

Factors influencing the Yield of Transgenic Mouse Embryos for Cryopreservation

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Summary

Cryopreservation of pre-implantation embryos is a powerful tool to save and handle the increasing number of transgenic mouse lines, often consisting of very small populations. However, the outcome of embryos following superovulation and mating varies and is one of the limiting factors for cryopreservation. Using the data received together with the cryopreservation of more than 100,000 mouse embryos, the role of embryo yield limiting and of yield raising factors was analyzed: annual rhythms, seasonal changes (possibly due to environmental effects, such as extreme weather conditions), infections of a mouse colony, or the mating frequency of males. Then again to increase embryo yields two pheromonal effects were investigated: Especially the housing of female donors in small groups (Lee-Boot effect), but also the exposure to the future males' bedding two days before mating (Whitten effect) resulted in remarkably higher embryo yields.

Introduction

Transgenic (tg) animals exhibit an enormous scientific potential and the number of mouse mutants is increasing rapidly. Small populations,

a continued danger of loss, moderate breeding success, the need to keep the mutants in stock, and frequent interchange of tg mice between different facilities are some of the major challenges when dealing with these unique mutants. To handle these issues cryopreservation of pre-implantation embryos or of spermatozoa is a valuable tool.

A common procedure is the cryopreservation of eight-cell-embryos, an oviductal stage exhibiting some protection against several infectious agents and qualifying for rederivation (Reetz *et al.*, 1988; van Keuren *et al.*, 2004). The minimum number of eight-cell-embryos to be frozen varies from 200 to 500 depending on the genotype of the genetically modified (GM) mouse line. Embryo yields often vary and subsequently they exhibit a major limitation for cryopreservation. High yields save animals as well as expense; yield-depressing factors should be identified and –if possible- eliminated.

In many cases embryos are produced by mating tg males with wild type (WT) females. Female donor mice can often be obtained at an age suitable for superovulation from commercial breeders. Therefore, age and mating frequency of males have to be considered as limitations. Repeated mating of males leads in time to a loss of activity. Earlier, we reported a major drop after the 13th mating if the males were mated once a week. Sporadic mating of males on the other hand leads to irregular results (Nagy *et al.*, 2003; Wayss *et al.*, 2005). A possibly optimal frequency of mating remains to be determined.

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Abbreviations

GM = genetically modified, hCG = human chorionic gonadotropin, IU = international unit, IVC = individually ventilated caging system, LN₂ = liquid nitrogen, PMSG = pregnant mare serum gonadotropin, SD = standard deviation, SPF = specified pathogen free, tg = transgenic, VP = vaginal plug, VP⁺ = vaginal plug positive, WT = wild type

Optimum housing conditions are a prerequisite for animal experimentation and satisfying breeding performance. Much effort is necessary to maintain these conditions, but there is a continued danger that these high standards can collapse. In general, animal facilities exhibit a strict day-night light cycle and are equipped with powerful air-conditioning to level out variations of the local climate and to suppress circannual behaviour of the animals. It remains to be investigated, if this artificial environment leads to a complete suppression of circannual effects and if the technical equipment is able to disguise unwelcome peaks under extreme external weather conditions (*Shirley et al., 1985; Perissin et al., 1998; Oster et al., 2002*).

Important for housing, breeding, and animal experimentation is their hygienic condition. Infections severely affect the welfare of animals. Yet, in case of rodents housed in a high density, infections may spread exponentially. Pharmacological treatment most often remains insufficient and subsequently the infected mouse colony has to be rederived under difficult conditions (*Nagy et al., 2003; Hedrich et al., 2004; Deb et al., 2005*). Infections might also affect embryo yields and breeding results.

Mice are reported to be very sensitive to certain signals, especially to pheromones. These are external hormones secreted *inter alia* in urine or excreta, transported through the air, and then received by another animal of the same species (*Karlson & Luscher, 1959*). Pheromones can lead to specific reactions in the receiving animal, among others influencing the oestrous cycle (*Choi & Anderson, 2005; Yoon et al., 2005*). The oestrus cycle of mice can be influenced by other females and by males (*van der Lee & Boot, 1955a, 1955b; Whitten, 1956; McClintock, 1983; Chung, 2003*). Especially in case of mating for high numbers of embryos, it remains to be elucidated whether those effects might trigger the number of embryos, even if hormonal superovulation is applied.

Using the data of the cryopreservation of about 100,000 tg mouse embryos within ten years, here

we report and discuss the complexity of those influences.

Materials and Methods

Animals and Housing

The mice were housed in the animal facilities of the German Cancer Research Centre, Heidelberg, FRG. For the cryopreservation of embryos of a common genetic background, female donors were bought from commercial breeders.

WT embryo donors (C57BL/6N, FVB/N, B₆D₂F₁/BDF, NMRI) were received in most cases at an age of five weeks. Imported mice were generally allowed to adapt to the facility for up to one week before superovulation, exceptions being mentioned in the main text. In the case of homozygously mutated GM or in-house bred mice, the age could vary more due to availability. For the SPF (barrier), a very few conventionally driven facilities were used as well as isolators. All of them used open caging systems. The age of male mice ranged between three and nine months.

