19. Silber SJ, Ord T, Balmaceda J, Patrizio P, Asch RH: Congenital absence of the vas deferens, The fertilizing capacity of human epididymal sperm. N Engl J Med 1990;323:1788–1792
20. Bustillo M, Rajfer J: Pregnancy following insemination with sperm aspirated directly from the vas deferens. Fertil Steril 1986;46:144–146
21. Brindley G, Scott GI, Hendry WF: Vas cannulation with implanted sperm reservoirs for obstructive azoospermia or ejaculatory failure. Brit J Urol 1987;58(6):721–723
22. Warner H, Martin D, Perkash I, Speck V, Nathan B: Electrostimulation of erection and ejaculation and collection of semen in spinal cord injured humans. J Rehab Res Dev 1986; 23:21–31
23. Bennett CJ, Seager SW, Vasher EA, McGuire EJ: Sexual dysfunction and electroejaculation in men with spinal cord injury: Review. J Urol 1988;139:453–457
24. Bennett C, Seager S, McGuire E: Electroejaculation for recovery of semen after retroperitoneal lymph node dissection: Case report. J Urol 1987;135:513–515.
25. Braude P, Ross L, Bolton V, Ockenden K: Retrograde ejaculation: A systemic approach to non-invasive recovery of spermatozoa from post-ejaculatory urine for artificial insemination. Br J Obstet Gynecol 1987;97:76–83
26. Mortimer, D: Semen Analysis and Sperm Washing Techniques. *In* Controls of Sperm Motility: Biological and Clinical Aspects, C Gagnon (ed). Boca Raton, FL, CRC Press, 1990, pp 263–284

Stacy Elliot[1]
George Szasz
Sexual Medicine Unit
University Hospital—Shaughnessy Site
4500 Oak Street
Vancouver, British Columbia
Canada V6H 3N1

Christo Zouves
Department of Obstetrics and Gynecology
Grace Hospital
Vancouver, British Columbia
Canada

[1]To whom correspondence should be addressed.

BOSTON, MASSACHUSETTS

Water-Escape Time in Adult Mice Derived from Manipulated Preimplantation Embryos

Submitted: May 17, 1991
Accepted: June 14, 1991

INTRODUCTION

The possibility that manipulation of preimplantation embryos can influence the postnatal and adult phenotypes of animals poses an important clinical question that has been studied recently in genetically identical mice. When control mice produced by natural mating, transferred-embryo mice, and half-embryo mice derived by transfer were evaluated by comparing the growth in body weight and tail length, early differences were found in body weight between the controls and the animals born following embryo transfer. These differences disappeared by 12 weeks of age due to compensatory growth (1,2). It seems likely that the initial differences in body weight were a consequence of smaller litters and a resultant longer gestation time in the transferred groups.

At 26 weeks of age all mice in this experiment were observed for other phenotypic characteristics including the ability to swim, which has been used frequently to test for the functional integrity of the nervous system. Such tests examined the function of the cerebellum in the study of the mutant staggerer (3), for example, and the vestibular system of the inner ear in manganese-deficient mice (4). The water-escape test has also been extensively used to examine for the ability to learn, a function of the cerebral cortex (5–11). We tested mice for the ability to escape from water based on the method used by Festing (6). The results, described in this paper, show no differences between the distributions of the time–response curves for water-escape time of the naturally mated control mice, the control mice resulting from transfer of two-cell embryos, and the mice produced by transfer of half-embryos after destroying one blastomere of the two-cell stage. Thus manipulation and transfer of the two-cell mouse embryos had no effect on the subsequent development of the nervous system as measured by this behavioral test.

MATERIALS AND METHODS

Animals. Water-escape times have been analyzed in three groups of genetically identical F_1 mice (C57/BL/6J × SJL/J)F_1: (1) controls—the natural offspring of timed matings (38 females, 41 males from 12 litters); (2) transferred controls—offspring from

embryos flushed at the two-cell stage and transferred to recipients 1 day asynchronous (38 females and 40 males from 14 and 16 litters, respectively); and (3) transferred half-embryos—offspring developing from one blastomere from the two-cell stage transferred to recipients 1 day asynchronous (39 females and 39 males from 15 and 16 litters, respectively). Half-embryos were produced by puncture lysis of one blastomere of the two-cell stage. Details of embryo handling and animal husbandry have been published previously (1,2).

Measurement of Water-Escape Time. At 26 weeks of age, each animal was tested on 5 consecutive days in a water-escape apparatus similar to that described by Festing (6). It consisted of an aquarium 31 cm high × 59 cm wide × 29 cm deep filled with room-temperature water to a depth of 10 cm and containing a 7 × 5-cm wire-mesh escape platform 9 cm from one end. Each animal was individually placed in the water at one end of the apparatus and timed, to the nearest 1/100 sec, while it attempted to locate and mount the escape platform at the opposite end. Animals failing to escape after 180 sec were removed from the water and given a score of 180 sec.

