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TITLE: Embryo transfer following IVF alters susceptibility to metabolic phenotypes in male mouse offspring compared to naturally conceived offspring

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Main Text

One in six European couples experience infertility, and global infertility rates continue to rise (Skerrett-Byrne et al., 2025). Consequently, *in vitro* fertilisation (IVF) is increasingly employed as an assisted reproductive method to circumvent these growing infertility issues. It is well established that IVF-derived offspring in both humans and mice exhibit differences compared to naturally conceived individuals (Guo et al., 2017; Rhon-Calderon et al., 2025). However, outcomes reported in animal studies are highly strain and experimental set up dependent, highlighting substantial unresolved uncertainty in the literature. Although many experimental designs use IVF to control for *in utero* effects and other confounders, the procedure itself introduces biological perturbations whose associated comorbidities must be carefully considered.

In this study, we comprehensively examined metabolic phenotypes in C57BL/6N mice offspring derived from natural conception (NC) and embryo transfer following IVF (Embryo Transfer; ET). Detailed methods are attached in the supplements (S1). CD1 foster mothers, commonly used due to their robust maternal behaviour, served as recipients for IVF-derived embryos. We used protocols of the European Mouse Mutant Archive (EMMA) for sperm collection and analysis, IVF and embryo transfer, publicly available under: https://www.infrafrontier.eu/emma/cryopreservation-protocols/. Phenotypic differences between NC and ET derived mice may originate from the IVF procedure, the embryo transfer or embryo culture conditions, *in utero* development in foster mothers as well as from differences in milk availability and differences in rearing behaviour.

At 9 weeks of age the offspring was challenged with high-fat diet (HFD) until organ withdrawal at 16 weeks of age, to enhance the development of potential metabolic phenotypes. All the tests were performed after the HFD challenge at 15 weeks of age, exceptionally activity measurement was performed during the 5th week of the HFD challenge, as well as body weight was measured weekly. Additionally, blood glucose was measured at 9, 12, 15 and 16 weeks of age. Beyond the known increases in body weight and glucose metabolism in IVF conceived offspring (Fig. S2 A-F) (Rhon-Calderon et al., 2025), we identified marked alterations in liver parameters (Fig. 1A-C), activity patterns (Fig. 1K, L, M), and inflammation markers (Fig. 1D-J). The litter size was just slightly increased in NC derived mice (Fig. S2 G) and now differences in sex distribution was observed (Fig. S2 H). Strikingly, there appears to be a sex-specific metabolic changes, with male IVF offspring more severely affected compared to their NC male counterparts. However, female IVF

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offspring exhibited a significantly increased body composition (% of fat mass) after HFD challenge (Fig. S2 D). This increased body fat could point towards a higher susceptibility to develop obesity. The observed elevation of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and liver weight, together with increased pro-inflammatory cytokines (TNF-α, IL-6 Keratinocyte chemoattractant (KC)/human growth-regulated oncogene (GRO)) and the compensatory anti-inflammatory IL-10 (Fig. 1A-J), suggests that male IVF offspring experience hepatic and systemic inflammatory stress (Al-Qahtani et al., 2024). The absence of these changes in female offspring points to possible sex-dependent mechanisms, potentially mediated by hormonal or epigenetic factors (Aljabali et al., 2024). Hyperactivity was observed in both sexes of the IVF offspring during their habitual sleep phase (Fig. 1L), which points toward a potential disruption of circadian regulatory mechanisms. However, this could also have other causes e.g. stress response or metabolic changes.

In conclusion, these findings underscore the potential impact of IVF and related confounding factors on offspring's metabolic health and emphasise the importance of carefully evaluating reproductive methods and their potential long-term consequences in experimental animal models.

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Conflict of Interest

The authors declare no conflicts of interest. David A. Skerrett-Byrne is an Associate Editor of Reproduction and Fertility and was not involved in the review or editorial process for this paper, on which he is listed as an author.

Author Contributions

Conceptualisation, K.L., D.A.S.B., and J.B.; Investigation, K.L., S.M., S.H., and K.R.; Formal analysis, K.L., D.A.S.B., and J.B.; Resources, M.H.A., R.T., and J.B.; Writing – Original Draft K.L., D.A.S.B, and J.B.; Writing – Review & Editing K.L., D.A.S.B, S.M., S.H., K.R., R.T., M.H.A, and J.B.; Visualisation, K.L., D.A.S.B., and J.B.; Funding Acquisition, D.A.S.B, and J.B.; Supervision, D.A.S.B, M.H.A., and J.B.