All units were equipped with air-conditioning maintaining –as far as possible– stable temperature (22-24°C) and relative humidity (55%), as well as providing a strict 12 hrs day-night light control (changing at 06.00h and 18.00h). All isolators were located within the same room. The actual parameters within the facilities were measured continuously leading to an alarm if limits of ±15% of temperature or humidity were hit. There are no charts available. Health monitoring of the animals was performed according to the FELASA recommendations (*Nicklas et al., 2002*).

Animals received a standard diet (Altromin, Lage, FRG 1324 NFF) and water *ad libitum*. If not mentioned otherwise, males were kept singly and females in groups. To get vaginal plug positive (VP⁺) mice, females were superovulated by standard procedures (*Nagy et al., 2003*) and mated 1:1 (i.e. one male with one female).

All barrier facilities were run under SPF-conditions, but in time some of them showed repeated infections with *Actinobacillus muris*, *Helicobacter* (different

species), *Pneumocystis carinii*, intestinal flagellates and pinworms. Animals in one barrier facility were never infected with viruses, parasites or bacterial agents as listed in the FELASA guidelines. Another barrier-facility suffered a parvoviral infection; for details see the Results section. The animals housed in isolators and in the conventionally operated facilities showed repeated infections with various agents and were frequently rederived or the facility was closed down.

All animal experiments were permitted by the Animal Welfare Department of the *Regierungspräsidium Karlsruhe*, FRG and were under the surveillance of the intramural animal welfare committee.

Special Housing

Separating Cages: A standard cage (Type II or Type III) was used, equipped with lids dividing the cage in two compartments by a middle grid allowing olfactory and visible contacts, but preventing copulation. A double set of water and food supply was needed. To terminate the separation the whole lid was replaced by a standard one.

Bedding: If females were to be exposed to their future males' bedding, a handful of the male's bedding was placed in the freshly prepared cage of the female. To omit any mix-up of cages, corresponding cages were placed in the same rack one below the other and the cage tickets were marked in an eye-catching manner.

Collection of Embryos

Donor females of the following lines were used and superovulated (B₆D₂F₁/BDF 12h PMSG 8 IU, 12h hCG 7 IU [two days later], C57BL/6 16h/12h 7 IU each, FVB/N 16h/12h 5 IU each, NMRI: 12h/12h 10 IU each). Hormones were prepared weekly and dissolved in phosphate buffered saline (PBS), portioned in 1 ml samples and stored at -20°C. These samples were thawed directly before use, and any remains were thrown away. VP⁻-females were separated at day 0.5 *post coitum* (p.c.) and oviducts were prepared at day 2.5 p.c. according to Nagy et

al. (2003). The oviducts were flushed with PBS, embryos were collected and washed twice each in PBS and M2 (Sigma-Aldrich), osmolarity 265-280 mOsm. Afterwards, only well-developed eight-cell-embryos with an intact morphology were selected for cryopreservation and used in this work.

Criteria of Selection

Eight-cell-embryos were selected microscopically (magnification 40x) as intact under the following conditions: round form, normal size (ca. 110 µm), normal cytoplasmic granulation, intact *zona pellucida*, eight cells, not shrunken, and (as far as it could be detected) microscopically- alive.

Freezing Procedure

Morphologically intact embryos were transferred into "freezing medium" (1.5 M glycerol [Sigma-Aldrich] in M2, 1960-1980 mOsm). Following equilibration the embryos were washed in "freezing medium" and intact embryos were selected for cryopreservation under the criteria mentioned above.

The embryos were then loaded into "French straws" (IMV et Cie., L'Aigle, France, Cat.-Nos. A201 and 101). A straw was filled with "thawing medium" (0.94 M sucrose [Sigma-Aldrich] in M2, 1700-1720 mOsm) over a length of 5 cm (ca. 100 µl) and, separated by air, for 1 cm (ca. 20 µl) with "freezing medium". About 20 embryos were transferred in a very small volume into the "freezing medium" section using a glass capillary. Separated by air, one or two additional drops (ca. 10 µl each) of "thawing medium" were aspirated and both ends of the straw were sealed with a plastic sealer. A schematic figure of this procedure is shown in the URL of the *European Mouse Mutant Archive* (<http://www.emmanet.org/sops/embryo.php>).

A larger straw was attached to the embryo-containing straw to serve as a handle. Following 10 min equilibration on ice (0°C), the sample was placed into an ethanol driven regulated freezer (Haake K controlled by Haake PG 41, Thermo Electron Karlsruhe, FRG) at 0°C for 10 min and