Statistical Methods. The distribution of the water-escape times for each day in each group was summarized using order statistics (median; 10, 25, 75, and 90 percentiles). These are plotted as either regular or notched box plots; the latter are used when the medians are independently determined, thus allowing valid tests for the significance of differences between the medians. If the notches of adjacent distributions do not overlap, the medians are statistically different at the $P = 0.05$ level.

When successive observations are made on the same individual, it is necessary to take account of the correlations between the data obtained on successive occasions (12). A particularly useful analysis of time–response data is obtained by replacing the data on each individual with a regression line expressed as a linear combination of orthogonal polynomials and analyzing the distribution of the individual regression coefficients. This method was first introduced by Wishart (13) and later refined by Rao (14), Grizzle and Allen (15), and Kenward (16). The computational procedures described by Grizzle and Allen were used by Festing (6–8) to estimate mean water-escape-time learning curves. Our procedure, outlined in the Appendix, is novel in that it combines Rao's approach with exploratory data analysis using box plots (17).

RESULTS

Overall Responses

The water-escape-time distributions of the control, transferred control, and half-embryo groups, classified according to sex and day of observation on days 1, 3, and 5, are summarized in the form of notched box plots in Fig. 1. Because the correlations caused by repeated observations on the same animal and by male and female littermates make comparisons complicated, the results are presented in separate panels to show valid tests of significance in which the requirement of independence between groups being compared is satisfied. Within all panels the notches overlap, indicating that the medians of the water-escape times for the control, transferred control, and half-embryo mice are not significantly different.

Initial Water-Escape Time and Body Weight

Figure 2 presents the individual water-escape times on the first trial of the male and female mice in the control, transferred control, and half-embryo groups as a function of body weight. There is no obvious effect of body weight on the initial water-escape times: a few animals (two females and six males) failed to escape by 180 sec and had to be rescued; another seven males were relatively slow to escape (>110 sec).

Estimation of the Water-Escape-Time Time–Response Curve

Figure 3 shows several individual water-escape-time time–response curves (learning curves) selected from the control female group to show the large variation in curve shape. A more thorough analysis is made by comparing the variation of the water-escape-time time–response curves in the various experimental groups. In Figs. 4 and 5 the distributions of the constant, linear, quadratic, cubic, and quartic components of the individual water-escape-time curves for both sexes of the three groups of animals are compared as notched box plots. The results indicate that the distributions of all these components are similar within each group. In all cases the median coefficient of the linear component is negative and the median coefficient of the quadratic component is slightly positive, while the

