ORIGINAL ARTICLE

Embryo survival and fertility differ in lines divergently selected for birth weight homogeneity in mice

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Abstract

The selection of animals for lower environment sensitivity around the optimum trait value can also provide benefits in productivity and welfare. A divergent selection experiment for birth weight environmental variability in mice was successfully conducted over 17 generations. Animals from low variability selected line (L-line) were more robust by having a higher litter size and survival at weaning in a common breeding environment, than those from high variability line (H-line). The objective of this study was to analyze the differences between those divergently selected lines for embryo and fetal survival and for fertility and prolificacy rate. To study embryo survival and ovulation rate, a total of 98 females (34 H-line and 50 L-line) were studied in four generations of the divergent experiment. To analyze fetal survival and fertility rate, 378 female mice (138 H-line and 240 L-line) in 10 generations or the divergent experiment were studied. Ultrasound scans were performed at day 14 of gestation to establish the number of total fetal and the embryo absorptions. Mortality was addressed as the difference between litter size at birth and the number of fetuses at 14 days of gestation. The number of pregnant females in the first 3 days after mating was used to measure fertility. A linear model was also fitted to analyze embryo mortality, litter size, and the number of embryos at 14 days of gestation. A categorical model was then used to study fertility, including line, generation, and its interaction as effects. Despite the fact that there were no significant differences in the ovulation rate, litter size at birth was significantly higher in the L-line than in the H-line (9.82 vs. 8.36 pups, $p < 0.001$). Moreover, embryo mortality was significantly lower in the L-line than in the H-line (1.39 vs. 2.87 fetuses, $p < 0.001$). L-line females were more fertile (53.49% vs. 23.26% for the H-line). According to these results, the line selected for low environmental variance would be preferable for robustness and animal welfare.

KEYWORDS
litter size, robustness, survival, uniformity

1 | INTRODUCTION

The pig industry has recently been facing a complicated challenge. The industry aimed at producing a quality product together with profits in a socially acceptable way (Rutherford et al., 2013). The selection performed over the years for increased litter size at birth has improved production efficiency by increasing the number of slaughtered animals produced per sow. Prunier et al. (2010) have expressed their concern about increasing litter size which may be detrimental to animal welfare. For piglets in particular, it has been demonstrated that larger litter sizes increased: mortality, lower birth weight (Rutherford et al., 2013), higher competition for teats (Andersen et al., 2011), lower physical size, immune function development, and lower strength (Tuchscherer et al., 2000). Increased litter size has led a high variability of piglet weight within the litter thus providing a competitive environment where the smallest ones lose out and die (Damgaard et al., 2003).

Phenotypic robustness can be defined as the ability of an organism to buffer the impact of internal (genetic variation) and external factors (environmental effects) on the phenotype (Varón-González et al., 2019). Homogeneity has also been associated with a reduction of handling and production costs in the animal production industry, giving homogeneous batches of animals, fewer cases of mortality in prolific species, and higher profits (Bolet et al., 2007). Selection for homogeneity has resulted in more robust animals that are better prepared to deal with environmental challenges (Broom, 2008). Pallares and Gonzalez-Bulnes (2010) argued that decreased embryo survival and intrauterine growth retardation may result from genetic factors inherent by the embryo itself (embryo genotype) or from deficiencies in uterine environment or function (maternal factors). Global survival during gestation in a population is a valid indicator of its robustness.

Formoso-Rafferty et al. (2016a) developed a divergent selection experiment for birth weight environmental variability in mice, and they concluded that it was possible to modify the genetic control of the birth weight environmental variability. Formoso-Rafferty et al. (2016b) also showed that this selection criterion had direct effects on other interesting traits in livestock. In the present paper, we describe the results obtained in two complementary experiments developed to evaluate survival along gestation during the embryo and fetal stages in two divergent selected lines in mice. The experiments were consecutive: the results from the precedent experiment evaluated the reproductive features such as the appearance of estrus, ovulation, fertility, and prolificacy rates. Therefore, the objective of this study was to compare two divergent selected lines for birth weight environmental variability analyzing their differences in embryo loss, fetal survival, and fertility rate.