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FIGURES

Figure 1. Liver, inflammation and activity assessment of offspring derived from natural conception (NC) and embryo transfer (ET). All the tests were performed after organ withdrawal at 16 weeks of age, exceptionally activity measurement was performed during the 5th week of the HFD challenge. (A) Liver weight of female and male NC and ET offspring and (B) liver weight expressed as a percentage of bodyweight. (C) Histological steatosis scores and (D) histological inflammation scores in livers from of NC and ET offspring. (E) Plasma levels of alanine aminotransferase (ALT) and (F) aspartate aminotransferase (AST) in NC and ET offspring. (G) Circulating levels of cytokines IL-10 and (H) IL-6, (I) Keratinocyte chemoattractant (KC)/human growth-regulated oncogene (GRO) and (J) TNF-alpha in NC and ET offspring. (K) Activity counts during the sleep and (L) active phases of female and male NC and ET offspring. (M) Visualisation of the average locomotor activity observed during the course of a high-fat diet (HFD) challenge. Data are presented as mean with SD and statistical significance is indicated as *p*-values: *<0.05, **<0.01, ***<0.001, ****<0.0001

SUPPLEMENTS

Methods S1. Detailed information about phenotypic procedures and statistics.

Figure S2. Phenotypic characterisation of offspring derived from natural conception (NC) and embryo transfer (ET). All the tests were performed after the HFD challenge at 15 weeks of age, exceptionally body weight was measured weekly. (A) Body weight of female and male NC and ET offspring at weaning and (B) during the course of a high-fat diet (HFD) challenge initiated at 9 weeks of age. (C) Body composition of NC and ET offspring prior to and (D) post HFD exposure.
(E) Glucose tolerance test was performed at 15 weeks of age, whilst (F) blood glucose measurement was taken at 9, 12, 15 and 16 weeks in NC and ET offspring. (G) Litter size from NC and ET pregnancies and (H) sex distribution of offspring across groups. Data are presented as mean with SD and statistical significance is indicated as *p*-values: *<0.05, ****<0.0001

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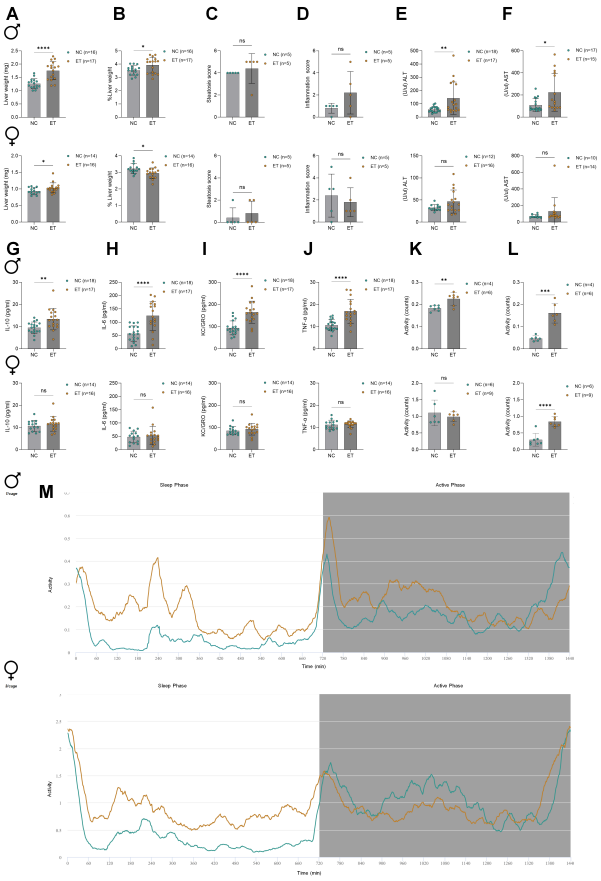
Lay Summary

The aim of this study is to highlight potential differences between embryos conceived naturally and those conceived via *in vitro* fertilization (IVF). While the current scientific literature generally assumes that differences between these two modes of conception exist, many studies do not provide detailed phenotypic data, e.g. bodyweight, blood glucose, and other observational metabolic health parameters. In addition, comparisons are often complicated by variations in choosing different strains of foster mothers to carry embryos.

By addressing these limitations, our short summary and overview provide a well-defined comparison within a controlled experimental setup including a high-fat diet feeding period as metabolic challenge. We make the resulting data publicly available to offer a reference point for researchers using a specific mouse strain as fosters and to improve transparency regarding the specific differences in metabolic health observed between natural conception and IVF in this context.