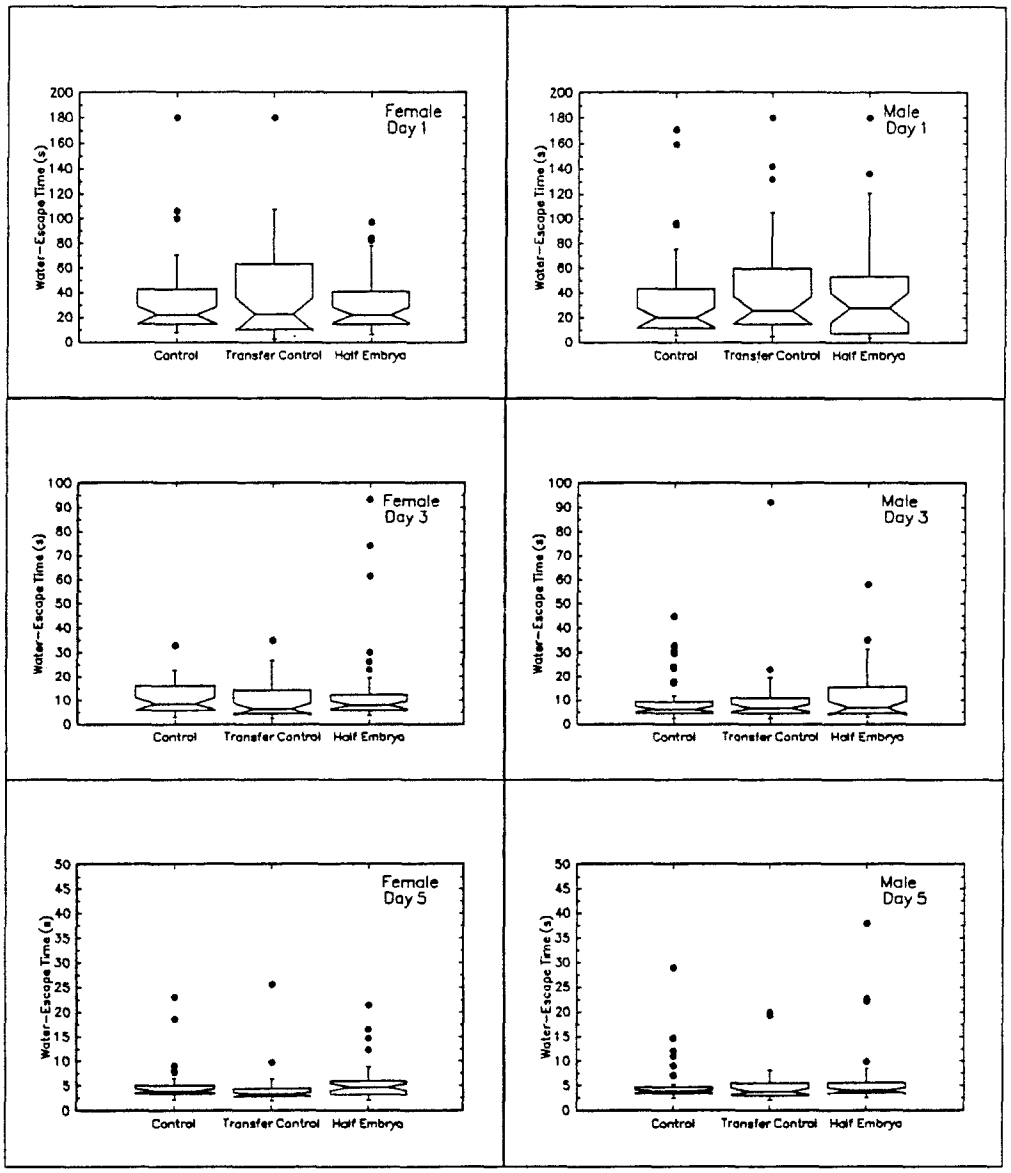

Fig. 1. Notched box plots of the water-escape-time distributions of the control, transferred control, and half-embryo groups, classified according to sex and day of observation. Horizontal lines represent the 10th, 25th, 50th (median), 75th, and 90th percentiles, so that 50% of the observations are within the boxes, and all values beyond the 10th and 90th percentiles are graphed individually. The notches are given by the median ± 1.57 (75th %ile − 25th %ile)/n. If the notches of two box plots do not overlap, the median is significantly different at the $P = 0.05$ level.

median coefficients of the cubic and quartic components are not significantly different from zero. Thus the variation in the location and shape of the water-escape-time time–response curves is similar in all groups, indicating that learning ability is unaffected by embryo manipulation and transfer. Estimates of the water-escape-time time–response curves of both sexes of the three groups of animals are shown in Fig. 6 (see Appendix).

DISCUSSION

The box plots summarizing the order statistics of the water-escape times for the first trial (day 1) of the female and male mice in all groups (Fig. 1) indicate that the distributions are skewed to the right, owing to the relatively long escape times of a few animals. Figure 2 shows that the individual initial water-escape times are not related to the body

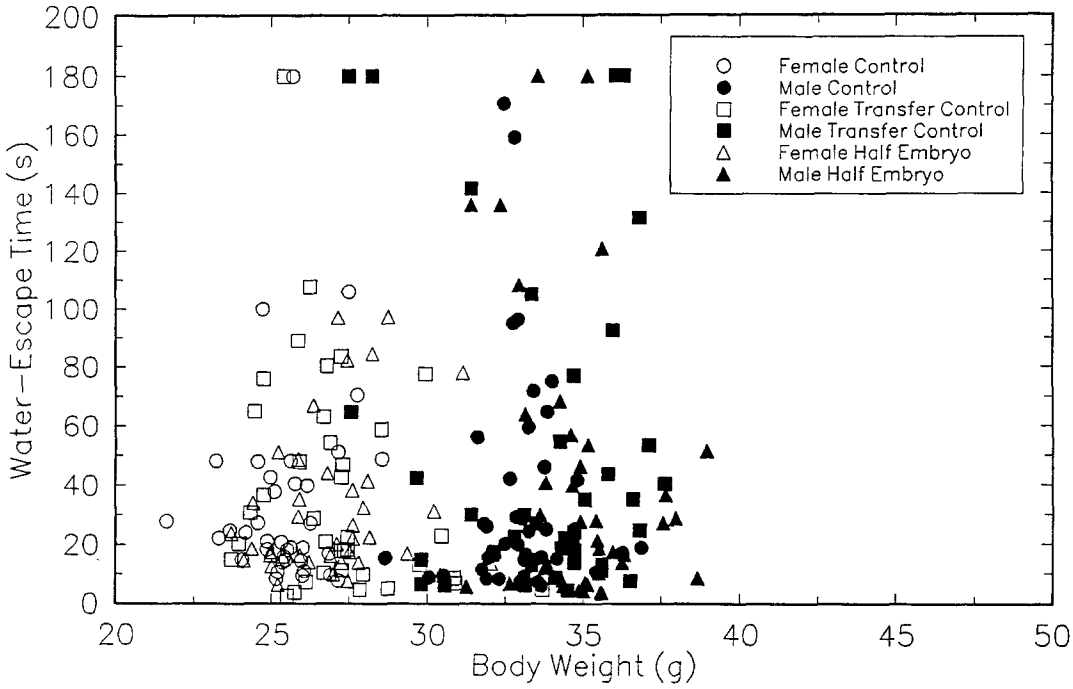

Fig. 2. Individual water-escape times as a function of body weight on the first trial (day 1) of all mice tested.

