancelled by the maternal effects, demonstrating the need for using designs that can estimate both factors.

The reasons why correlated genetic responses occur in a population initially in genetic equilibrium are genetic drift and/or pleiotropy. Because no selection replicates were used in the litter size line, these two factors were confounded; although both consitituted genetic change, the direction for drift is random while that for pleiotropy is expected to be driven by the sign of the genetic correlation between PTS and the selected trait.

The present study found no evidence of significant embryo heterosis for PTS in crosses between high- and low-fat selected lines. Reciprocal crosses between a line selected for high litter size and a randomly selected line and between those selected for large body weight and selected randomly also failed to yield embryo heterosis (Pomp and Eisen 1990). Further, crosses of inbred strains showed no heterosis (Schmidt et al. 1985), but failure to generate reciprocal crosses have confounded embryo heterosis with maternal effects (Pomp and Eisen 1990). Thus, there is at present no evidence that PTS is affected by nonadditive genetic effects of the embryo. Estimates of maternal heterosis for PTS have not been reported.

Genetic influences on cryosurvival in unselected inbred and crossbred lines also have not been widely investigated. Whittingham and Whitten (1974) noted a difference in survival between two inbred strains, but their sample sizes were small. They felt the difference could possibly be due to the freeze-thaw process or to a genotype-by-cryoprotectant interaction. Differences in PTS in mutant and inbred strains have been found although unfrozen control embryos were not concurrently cultured, and it could not be determined if the differences were the result of freezing or culturing (Whittingham et al. 1977). Significant genotypic effects were found in PTS for three outbred and two inbred strains, and survival differences were independent of whether the strain was inbred or outbred (Schmidt et al. 1985, 1987).

Cryoprotectant type profoundly affected the ability of embryos to survive the freeze-thaw process in the present study. The freezing of embryos in GLY resulted in a greater percentage of embryos surviving to the blastocyst stage than freezing in PG. Although no significant genotype-by-cryoprotectant interactions were found for PTS, a cautionary note is issued because of the relatively low statistical power of detecting an interaction even in an experiment of the present magnitude (Wahlsten 1990).

Considering the large array of freezing protocols that have been studied, few reports have addressed the importance of genotype-by-freezing variable interactions. Genotype-by-cryoprotectant [GLY *vs* dymethyl sulfoxide (DMSO)] interaction was found to be more important than genotype-by-container (ampule vs straw) interaction (Schmidt et al. 1987); no statistical tests of interactions were carried out, but the inference was made that interactions involving both freezing variables were biologically important. Pomp and Eisen (1990) found no genotype-by-freezing method (slow-cool vs ultrarapid) interaction for PTS. Interactions are likely to be more important when dealing with large genetic and/or large freezing variable differences, but insufficient estimates of genotype-by-freezing variable interaction are available to draw any conclusions as to their general biological importance.

Conflicting results on the significance of genotype on cryosurvival could be due to the failure to consider donor female variation. Most studies, including those of Schmidt et al. (1985, 1987), pooled the embryos of donor females, which could lead to increased risk of false rejection of the null hypothesis of no genetic effect (Pomp and Eisen 1991). Pomp and Eisen (1990) accounted for donor female variation in their cryropreservation study, which led to a more conservative test of the genotypic influence on cryosurvival. The same argument can be made in testing effects of freezing variable and genotype-by-freezing variable interactions. The present study used the statistical methodology recommended by Pomp and Eisen (1990, 1991), taking into account donor female variation. To our knowledge, no investigations have attempted to partition the phenotypic donor female variance in PTS into genetic and environmental components within a randombred population.

The present study demonstrated significant genetic effects for IVD to hatched blastocyst, indicating differential survival at this stage of development. The results suggest that selection for high fat content has improved the ability of embryos to survive in vitro to the hatched blastocyst compared to selection for low fat. The embryonic genome seems to influence in vitro survival at later stages of development because lines were not significantly different at IVD36 and IVD48. Asymmetry was positive and large; HF and LF had greater IVD rates than RC, although HF reponded to a greater extent than LF. Thus, selection for both high fat and low fat content resulted in improved IVD rates over controls. It remains to be determined, however, whether this phenomenon also occurs in normal development or is strictly an in vitro occurrence.

In an attempt to replicate, in part, the experiment of Renard and Babinet (1984), four- and eight-cell mouse embryos were frozen in 1.5 *M* GLY or PG. The results of the present investigation clearly show that PG is a much poorer cryoprotectant than GLY. The reason for the lower survival rate with PG is unclear. Differences in the freeze-thaw protocol may account for some of the difference in survival. Renard and Babinet (1984) cooled embryos at 2 °C/min to − 7 °C, seeded, and then further cooled embryos at 0.30 °C/min to a temperature of − 30 °C before plunging the embryos into liquid nitrogen. Although their freezing protocol varied slightly from the protocol used here, freezing rates and plunge temperature were well within the demonstrated rates needed for survival of mouse embryos (Schneider et al. 1983).

In summary, these studies have further tested the hypothesis that cryosurvival is affected by genotype. No

significant genotype or genotype *x* cryoprotectant interactions were found. Results recorded in the literature have been variable, and only two studies have investigated the importance of genetic effects and their interaction with cryoprotectants after taking into account donor female variation. Cryoprotectant type played a significant role in cryosurvival. Their effects can be wide and varied depending upon freeze-thaw conditions and cell characteristics as evidenced in this study by the existence of possible interactions between thawing and dilution conditions. The study of interactions between plasma membranes and cryoprotectants may allow a greater understanding of the protective action of the latter and possibly lead to the discovery or design of better cryoprotectants. In order to extend cryopreservation to many animal species, increase efficiency and reduce costs, the role of genetic variables among families, lines and species needs further investigation. Further studies should also concentrate on genetically determined plasma membrane characteristics and cytoplasmic components like lipid content, which could affect cryosurvival.

**Acknowledgements** The authors wish to thank William Atchley, Charlotte Farin, Robert Petters and Daniel Pomp for helpful comments on an earlier version of this manuscript. The technical assistance of Beth Johnson and Linda Hester is appreciated.

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