2 | MATERIALS AND METHODS

2.1 | Divergent selection experimental frame

Individuals from two divergent mice lines were selected for birth weight (BW) environmental variability (Formoso-Rafferty et al., 2016a). The experiment was successful and showed a dramatic divergent response for BW residual variance, but also with a favorable correlated response on traits related to welfare and robustness. The process involved a selection intensity equivalent to 30% of individuals in each line during 30 generations, and optimizing the selection response carefully by controlling the increase in inbreeding. These lines will be referred to as H-line and L-line for high and low variability lines, respectively, across the rest of the present article. Details of the selection process are described in Formoso-Rafferty et al. (2016a).

Animals were housed at the experimental facility of the Department of Animal Production of the Veterinary Faculty of the Complutense University of Madrid. The housing and management conditions of the animals were according to Spanish legislation RD 53/2013, on the basic rules for the protection of animals used in experimentation and other scientific purposes (BOE, 2013).

2.2 | Experimental procedure

The present study as a whole was divided into two experiments: from the conception to the 14th day of gestation and from the 14th of gestation to birth.

2.2.1 | Experiment 1: Embryo survival and ovulation rate

In this experiment, a total of 98 selected females (48 H-line and 50 L-line) from four consecutive generations (from 23 to 26) were mated again after the mating schedule to have a third gestation. The males were separated in the morning when a vaginal plug was detected and this procedure was repeated over a 3-day period. A total of 80 females were finally pregnant (34 H-line and 45 L-line). At 14 days of gestation, females were anesthetized and euthanized by cervical dislocation. A midline laparotomy was performed to expose the uterus, which was removed together with all gestational sacs and placed in a Petri dish (NunclonTM; Nunc International); the fetuses were counted (NF) and

the total number of moles present in both uterus horns (NM). The genital tract was collected, and both uterine horn lengths were measured from the utero-tubal junction to the uterine bifurcation (HL, in mm). The ovaries were examined in a stereo microscope, and the corpora lutea in the two ovaries (CL) were counted as an ovulation rate indicator. The ratio between NF and CL (embryo survival) and the difference between CL and NF (number of embryo losses) were also analyzed.

2.2.2 | Experiment 2: Fetal survival and fertility rate

This experiment included the specific registered information of 43 females per line from 10 generations (from generation 16 to 26) of the selection experiment. Animals were mated one male to one female until the appearance of the vaginal plug by visual inspection every morning for 3 consecutive days after overnight mating. The male was separated in the morning when a vaginal plug was detected. The presence of a vaginal copulation plug the following morning implied that mating had occurred, but it did not mean that a pregnancy will result even if fertile males were used. It is important to check vaginal plugs early in the morning because the plug usually falls out or no longer detectable approximately 12 h after mating and sometimes even earlier (Behringer et al., 2016).

A total of 378 female mice (138 H-line and 240 L-line) were anesthetized at day 9 of gestation (females from the first seven generations) and at 14 days of gestation in an enclosed induction chamber with isoflurane vapor (Forane, Baxter) delivered in 100% oxygen using a precision vaporizer with appropriate scavenging of waste gas. Isoflurane vapor was delivered at 5% during induction and at 1% via a facemask during imaging minimizing stress and breathing movements during scans. The abdomens of the females were shaved, and heated coupling gel was applied. The animals were placed supine on a table and held gently by the operator performing the imaging and applying the anesthesia. Transabdominal ultrasound scan was carried out with a multifrequency (14–18 MHz) linear array transducer (Hitachi Aloka Noblus) and registered the number of fetuses at 9 and 14 days of gestation (LS_9 and LS_{14}) in both uterine horns. This procedure was quick, approximately 10 min for each female, with easy recovery of consciousness. Ultrasonography can be accurately used as an alternative reliable non-invasive technique for pregnancy diagnosis and establishing litter size in prolific animals from the very early stages of gestation. Thus, the need for such a tool as ultrasonography has become increasingly important (Brown et al., 2006). A total of 348 females were confirmed to be pregnant (121 H-line and

227 L-line). All the females were checked daily during the parturition period, and all the newborns from the females giving litters were noted to register the litter size at birth (LS_B) of the female. The evaluated traits were:

(i) The fertility (F) measured as the percentage of females presenting vaginal plug in the 3 days after mating;
(ii) The total number of fetuses at 9 and 14 days of gestation (LS_9 and LS_{14});
(iii) The asymmetry between uterus measured as the difference in embryos or fetus between horns at 9 and 14 days of gestation (AS_9 and AS_{14}, in pups);
(iv) Prenatal mortality from 9 to 14 days gestation and from 14 days to birth ($M_{9–14}$ and $M_{14–B}$, expressed in percentage and in number of pups lost);
(v) The number of moles in the uterus horns (NM);
(vi) And litter size at birth (LS_B, in pups).