weights of the animals. Further, there is no apparent difference in the initial water-escape times between the sexes. Although the distributions of the water-escape times in subsequent tests are also skewed, the median times are shorter, and associated with this, the variance is lower. In other studies on water-escape times the statistical effects of the skewness of the distributions and dependence of the variance on the mean have been reduced by transforming the data to the logarithmic scale (6), or the data have been analyzed by nonparametric techniques (18). Our work shows that the shapes of the individual water-escape time time–response curves are very variable (Fig. 3). Thus, to demonstrate convincingly that embryo manipulation has little effect on water-escape-time, it is necessary to compare the variation in shape of the time-response curves as well as their average location. Other stud-

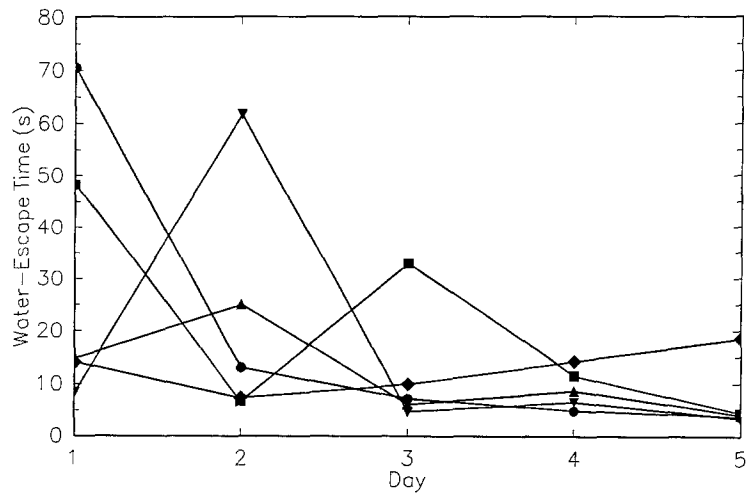

Fig. 3. Selected individual water-escape-time time–response curves from the control female group.

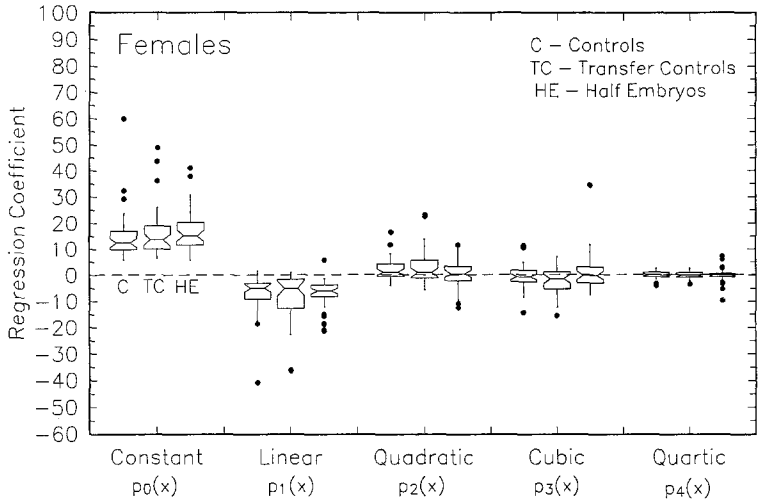

Fig. 4. Notched box plots of the distributions of the regression coefficients of the constant, linear, quadratic, cubic, and quartic components of the individual female water-escape-time curves.

ies such as those by Festing (6) and Tomaz *et al.* (18) have not explicitly taken into consideration the variation of the time–response curves.

The comparison of the variation in shape of the water-escape-time time–response curves in the three experimental groups can be summarized by the distributions of the regression coefficients of the orthogonal polynomials that give the shape of individual time–response curves. These distributions can be usefully depicted in the form of box plots (Figs. 4 and 5). No significant differences were detected among the distributions of the water-escape times in 26-week-old naturally mated control male or female mice, transferred control male or female mice, or transferred half-embryo male or female mice. Further, there were no detectable differences among the average learning curves estimated from the data in each of the groups. The results suggest that the transfer of preimplantation embryos between mothers has no major influence on the development and functions of the inner ear, cerebellum, or cerebral cortex. Additionally, the blastomeres of