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METHODS

All mice were housed in digital ventilated cages on a 12-h light/12-h dark cycle at constant temperature (22 ± 1 °C) and under controlled humidity. Mice had ad libitum access to water and food. Mouse experiments were conducted in compliance with the Federation of European Laboratory Animal Science Association (FELASA) protocols. All mouse experiments were performed according to ROB-55.2-2532.Vet_02-23-59 approved by the Regierung von Oberbayern.

In vitro fertilization and embryo transfer

In vitro fertilization (IVF) was conducted with gametes of C57BL/6N mice at the age of 17-18 weeks. In parallel animals were mated at the same age. Female oocyte donors were stimulated with 7.5 U of pregnant mare serum gonadotropin (PMSG) and 7.5 U of human chorionic gonadotropin (hCG) 64 and 14 hours prior to oocyte collection, respectively. Fresh sperm was obtained from the cauda epididymis and cultured in preincubation medium for 1 hour to allow capacitation. Oocytes were collected from the ampulla and co-cultured with 5 µL of capacitated sperm in human tubal fluid (HTF) medium at 37 °C and 5% CO2. After incubation for 4-6 hours, fertilized oocytes were washed in HTF to remove sperm and cumulus cells, transferred into potassium simplex optimization medium (KSOM) medium and incubated overnight at 37 °C and 5% CO2. In order to obtain foetuses and living offspring, 2-cell embryos were subjected to embryo transfer. Therefore, 2-cell embryos were transferred into pseudo-pregnant foster mothers of the CD-1 strain that have been mated with vasectomized males on the previous day and displayed a vaginal plug. CD-1 females were anaesthetized with a mixture of ketamin (100 mg/kg) and xylazin (16 mg/kg) before surgery and embryos were transferred bilaterally into the oviducts through two minor incisions. Postsurgical pain management was ensured by oral application of Novalgin (200 mg/kg). We used protocols of the European Mouse Mutant Archive (EMMA) for sperm collection and analysis, IVF and embryo transfer, publicly available under: https://www.infrafrontier.eu/emma/cryopreservation-protocols/.

Offspring were maintained on an identical dietary regimen. Standard chow was fed until 9 weeks of age. At 9 weeks of age, they were switched to a high-fat diet (HFD) for 6 weeks as a metabolic challenge.

DVC

Spontaneous locomotor activity and behaviour were monitored using the DVC™ (Digital Ventilated Cage) home cage monitoring system (Tecniplast, Buguggiate, Italy). Mice were housed in standard individually ventilated cages equipped with the DVC™ sensor system under controlled temperature and humidity conditions with a 12 h light/dark cycle. Food and water were available ad libitum.

The DVC™ system continuously recorded animal activity through capacitance-based sensing of movement within the cage floor plate. Activity data were collected and processed using the manufacturer's software. Parameters including locomotor activity patterns and circadian rhythmicity were extracted.

Litter Size

From animals derived with natural conception there were in total 5 litters to analyse, for embryo transfer followed by IVF derived offspring 7 litters. The litter size of NC derived offspring was slightly increased. The sex distribution was evenly between both groups.

Analysis

For NC offspring 14 females and 18 males were analysed, depending on the test the n-numbers could vary, indicated in the figure panels. The tests were performed per animal. For the activity analysis we monitored 4 male animals of NC and 6 male animals of ET analysed over 7 following days during the 5th week of the HFD challenge. For the females the animals were group caged in groups of 3, therefore 6 animals of NC and 9 animals of ET were analysed. All tests were performed after the HFD challenge at 15 weeks of age, exceptionally activity measurement was performed during the 5th week of the HFD challenge, as well as body weight was measured weekly.

Glucose tolerance test. Mice were fasted for 4 h during light phase before administration of 2 g of glucose per kg body weight by intraperitoneal injection. Blood was collected from the tail vein at 0, 15, 30, 60 and 120 min and blood glucose concentrations were measured using a Contour Bayer glucometer. The test was performed at 15 weeks of age.

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Fasting blood glucose. Mice were fasted for 4h during light phase. Blood was collected from the teil vein and blood concentrations were measured using a Contour Bayer glucometer. The test was performed at 9, 12, 15 and 16 weeks of age.

Statistical analysis of physiological parameters from mice GraphPad Prism v.10.4.1 was used to analyse physiological data from mice. Graphs are shown with SD. Unpaired t-test for all bar graphs. Mixed models with multiple testing correction for bodyweight, glucose tolerance test and blood glucose measurement. *$p \leq 0.05$, **$p \leq 0.01$, *** $p \leq 0.001$.