2.3 | Statistical analyses

All the considered traits were compared between lines via a generalized mixed linear model with equation:

$$y = \text{generation} + \text{line} + \text{generation} \times \text{line} + \text{female} + \text{error}$$

where y was the traits NF, NM, HL, CL, embryo survival and number of embryo losses, in Experiment 1, and LS_9, LS_{14}, LS_B, AS_9, AS_{14}, $M_{9–14}$, $M_{14–B}$, NM, and F in Experiment 2, one at a time. Including the generation (23–26 in Experiment 1 and 16, 17, 18, 19, 20, 22, 23, 24, 25, 26, or 27 in Experiment 2), line (H-line or L-line), and its interaction as fixed effects and the female as random effect besides the residual in both experiments. The means were compared by the Tukey's test under different significance levels: $p < 0.05$; $p < 0.01$; and $p < 0.001$. The least square means were computed to show the observed differences between levels of relevant effects. Female fertility was compared based on single statistical chi-squared test using the proc frec procedure. The Statistical Analysis System software was used for the statistical analysis of the data (SAS Institute, 1990).

3 | RESULTS

3.1 | Experiment 1: Embryo survival and ovulation rate

Table 1 shows the means and standard error (in brackets) for NF, NM, CL, HL, embryo survival, and losses with the significance between lines, generation, and its interaction. Results for NF were similar between lines, but there were differences between lines in the rest of traits considered.

TABLE 1 Least square means and standard deviation (in brackets) with their significance in both variability lines, generations, and its interaction.

			Significance level		
	High line	**Low line**	**Line**	**Generation**	**Line*Generation**
NF	7.54 (0.11)	8.89 (0.07)	n.s.	n.s.	n.s.
NM	4.00 (0.09)	1.27 (0.03)	***	n.s.	n.s.
CL	13.91 (0.10)	11.56 (0.07)	**	n.s.	n.s.
HL	62.04 (0.49)	69.02 (0.47)	*	**	*
Embryo survival	0.54 (0.01)	0.78 (0.01)	***	n.s.	n.s.
Embryo losses	6.37 (0.10)	2.67 (0.07)	***	*	n.s.

Abbreviations: CL, number of corpora lutea; HL, uterus horns length (mm); NF, number of fetuses; NM, number of moles; n.s., not significant.

$*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

TABLE 2 Least square means and standard error (in brackets) for asymmetry between uterus horns at 9 and 14 days of gestation, total litter size at 9 and 14 days of gestation and at birth with their significance by line.

	Line		
	High line	**Low line**	***p***
AS_9	0.25 (0.02)	0.18 (0.02)	**
LS_9	9.53 (0.31)	9.84 (0.20)	n.s.
AS_{14}	0.22 (0.02)	0.18 (0.01)	n.s.
LS_{14}	9.84 (0.24)	10.28 (0.16)	n.s.
LS_B	6.35 (0.24)	9.24 (0.16)	***

Abbreviations: AS_9, asymmetry at 9 days of gestation (pups); AS_{14}, asymmetry at 14 days of gestation (pups); LS_B, litter size at birth (pups); LS_9, litter size at 9 days of gestation (pups); LS_{14}, litter size at 14 days of gestation (pups); n.s., not significant.

$**p < 0.01$; $***p < 0.001$.

NM and CL were significantly bigger for H-line with a uterus horns slightly shorter than L-line. Results for embryo survival were significantly better for L-line (0.54 vs. 0.78). As a result, L-line presented lower embryo losses of 3.70 embryos less than the H-line. Generation was not or slightly significant and differences were no relevant.