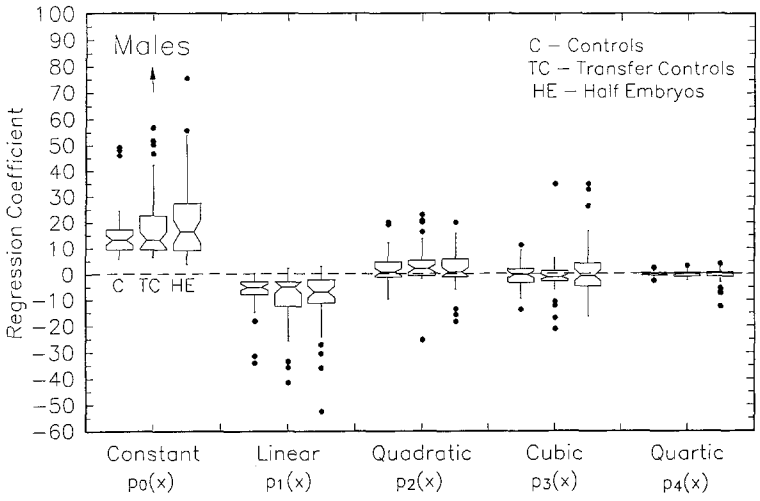

Fig. 5. Notched box plots of the distributions of the regression coefficients of the constant, linear, quadratic, cubic, and quartic components of the individual male water-escape-time curves. The arrow indicates an extreme outlier that never escaped from the water on any day and therefore had a repeated escape time of 180 sec.

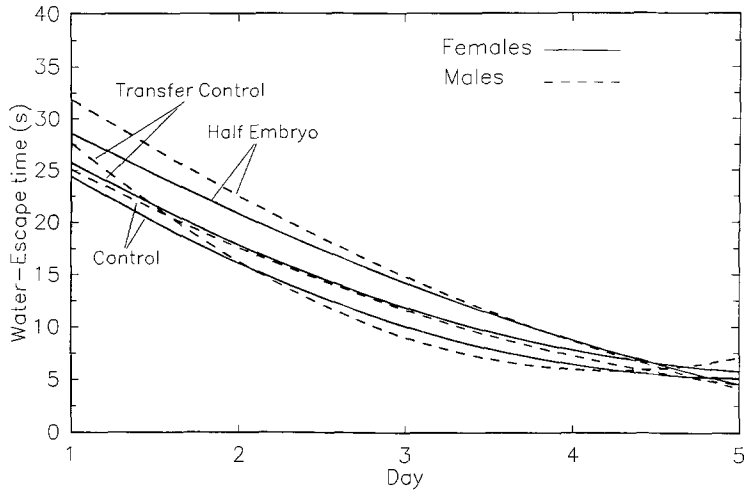

Fig. 6. Estimation of the average water-escape-time time–response curves of each group based on the medians of the constant, linear, and quadratic components in Figs. 4 and 5.

the two-cell mouse embryo are fully totipotent in relation to these measures of central nervous system function.

APPENDIX

Let the water-escape time be determined at n successive times $[t_j; j = 0, 1, 2, \ldots, (n - 1)]$. Associated with these times is a set of n orthogonal polynomials $[p_j(x)]$. In our work five determinations were made at daily intervals $(t_1, t_2, t_3, t_4, t_5)$. The associated five orthogonal polynomials were derived, using the method described by Kennedy and Gentle (19), and they are given by

$$p_0(x) = 1$$
$$p_1(x) = x$$
$$p_2(x) = x^2 - 2$$
$$p_3(x) = 5(x^3 - 3.4x)/6$$
$$p_4(x) = 35(x^4 - 4.43x^2 + 2.06)/12$$

where $x = t - 3$. These polynomials are illustrated in Fig. 7 (inset).

Let the vector $(\mathbf{y} = \{y_1, y_2, y_3, y_4, y_5\})$ represent the water-escape times observed on 5 successive days on each individual. This vector of correlated observations can be fitted exactly by a fourth-degree polynomial $[F(t)]$, which can be expressed as a linear combination of the five orthogonal polynomials $[p_i(x)]$, given by

$$F(t) = b_0p_0(t) + b_1p_1(t) + b_2p_2(t) + b_3p_3(t) + b_4p_4(t)$$

where $b_i = \Sigma_j y_j \phi_{ij}$, and ϕ_{ij} is the value of the ith degree orthogonal polynomial at the jth time point (Rao, 1965). Values of ϕ_{ij} are available in tables of orthogonal polynomial coefficients (20). Thus the information in the water-escape-time time–response data of all individuals can be completely represented by different linear combinations of the set of orthogonal polynomials. Two examples of these representations are shown in Fig. 7. No information is lost by expressing the observations obtained on each animal in this form. Since the orthogonal polynomials are independent, the complete information on each individual given by a vector $(\mathbf{b} = \{b_0, b_1, b_2, b_3, b_4\})$ of uncorrelated regression coefficients. The elements of b can then be used in independent statistical analyses to examine the constant, linear, quadratic, cubic, and quartic components of polynomials obtained from a population of individuals. These distributions can be used to compare the variation in the shape of the time-response curves in different experimental groups.