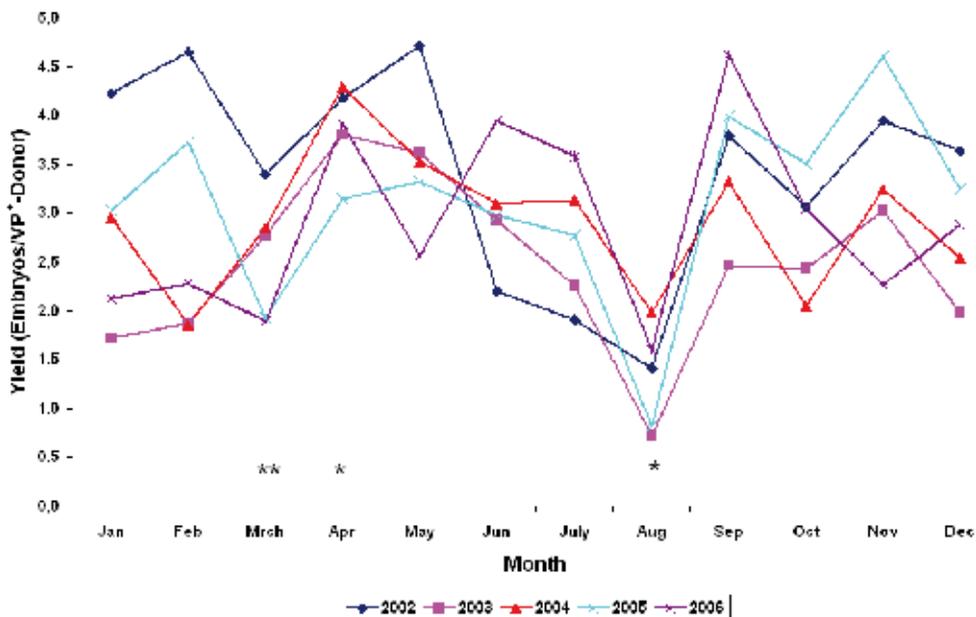


Figure 1. Seasonal variations on embryo yield: Eight-cell embryos/VP⁺-donor obtained during a five years period (2002-2006) were sorted by the month of preparation. 108 tg lines with 31689 embryos from 10279 VP⁺-donors were introduced in this analysis. An ANOVA showed significant monthly (*) patterns (p=0.00017). A significant increase was calculated in the embryo yields of April (*) of all years, a significant drop in August (*) of all years and in March 2005 (**). The data used are shown in Table 1. The asterisks indicate significance.

was afterwards frozen to -6°C with a cooling rate of 1°C/min. During a 10 min equilibration time, the liquid in the straw was seeded using liquid nitrogen (LN₂) cooled metal forceps. With a cooling rate of 0.4°C/min the embryos were frozen to -32°C, 10 min equilibrated and afterwards plunged directly into the liquid phase of LN₂.

Storage of Embryos

The embryos containing part of the straw was stored in the liquid phase of LN₂. For identification each “handle-straw” was labelled individually and registered in a data base.

Revitalization

Straws were transferred directly from LN₂ to room temperature according to *Leibo (1986)*. To remove the glycerol from the embryos, both media within

the straw were mixed by shaking directly after thawing and were afterwards transferred into a Petri dish (tissue culture quality). Following repeated washing in M2, the revitalised embryos were selected by their morphology and were ready for further use (*Wayss et al., 2005*).

External weather

External weather data for the location of the animal facility (humidity, temperature) were made available by the meteorological services.

Statistics

For the major part of this study data received were analysed retrospectively. Subsequently “case studies” were performed and a *post hoc* statistical analysis was not possible in all cases. A possible statistical significance between experimental and

Table 1. Data basis for Figure 1, the yields (eight-cell embryos/VP⁺-donor [E/VP⁺]) are shown for the years 2002 to 2006 and the statistically analyzed block effect. ANOVA showed a highly significant (*) month effect (p=0.00017). The highest absolute deviation was calculated for April (gain) and August (drop) of all years (*), as well as a drop in March 2005 (**). The asterisks indicate significance.

Year Month	2002		2003		2004		2005		2006		block effect
	E/VP ⁺	±SD									
											*
Jan	4.23	2.39	1.72	1.77	2.96	2.30	3.02	2.89	2.13	2.05	-0.15
Feb	4.65	3.30	1.88	3.78	1.87	1.91	3.72	2.91	2.28	2.70	-0.081
Mrch	3.40	4.74	2.78	3.80	2.86	2.29	1.93	2.27	1.89	3.25	-0.392**
Apr	4.18	6.31	3.80	3.52	4.30	3.17	3.15	1.96	3.92	2.77	0.909 *
May	4.72	3.02	3.62	3.80	3.53	3.09	3.32	2.89	2.56	2.37	0.59
Jun	2.21	1.62	2.93	1.75	3.10	2.73	2.98	2.26	3.95	2.65	0.072
Jul	1.91	1.93	2.26	2.07	3.14	2.73	2.77	2.23	3.58	2.67	-0.229
Aug	1.41	1.66	0.73	0.94	1.99	1.82	0.83	1.30	1.60	0.88	-1.65 *
Sep	3.79	3.62	2.46	3.15	3.32	2.79	3.99	3.46	4.62	4.45	0.674
Oct	3.08	2.10	2.44	2.25	2.05	2.69	3.51	2.70	3.05	5.12	-0.136
Nov	3.94	3.98	3.02	2.44	3.24	2.87	4.60	3.86	2.27	1.84	0.495
Dec	3.64	4.33	1.99	2.07	2.54	3.80	3.25	2.56	2.88	1.34	-0.101

control group or between two groups of data was calculated by a *t*-test for random samples with different variations; $p < 0.05$ is significant, $p < 0.001$ is highly significant.