3.2 | Experiment 2: Fetal survival and fertility rate

Table 2 shows the least square means and the standard error of traits within lines and the significance between them. There were significant differences between lines in AS_9 ($p < 0.01$) and in LS_B ($p < 0.001$). No differences were observed between lines in LS_9, AS_{14}, and LS_{14}. Note that LS_{14} is a measure of NF obtained indirectly from ultrasound images and that there were no differences between lines, as found for NF in Experiment 1 (Table 1). Thus,

both lines were similar for LS_9 and LS_{14}, with no differences between lines. Table 3 includes the least square means and the standard error for the same traits across generations and the significance. There were significant differences in AS_9, LS_9, LS_{14}, and LS_B. The L-line presented more symmetric uterus horns and LS_B higher. There were only significant differences for the interaction line and generation in LS_B ($p < 0.01$).

Figure 1 shows the differences between lines across generations in LS_{14} and LS_B with LS_B in L-line in all generations being markedly higher. Table 4 shows the means and significant differences between lines in the detection of the vaginal plug, which was 55.35% in L-line over the total of females mated in the 3 days following mating, and 32.09% in H-line ($p < 0.001$). Differences between lines in parturition rate were significant and also better for L-line females than H-line (94.54% vs. 86.23%, $p < 0.01$).

4 | DISCUSSION

The present study included two experiments, Experiment 1 focused on the evaluation of ovulation rate inferred by the number of CL and the implantation success assessed by the NF found at day 14 of gestation. This revealed no differences between lines in both traits but with NF relevantly higher in the L-line, while CL was relevantly higher in the H-line. Experiment 2 was a study on the survival from day 14 of gestation to birth by studying the differences between lines in litter size on the 14th day of gestation and at birth: this resulted in a higher LS_B but with no differences in LS_{14}. The studies showed that selecting for homogeneity improved the robustness in better reproductive performance of the females and the reduction of embryo losses, thus resulting in bigger litter size at birth. The observed differences among lines in litter size at birth could have been established during the ovulation, as reported in rabbits (Argente et al., 2017). However, in the

TABLE 3 Least square means and standard error (in brackets) for asymmetry between uterus horns at 9 and 14 days of gestation, total litter size at 9 and 14 days of gestation and at birth with their significance by generation.

| | Generation | | | | | | | | | | |
	16	17	18	19	20	22	23	24	25	26	p
AS_9	0.10 (0.03)	0.14 (0.03)	0.16 (0.04)	0.37 (0.05)	0.25 (0.04)	0.23 (0.04)	0.28 (0.03)				***
LS_9	10.91 (0.44)	11.22 (0.43)	10.50 (0.52)	9.06 (0.69)	9.04 (0.48)	8.29 (0.44)	8.80 (0.39)				***
AS_{14}	0.14 (0.03)	0.18 (0.03)	0.20 (0.04)	0.25 (0.05)	0.14 (0.03)	0.19 (0.03)	0.24 (0.03)	0.19 (0.03)	0.25 (0.03)	0.23 (0.03)	n.s.
LS_{14}	11.40 (0.43)	12.09 (0.42)	10.21 (0.51)	8.96 (0.68)	11.11 (0.47)	9.22 (0.43)	9.56 (0.38)	10.10 (0.41)	8.85 (0.37)	9.11 (0.45)	***
LS_B	9.69 (0.43)	9.64 (0.42)	8.07 (0.51)	5.24 (0.68)	7.80 (0.47)	6.73 (0.43)	7.81 (0.38)	7.42 (0.41)	9.11 (0.45)	7.83 (0.45)	***

Abbreviations: AS_9, asymmetry at 9 days of gestation (pups); AS_{14}, asymmetry at 14 days of gestation (pups); LS_B, litter size at birth (pups); LS_9, litter size at 9 days of gestation (pups); LS_{14}, litter size at 14 days of gestation (pups); n.s., not significant.

***$p < 0.001$.

present study, homogeneous females were found to be no different from heterogeneous ones in the ovulation rate and litter size at 14 days, but had a bigger litter size at birth suggesting fewer embryo losses: it seems that the causes of the losses would occur during late gestation. Argente et al. (2017) in a divergent selection experiment for environmental variability of litter size in rabbits found differences in ovulation rate in favor of the homogeneous line with more embryo losses in the heterogeneous line. These authors hypothesized that the heterogeneous line could be more sensitive to stress and diseases than the homogeneous line. In this context, Argente et al. (2014) found a lower immune response to pathogenic agents in females from the heterogeneous line, thus showing a greater vulnerability to diseases. Moreover, there is evidence that maternal stress around the time of implantation increased the failure rate in blastocyst implantation (Burkus et al., 2015). So, this would be in agreement with a large number of embryonic losses around implantation in this heterogeneous line.