Figure 8 shows the distribution of the water-escape times of the control female group in the form of regular box plots. Figure 8 (inset) is a transformation of the same data represented by the distributions of the constant, linear, quadratic, cubic, and quartic orthogonal polynomial coefficients in the form of notched box plots. The results demonstrate that the median value of b_0 is significantly positive, the median value of b_1 is significantly negative, the median value of b_2 is marginally significant, and the values of b_3 and b_4 are not significantly different from zero. Thus the data can be

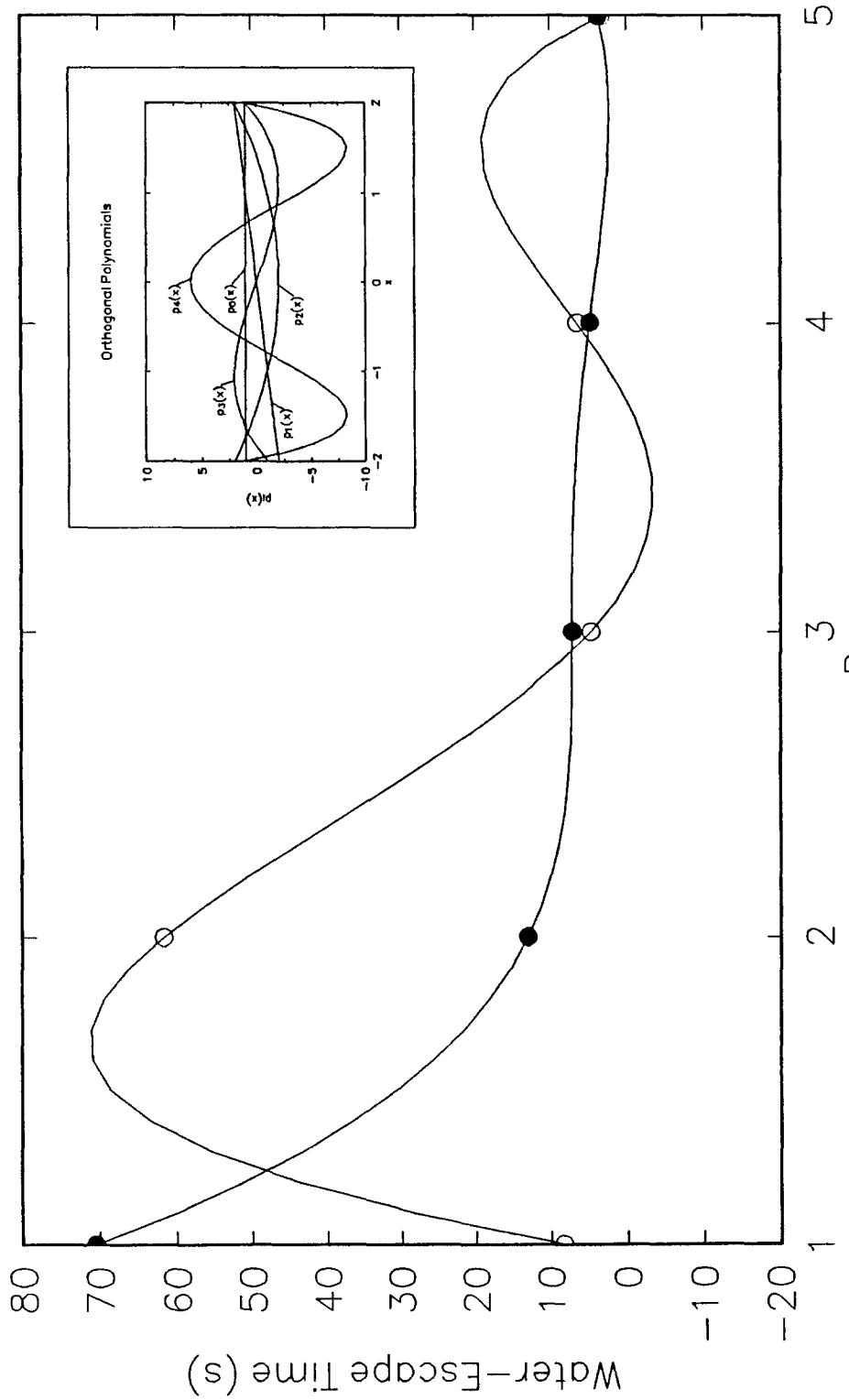

Fig. 7. Fourth-order polynomial fit of two individual water-escape-time time–response curves determined by five trials. The inset shows the five orthogonal polynomials that were combined using Eq. (1) to obtain these individual water-escape-time time–response curves. The equations are as follows. Open circles: $y = 16.90p_0(x) - 6.53p_1(x) - 3.87p_2(x) + 10.52p_3(x) - 3.33p_4(x)$. Filled circles: $y = 19.81p_0(x) - 14.18p_1(x) + 8.30p_2(x) - 5.04p_3(x) + 0.65p_4(x)$, where $x = t - 3$.