The Gaussian distribution was examined by the d'Agostino-Pearson-Test. The significance of the yields presented in Figure 1 and Table 1 was calculated by a two-way ANOVA. The test for the block designs is based on the model equation $X_{ij} = m + a_i + b_j + \varepsilon_{ij}$, which directly corresponds to our data structure. X_{ij} are the mean numbers of embryos per VP⁺-donor for each month (counted by *i*) and each year (counted by *j*). *m* is the overall means of all X_{ij} , a_i is the effect due to month *i*, b_j is the effect due to year *j*, and ε_{ij} is a random residual term.

A trend line was calculated using the smallest squares method. The course of a trend line was determined by a regression analysis leading to R^2 values between 0 and 1. A higher R^2 value describes a higher reliability of the trend line.

Results

Annual rhythms and environmentally influenced patterns

Mice react very sensitively to changes in their environment leading to annual rhythms in behaviour. To overcome those rhythms negatively

influencing animal experimentation, the artificial environment in an animal facility should be kept very stable. To learn more about a possible seasonal pattern all yields of embryos served as a parameter over a time span of five years (2002 to 2006 [Table 1]) and were analyzed in monthly outcomes (108 tg lines with 31689 embryos from 10279 VP⁺-donors). As demonstrated in Figure 1, it becomes obvious that the embryo yields increase in April and drop in August. Statistical analysis was calculated by an ANOVA as described above. The a_i s as well as the b_j s are scaled so that their sum is 0, and the ε_{ij} are scaled that their expectation is 0, too. Using these conditions the model equation is unique. The a_i s point to the deviation from the overall means due to a month effect and the b_j s show the deviation from the overall mean due to a year effect. The applied ANOVA test indicates that the month effect is highly significant ($p=0.00017$). As demonstrated in Figure 1 and Table 1, the a_i s show the largest absolute deviation from the overall means in August (drop), while an increase was observed in April (Table 1, Figure 1). In addition, single month analyses March 2005 also showed an absolute deviation (drop). As a prerequisite to perform an ANOVA, the d'Agostino-Pearson Test of the residuals does not indicate anything contrary to the Gaussian distribution.

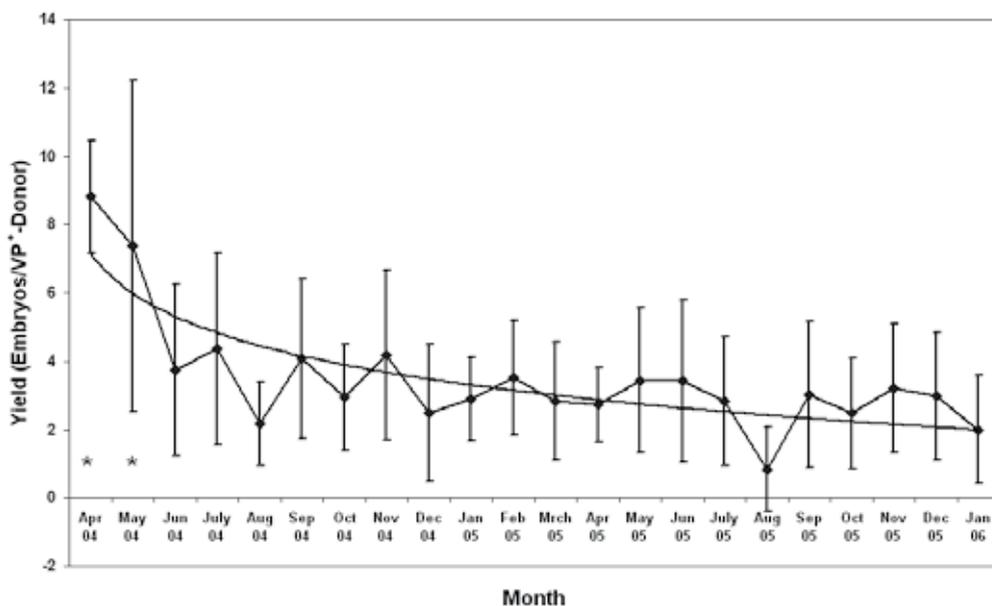


Figure 2. Influence of a parvoviral infection: The outcome of all eight-cell embryos per VP⁺-donor in a mouse facility with a parvoviral infection was sorted by the month of preparation. The infection was detected in early July 2004. 4412 embryos were delivered from 1252 VP⁺-donors. The trend line was calculated using the method of the smallest square, $R^2 = 0.6481$. Only the embryo yields in April 2004 and May 2004 differ significantly from the embryo yields of other months ($p=0.0012$ for April 2004, $p=0.05$ for May 2004). The asterisks indicate significance.