These differences between divergent mice lines in the litter size at birth were already shown to be maintained throughout lactation resulting in bigger litter size at weaning (Formoso-Rafferty et al., 2017). Also, important was higher reproductive longevity reported with a threefold probability of performing a new parturition in the homogeneous line relative to the heterogeneous line (Formoso-Rafferty et al., 2022). The advantage of the L-line is added to the ones already found, such as litter size, weaning weight, and survival, presenting benefits in production (Formoso-Rafferty et al., 2016b), animal welfare (Formoso-Rafferty et al., 2016a), heritability (Formoso-Rafferty et al., 2016b), and robustness traits (Formoso-Rafferty et al., 2018, 2019).

Mice are considered a suitable animal model for livestock prolific species like pigs or rabbits (Hill & Caballero, 2000). Driven by an objective to improve production efficiency, litter size through genetic selection and management techniques has been a selection criterion for the pig industry (Spötter & Distl, 2006; Webb, 1998). However, concerns were recently raised regarding the consequences of production efficiency on animal welfare (Baxter et al., 2013; Prunier et al., 2010). Welfare issues related to litter size in the domestic pig are complex, affecting both sows and piglets. Pig production is also an economic activity whose profitability relies largely on the efficient reproductive management of sows (Knox, 2016; Lopes et al., 2000; Roca et al., 2016). Achieving such efficiency involves reducing non-productive days, which will allow more piglets to be weaned per sow and per year (Knox, 2016). Sows with one or more farrowings represent around 80% of the total sow population in breeding pig farms.
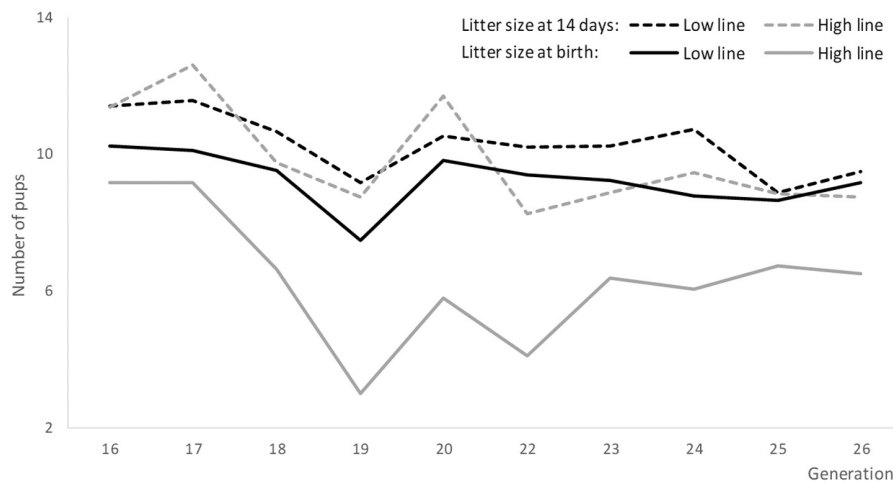
FIGURE 1 Least square means of litter size at 14 days of gestation (discontinuous lines, LS_{14}, in pups) and at birth (continuous lines, LS_B, in pups) by line and generation.

TABLE 4 Means and differences in the appearance of the vaginal plug ($n = 860$ females) and parturition rate ($n = 378$ females) between lines in Experiment 2.

	High line	Low line	p
Appearance of vaginal plug (%)	32.09	55.35	***
Parturition rate (%)	86.23	94.54	**

**$p < 0.01$; ***$p < 0.001$.

Litter size is one of the most important economic traits in pig production (He et al., 2016). It is a composite trait influenced by multiple factors including ovulation rate, embryonic/fetal survival rate, uterine capacity, and placental efficiency (Biensen et al., 1998; Spötter & Distl, 2006; Wilson et al., 1999). Moreover, the heritability of litter size is known to be low (0.09) for both the total number born and number born alive (Schneider et al., 2012), and thus, direct selection achieves slow progress in increasing litter size.