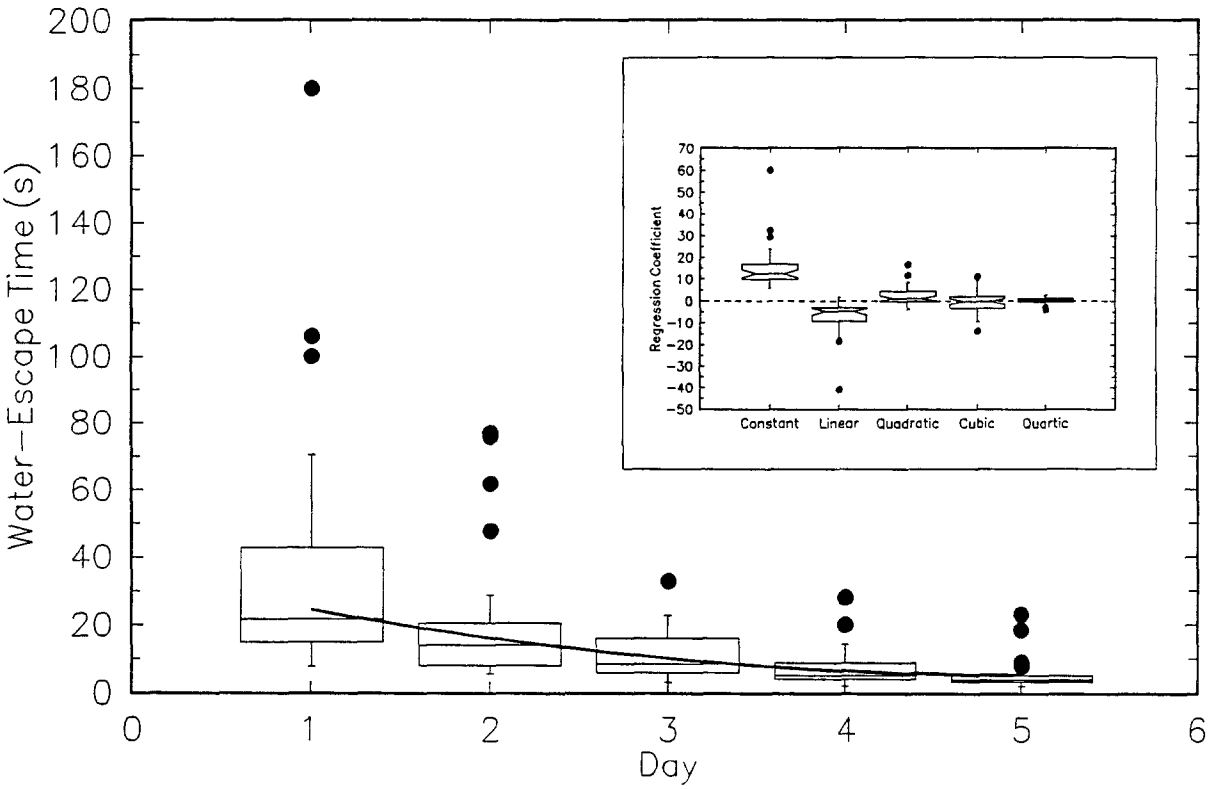

Fig. 8. Regular box plots of the distribution of the water-escape times of the control female group together with the fitted average water-escape-time curve. The inset shows the notched box plots of the distributions of the regression coefficients of the constant, linear, quadratic, cubic, and quartic components of the water-escape-time curves of the control females used to compute the average water-escape-time time–response curve.

fitted by the quadratic equation shown in Fig. 8. The medians of b_0, b_1, and b_2 have been used as robust estimates of the regression coefficients of the orthogonal polynomials $p_0(x)$, $p_1(x)$, and $p_2(x)$ because of the skewness to the left of the distributions of b_0 and to the right of b_1 and b_2.

ACKNOWLEDGMENTS

We wish to thank John Mkandawire for dedicated technical assistance and Carol Kountz for help with preparation of the manuscript. Animals used in this study were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council [DHHS publication No. (NIH) 85-23, revised 1985]. This work was supported by NIH Grants 2 R24 RR02510 and 5 RO1 HD25225 (V.E.P.).