This pattern lead us to think about possible (external) influences on the housed animals. However, meteorological data measured close to the facility were available for the time span from January 2003 to April 2005. Even with only a few data about the internal climate of the facilities, these data indicated that extreme weather conditions might coincide with the remarkable drop of yield e.g., in August 2003 the average temperature in Heidelberg was 24.2°C, i.e. about 5°C higher than the long-term mean and the drop of yield in this month was more than in the other years, e.g. the average temperature of August 2004 was 20.6°C. In March 2005 the environmental humidity was very low leading to a humidity of not more than 40% within the facility over two weeks. This is in parallel with the significant drop of yields in this month.

Hygienic status of the facility

Aprerequisite of animal housing and experimentation is a sufficiently hygienic environment and much effort is necessary to maintain these conditions. On the other hand, the generation of pre-implantation embryos with mice housed in an infected facility is sometimes necessary, e.g. for rederivation or cryopreservation purposes. Yet, we have had the unfortunate opportunity to work with animals housed in an originally SPF-facility suffering a parvoviral infection. The infection was detected initially by routine health checks in early July 2004. The affected facility has space for about 7000 mice. Investigating the outcome of embryos from this facility as shown in Figure 2, we retrospectively have learned that the embryo yield was reduced remarkably and can be equated with the date of the

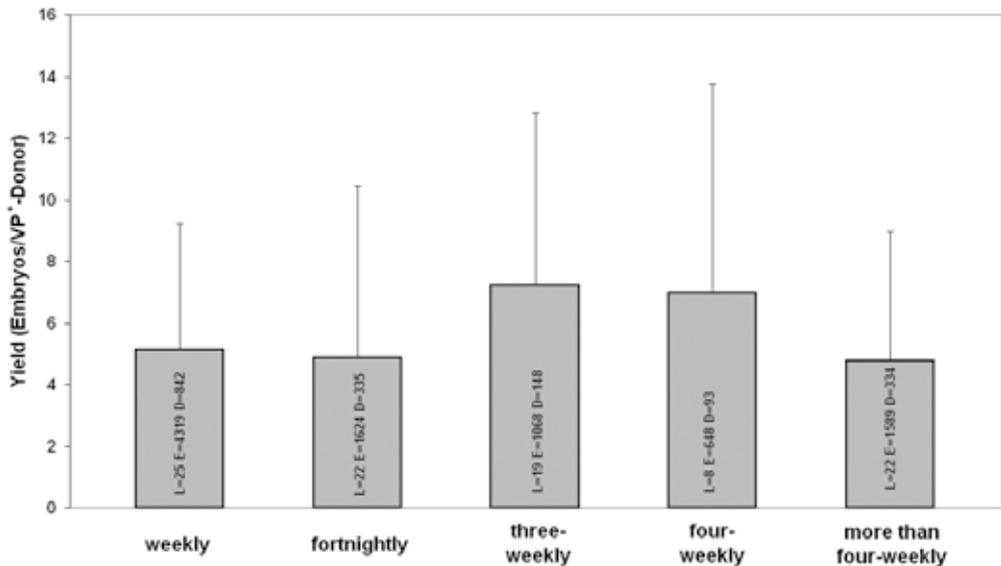


Figure 3. Influence of the frequency of mating males on the embryo yield. Eight-cell embryos/VP⁺-donor obtained were sorted by the frequency of the males' mating (1:1). No significant differences were calculated. L = number of tg mouse lines, E = number of embryos, D = number of VP⁺-donor females.

infection. However, in the time period shown in Figure 2 altogether nine lines were cry preserved with a maximum of four in parallel. All of them were hemizygotously mutated tg mice on the C57BL/6 genetic background. In summary 4412 embryos were delivered from 1252 VP⁺-donors.

A trend line of embryo yield was calculated. The regression coefficient of a linear trend line was $R^2 = 0.3938$; in the case of a logarithmic trend line $R^2 = 0.6481$. However, all trend lines show a continued drop of embryo yields. Only the differences between the yields of April 2004 or May 2004 and those of the other months investigated are statistically significant ($p=0.0012$ for April 2004, $p=0.05$ for May 2004). Before the infection was detected in 2004, the outcome of embryos in this facility exhibited only similar variations as demonstrated in Figure 1; the average yield (from 1996 to 2003) was 7.01 embryos/VP⁺-donor. The embryo production in this facility was discontinued afterwards.

Frequency of mating

As published earlier (Wayss *et al.*, 2005), the activity of males (embryos/VP⁺-donor) dropped after about 13 weekly matings. As demonstrated by the embryo yield per VP⁺-donor in Figure 3, there is a tendency that one mating every three weeks might be more efficient. The mating scheme was 1:1. 9248 embryos from 1752 VP⁺-donors and 27 tg lines were evaluated for this analysis but no statistically significant differences were detected.

Pheromonal effects

To increase the yields of embryos the influence of pheromones on mice housed in large groups (Lee-Boot effect) and the exposure of the males' pheromones to the donor females before mating (Whitten effect) were investigated. Lines under current cryopreservation were selected for these purposes. These mice were housed in SPF facilities.