Furthermore, an increase in litter size has resulted in a simultaneous increase in its variability (Dobrzanski et al., 2020; Sell-Kubiak et al., 2015), affecting profitability of production by increasing management costs (Freyer, 2018) and negatively the animal welfare (Prunier et al., 2010; Rutherford et al., 2013). Hence, litter size in pigs has increased during the past decades, causing uterine crowding and a higher demand for nutrients during gestation (Foxcroft et al., 2009; Lynegaard et al., 2020; Town et al., 2004). Also, an increase in mortality as a consequence of the selection aimed at increasing litter size, which is linked to a greater heterogeneity of piglet weights at birth (Damgaard et al., 2003). Consequently, birth weight of the individual piglet has decreased (Quiniou et al., 2002), and approximately 30% of neonatal piglets in Denmark are intrauterine growth restricted (Amdi et al., 2013; Hales et al., 2013),

resulting in reduced postnatal growth not entirely explained by decreases in BW (Hansen et al., 2018; Huting et al., 2018).

Prolific species have a natural propensity to conceive large numbers of offspring: issues relating to fetal litter size that were reviewed and discussed previously (Ashworth et al., 2001; Foxcroft et al., 2006; Rutherford et al., 2013). The first stage at which the litter size could be expected to affect newborn biology is in the uterus.

Studies based on post-mortem examinations performed at a specifically gestational age showed discrepancies associated with the chronology of the events causing differences in litter size and fetal development in mice. Pallares and Gonzalez-Bulnes (2010) reported that embryo losses and retardations occurred around the time of implantation and early embryo development (Gagioti et al., 2000; Gouge et al., 1998). Other authors stated (Hefler et al., 2001; Van der Heijden et al., 2005) that differences in fetal growth were established not earlier than during late pregnancy (day 17 post-coitum). Recent studies using real-time ultrasound imaging showed a first peak of embryo losses at implantation and a second peak between days 8.5 and 13.5 in sows (Auvigne et al., 2010), i.e.: from the beginning of gastrulation to the transition of late embryo to early fetus. In the conceptus showing IUGR (the failure of fetuses to reach their growth rate is known as Intrauterine Growth Restriction), retardations in the development of the embryo and the entire gestational sac were also found to occur after day 8.5 (Bertoldo et al., 2011). In rabbits, the experiment selection criteria was litter size variability, and probably this could affect the ovulation rate (Argente et al., 2014). In the present mice experiment, BW variability was used as a trait not having an influence on the ovulation rate but on the embryo and fetal survival. Our results showed that BW variability seems to be more related to survival mainly in the late stage of gestation.

There was a negative correlation between litter size variability with both the number of implanted embryos and litter size in rabbits (Argente et al., 2014). Moreover, a negative correlation between uterine capacity and its residual variability was reported (Ibáñez-Escriche et al., 2008), which was highly correlated with litter size (Argente et al., 2000). These authors concluded that selection for litter size variability showed a negative correlated response in embryonic survival and also had an influence on litter size at birth.

According to the results of the present study, the line selected for low variability presented important reproductive advantages. Higher longevity was previously demonstrated in homogeneous animals suggesting higher robustness and better animal welfare (Formoso-Rafferty et al., 2022). The results of our study suggest that selection for homogeneity may provide a valuable tool for optimizing litter size in commercially important prolific breeds. Also important is that selection for litter size at birth in piglets has led to an increase in the number of non-viable piglets at birth (Rutherford et al., 2013). It was reported that the selection for homogeneity improved the survival at birth and weaning, even during the gestational period. Thus, animal welfare indicators are higher survival throughout gestation, at birth, and at weaning. Moreover, homogeneity is an economic trait in livestock production (Mulder et al., 2008). Our results suggest that selecting for birth weight homogeneity could potentially increase animal welfare. Based on the findings presented here, we argue that selection for uniformity could be useful to improve robustness and animal welfare.

CONFLICT OF INTEREST STATEMENT
The authors declare that there have no conflicts of interest to this publication.

DATA AVAILABILITY STATEMENT
The datasets generated and/or analyzed during the present study are available from the corresponding author upon reasonable request.

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