REFERENCES

1. Papaioannou VE, Mkandawire J, Biggers JD: Development and phenotypic variability of genetically identical half mouse embryos. Development 1989;106:817–827
2. Biggers JD, Papaioannou VE: Postnatal compensatory growth of manipulated mouse embryos. Hum Reprod 1991; 6:36–44
3. Goodall G, Guastavino J-M, Gheusi G: The swimming activity of the staggerer mutant mouse. Behav Process 1986; 13:287–299
4. Erway L, Hurley LS, Fraser AS: Congenital ataxia and otolith defects due to manganese deficiency in mice. J Nutr 1970;100:643–654
5. Essman WB, Jarvik ME: A water-escape test for mice. Psychol Rep 1961;8:58
6. Festing MFW: Water escape learning in mice. I. Strain differences and biometrical considerations. Behav Genet 1973; 4:13–24
7. Festing MFW: Water escape learning in mice. II. Replicated

selection for increased learning speed. Behav Genet 1973;3: 25–36
8. Festing MFW: Water escape learning in mice. III. A diallel study. Behav Genet 1974;4:111–124
9. Lassalle J-M, Médioni J, Le Pape G: A case of behavioral heterosis in mice: Quantitative and qualitative aspects of performance in a water-escape tank. J Comp Physiol Psychol 1979;93:116–123
10. Lassalle J-M, Le Pape G: Differential effects of the albino gene on behavior according to task, level of inbreeding, and genetic background. J Comp Physiol Psychol 1981;95:655–662
11. Lassalle J-M, Le Pape G: Measurements of the behavioural effects of albino mutation in mice (*Mus musculus*): Comparisons of coisogenic inbred and hybrid lines. J Comp Physiol Psychol 1983;97:353–357
12. Crowder MJ, Hand DJ: Analysis of Repeated Measures. London, Chapman and Hall, 1990, pp 1–257
13. Wishart, J: Growth-rate determinations in nutrition studies with the bacon pig, and their analysis. Biometrika 1983;30: 16–28
14. Rao CR: The theory of least squares when the parameters are stochastic and its application to the analysis of growth curves. Biometrika 1965;52:447–458
15. Grizzle JE, Allen DM: Analysis of growth and dose response curves. Biometrics 1969;25:357–381
16. Kenward MG: The use of fitted higher-order polnomial coefficients as covariates in the analysis of growth curves. Biometrics 1985;41:19–28
17. Chambers JM, Cleveland WS, Kleiner B, Tukey PA: Graphical Methods for Data Analysis. Belmont, CA, Wadsworth, 1983
18. Tomaz C, Okada CT, De Faria MCF, Castreghini JL: Swimming escape learning in rats with telencephalic lesions. Brazil J Med Biol Res 1989;22:61–64
19. Kennedy WJ, Gentle JE: Statistical Computing. New York, Dekker, 1980, pp 343–347
20. Fisher RA, Yates F: Statistical Tables for Biological, Agricultural and Medical Research, 6th ed. New York, Hafner, 1963, p 98

John D. Biggers[1]
Laboratory of Human Reproduction and Reproductive Biology
Department of Cellular and Molecular Physiology
Harvard Medical School
Boston, Massachusetts 02115

Virginia E. Papaioannou
Department of Pathology
Tufts University School of Medicine and Veterinary Medicine
Boston, Massachusetts 02111

[1] To whom correspondence should be addressed at LHRRB, HMS, 45 Shattuck Street, Boston, Massachusetts 02115.

SANTIAGO, CHILE

Experience with Intravaginal Culture for in Vitro Fertilization (IVF)

Submitted: June 27, 1991
Accepted: July 8, 1991

INTRODUCTION

The use of intravaginal culture (IVC) for in vitro fertilization (IVF) has been described as a procedure that can simplify and reduce costs in assisted reproduction yielding similar conception rates when compared with IVF using fully equipped laboratory (1). This is especially important for developing countries, where such laboratories are difficult or impossible to finance. Our group, with experience in IVF since 1983 (2,3), decided to assess this new method.

MATERIALS AND METHODS

During the months of April and May 1989, 21 patients were admitted for IVF performed with an IVC procedure. A total of 23 cycles was initiated: 12 with leuprolide acetate plus human menopausal gonadotropins (hMG), follicle stimulating hormone (FSH), and human chorionic gonadotropin (hCG) and 11 with hMG, FSH, and hCG.

Oocyte retrieval was performed using a transvaginal ultrasound (General Electric RT 3000)-guided aspiration needle. Subsequent to the aspiration of the follicles the oocytes were identified and up to five were placed in a 3-ml cryopreservation tube (Nunc, Denmark) filled with Menezzo B2 culture medium containing 10,000 to 20,000 motile spermatozoa per ml. The sperm was prepared with the swim-up technique (2). All Menezzo B2 batches were previously tested in a mouse embryo culture system with more than 80% of two-cell embryos reaching the blastocyst stage. The tubes containing the oocytes and the spermatozoa were covered tightly with a cryoflex envelope, subsequently introduced into the vagina, and held in place with a