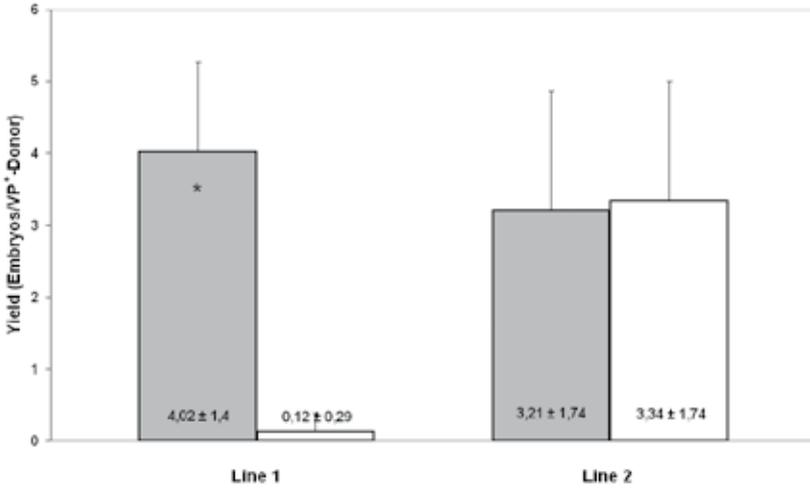


Figure 4. Phomonal effects (Lee-Boot effect): Embryo yields per VP⁺-females in the experimental groups (shaded columns) as compared to the controls. “Lee Boot” female mice were housed in groups of four animals per cage, the control in groups of ten animals per cage. Both lines were on the C57BL/6 genetic background. Line 1 exhibited highly significant differences between experimental and control group ($p < 0.0001$). Eight-cell embryos/VP⁺-donor are shown. The asterisks indicate significance.

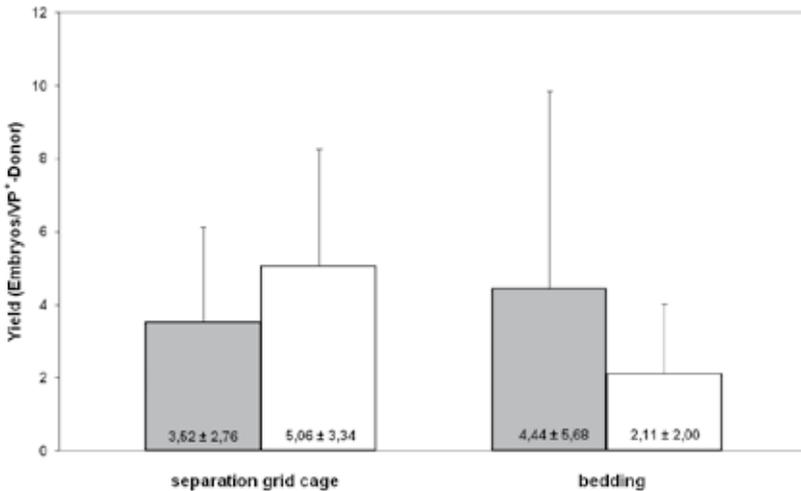


Figure 5. Phomonal effects (Whitten effect): “Whitten-Separation” mice were kept in separation cages for two days and were afterwards exposed to the male in standard cages following hCG-administration; the controls were exposed to the males following hCG-administration. “Whitten-Bedding” female mice were kept for two days on the bedding of their future males and were mated with the male following hCG-administration; the controls were exposed directly to the males following hCG-administration. Eight-cell embryos/VP⁺-donor are shown. In both approaches a trend was detected, but no statistically significant difference.

Lee-Boot effect: The Lee-Boot effect describes an oestrous suppression following bulk housing of females, while the oestrous is delayed but its cyclicity is maintained. The degree of suppression is determined by group size and strain. Here, the influence of a group size of four females per cage (for one week) was compared with housing in groups of ten females per cage (as shipped by the breeder). Two hemizygotously mutated GM lines without a transgene-dependent phenotype were selected for this approach; both lines were on the C57BL/6 genetic background (altogether 693 embryos). As demonstrated in Figure 4, 4.02 ± 1.4 embryos/VP⁺-donor were collected in the experimental group of line 1 as compared (highly significantly) to the control group with 0.12 ± 0.29 embryos/VP⁺-donor ($p < 0.0001$). Line 2 delivered 3.21 ± 1.74 embryos/VP⁺-donor, their controls 3.34 ± 1.73 embryos/VP⁺-donor ($p = 0.859$). No significant differences were observed if their development-delayed (e.g. two-cell embryos at day 2.5 p.c.) or non-developed embryos were compared between experimental and control group of both lines. It is noteworthy that this pheromonal effect did not lead to an increase of VP⁺-donors, but to a higher yield of eight-cell-embryos/VP⁺-donor. A negative effect was never observed.

Whitten effect: The Whitten effect describes the induction of ovulation due to a 48 hrs exposure to the male. Hemizygotously mutated tg lines (without a transgene dependent phenotype on C57BL/6 or FVB/N genetic background) were investigated. In a first approach 210 embryos, housed in separating cages, were used. Following the administration of pregnant mare serum gonadotropin (PMSG), each female was exposed to a male for two days. Separation was terminated following the administration of human chorionic gonadotropin (hCG). Under these circumstances, the outcome of embryos/VP⁺-donor was reduced (but without statistical significance) to 70% of the control group (3.52 ± 2.76 embryos/VP⁺-donor in the experimental group as compared to the control group with 5.06

± 3.34 embryos/VP⁺-donor, Figure 5). Intensive surveillance indicated the animals were stressed due to the separating grid allowing olfactory and visible contacts but omitting body contacts and copulation. Using another experimental set-up, females were exposed to their (future) males' bedding from the time when PMSG was administered and after then handled by standard superovulation procedures. In this case the outcome of embryos (altogether 201 embryos) rose by the factor 2.1 as compared to the controls (4.44 ± 5.68 embryos/VP⁺-donor in the experimental group versus 2.11 ± 2 in the control group). However, these differences were not significant (Figure 5).

Also in case of the Whitten effect no significant differences in the number of non-developed or delayed embryos or VP⁺-donors were detectable. The effect led to a higher yield of eight-cell-embryos/VP⁺-donor. A negative effect was never observed when using this set-up.

Revitalization

To assess the quality of cryopreserved embryos, control revitalization was performed with embryos of all lines and groups mentioned here (data not shown in detail). The revitalization rate was at about 80%, not varying from our earlier observations.

Discussion

Cryopreservation of early embryonic stages is a valuable tool to handle the increasing number of GM mouse lines, often exhibiting small populations. Applying this technique, the embryo yield is one of the most limiting and time/resources-consuming factors, with an enormous variance from line to line. An influence of the genetic background on breeding has been repeatedly reported as well as the age of the animals used for superovulation and mating (Zarrow & Wilson, 1961; Taketo *et al.*, 1991; Lasserre *et al.*, 1998; Rall *et al.*, 2000; Auerbach *et al.*, 2003; Nagy *et al.*, 2003; Bothe *et al.*, 2004; Dorsch, 2004).

Environmentally influenced patterns

The environmental circumstances and their changes (day-night cycle, succession of seasons) exert a major influence on the physiological and sexual behaviour of free living beings and result in the development of annual/seasonal rhythm probably induced by environmental and endogenous oscillators (Shirley *et al.*, 1985; Miyoshi *et al.*, 1993; Perisin *et al.*, 1998; Oster *et al.*, 2002). To suppress these influences, and subsequently rhythms, most animal facilities are equipped with a strict day-night cycle and air-conditioning permitting standardized conditions for housing, breeding, and animal experimentation the whole year round, even if a possible genetically induced annual pattern will not be fully eliminated. In this study, the outcome of embryos was analyzed by the month of preparation over a five years period. Most impressive is the drop of yield in August (Figure 1). When comparing these data with the data being available from the meteorological services it became obvious that the embryo yield dropped in single months in parallel with extreme weather conditions, e.g. the high temperatures in August. In August 2003 when the average outside temperature was about 5°C higher for more than four weeks compared to the long-term average the yield dropped more than in other years. Repeated attempts to control the climate conditions in the facilities showed that air conditioning cannot fully cover those extreme external weather conditions. It is possible that annual variations are herent and only partly suppressed by the artificial environment. The annual rhythms will become more obvious if extreme weather conditions are allowed to influence the internal climate of an animal facility.

Similar behaviour was observed repeatedly at other dates when the relative humidity in the animal facility was seriously affected; e.g. in March 2005 when a low humidity was observed leading to a drop of yields. Statistically significant is also the increase of yields in April, a month with moderate weather conditions. The increasing yields in September may have additional reasons, too: Possibly the end of

the holiday season and the restart of work after the summer break, allowing also a break to the males, might play a role (see section frequency of mating). Other facilities also report an annual rhythm effect on yields, even if they are equipped with powerful air-conditioning-supply, too. This results in the assumption that each animal facility develops its own pattern, in embryo yield and probably other parameters, too.

Taken together, our data show a varying pattern of yields over the year as well as a possible influence of extreme weather conditions, which are obviously not covered by the air-conditioning.

Hygienic status

Animal housing and animal experimentation must be done under healthy hygienic conditions, otherwise populations might fall or non-reproducible results will be obtained (Deb *et al.*, 2005; Brielmeier *et al.*, 2006). A direct influence of parvoviral infections on the embryo yield has not yet been proven, but we found the embryo yield dropped following the detection of the viral infection (Figure 2), even if embryonic pre-implantation stages are used to protect against viral infections (Kajiwara *et al.*, 1996; Pullium *et al.*, 2004). There is, however, evidence that all parts of the reproductive tract of mice are affected by MPV and the rederivation of MPV-infected animals by embryo-transfer is difficult (van Keuren & Saunders 2004; Agca *et al.*, 2007; Dr. Rita Sanchez-Brandelik *personal communication*) and can lead to a non-successful result.

Due to the frequency of health examinations, the infection was probably detected early in our work. The high yield variance in May 2004 leads to the assumption that the infection might have spread widely that time. The time course of yields as shown in Figure 2 allows two interpretations (or a combination of both): The logarithmic course of the trend line seems to be most appropriate and could lead to the conclusion that all yields drop over the time, but probably not to a complete collapse. If the monthly outcomes are examined one can also

conclude that following the infection the yield dropped rapidly and then stabilized on a lower level. The fact that only males were kept over a longer time within this facility but the females were imported and subjected within one week to superovulation can argue for the first interpretation: The females remain on a certain health level but the males, even if different sets of males were used for mating purposes, became more and more inactive due to the progressive infection. An argument for the second version might be that the fall-off of yields happened close to the infection and the outcomes were afterwards stabilized. There are good reasons hypothesizing that the time course from early summer 2004 on is, on a lower level, similar to the time course observed for all facilities over many years (Figure 1). All additional interpretations including the accumulation of yield reducing factors are rather speculative. But it was very conspicuous that in parallel to the advent of the infection a fall-off in the embryo yields was observed. The present state of discussion is that C57BL/6 mice can be infected by parvovirus relatively easily, but antibodies are only detectable in the serum following a longer incubation time (*Dr. Werner Nicklas, personal communication*). Subsequently investigations for antibodies in the donor females did not make any sense.

Taken together, these data show that a parvoviral infection can *inter alia* lead to impairment of breeding results and, in the very worst case, to a collapse of the whole animal population. This underlines the enormous need to maintain proper hygienic conditions within a mouse colony.

Frequency of mating

Laboratory manuals recommend a frequency of weekly mating or more to generate early stage embryos (*Nagy et al., 2003*). Our data indicate – without significance- that breaks of three weeks might lead to better results (Figure 3) or that in some cases a weekly mating might not be the most successful procedure. But it has to be considered that such a breeding regime needs three sets of

males if weekly cryopreservation will be performed or the complete preservation process will take a very long time. This is in congruence with other publications indicating that longer breaks result in a higher breeding success (*Sapp & Martin-DeLeon, 1992; Laing et al., 2000*). However, in practice one has to compromise individually between the optimized mating frequency and the availability of space and animals, respectively, as well as the activity of males for each line.

Pheromonal effects

Pheromones play a major role for the (chemical) communication between animals, including rodents (*Karlson & Luscher, 1959; McClintock, 1983*). The use of hormones to superovulate females is a powerful tool, but not all mouse lines respond to superovulation and, especially in case of mutant animals, a sufficient number of females with an age susceptible for superovulation is often not available (*Nagy et al., 2003*). In addition, transportation stress, the new facility, housing in large groups and other factors can negatively influence the outcome of mating (*Chapman et al., 2000*). We tried to understand if pheromonal effects can be used at least to supplement the superovulation.

As demonstrated in Figure 4 the efficiency of superovulation results in one of the lines investigated in a significantly higher number of embryos if donor females were kept in groups of four animals. In the other case no negative influence was observed. This is in good agreement with observations published elsewhere for the Lee-Boot effect (*van der Lee & Boot, 1955a, 1955b; McClintock 1983; Ma et al., 1998*).

Basic to our experiments to predispose females to the pheromones of their future male (Whitten effect) were reports by Whitten (*1956*), McClintock (*1983*), or Coquelin (*1992*). The use of separation grids was preferred as a relatively simple experimental approach for this exposure. However, hindrance of body contacts in the presence of visible and odorous contacts resulted obviously in stress leading to reduced numbers of well-developed

eight-cell-embryos as compared to the controls. This phenomenon could be omitted, if a female was exposed to the bedding of her future male for two days. Handling these mice needs care since mixing up the cages and subjecting females to the bedding, i.e. pheromones, of the “wrong” male will negatively influence the yield (*McClintock, 1983; Chung et al., 1997*). However, both approaches (bedding, separation grid) showed only trends, but no statistically significant differences.

The major effect of pheromones in combination with hormonal superovulation might be an increase of embryonic development, since the number of embryos/donor was positively influenced, but the outcome of VP⁺-animals did not change. However, a wrong experimental set-up did lead to a negative effect. Our data suggest that the utilisation of pheromonal effects in combination with hormonal superovulation is a valuable tool to trigger the embryo yield. The Lee-Boot approach (relatively easy to practise) seems to be the more successful technique. The more complex Whitten approach leads only to a small effect. Here, open caging systems were used; one can speculate that these effects might be rather smaller if IVC housing systems are used.

Revitalization

To complete the whole cryopreservation procedure the capacity to revitalize the frozen embryos of all groups was assessed. However, the same percentage of successfully revitalized embryos was obtained as previously. Subsequently the positive effects discussed here do not influence the revitalization capacity of frozen material.

Taken together the outcome of embryos following superovulation depends on many parameters, most likely in combination. Annual and seasonal effects can be suppressed by housing conditions, but remain detectable at least under climate extremes. There are only limited possibilities to influence this. Our data show also that proper hygienic conditions are a prerequisite to produce embryos successfully. An infection reduces embryo yields. But there

are additional tools to optimize the outcome of mating. The use of pheromonal effects or a suitable frequency of mating.

Even if statistically significant data could not be presented for all parts of this work, this study addresses factors depressing the efficiency and exhibits possibilities to get higher embryo yields. Subsequently it contributes to the “3R” postulations of Russell & Burch (*1959*) to reduce the number of animals needed for experiments whenever possible.

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