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Postnatal phenotype of dairy cows is altered by in vitro embryo production using reverse X-sorted semen

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ABSTRACT

Abnormal fetuses, neonates, and adult offspring derived by assisted reproductive technologies have been reported in humans and mice and have been associated with increased likelihood of certain adult diseases. To test the hypothesis that bovine females derived by assisted reproductive technologies have altered postnatal growth and adult function, a retrospective cohort study evaluated survival, growth, and production traits of offspring derived by in vitro embryo production (IVP) with conventional (IVP-conv) or reverse X-sorted semen (IVP-sexed), multiple ovulation and embryo transfer, and artificial insemination (AI) in a large dairy herd. Live calves produced by IVP were born slightly heavier compared with AI calves. In addition, IVP-sexed calves had a higher cumulative mortality from 90 to 180 d of age compared with AI offspring. Mortality of IVP-conv and multiple ovulation and embryo transfer offspring was intermediate and not different from AI or IVP-sexed offspring. The altered phenotype of offspring from IVP-sexed extended to adult milk production. Cows derived by IVP-sexed produced less milk, fat, and protein in their first lactation compared with dairy cows derived by AI. Additionally, females born to nulliparous dams had a distinct postnatal phenotype compared with offspring from parous dams even when data were restricted to offspring of surrogate females. In conclusion, procedures associated with in vitro production of embryos involving use of reverse-sorted spermatozoa for fertilization result in an alteration of embryonic programming that persists postnatally and causes an effect on milk production in adulthood. Thus, some benefits of reverse-sorted semen for genetic improvement may be offset by adverse programming events.

Key words: in vitro fertilization, reverse-sorted semen, developmental programming, milk yield, bovine

INTRODUCTION

The theory that maternal environment during gestation modulates offspring health and disease has been repeatedly demonstrated (Wadhwa et al., 2009; Schulz, 2010). Programming of adult phenotype can occur at the earliest stages of embryonic development, during the preimplantation period [see Fleming et al. (2015) and Hansen et al. (2016) for review]. Furthermore, epigenetic changes in male gametes can also play a role in programming offspring phenotype [see (Rando and Simmons, 2015) and (Schagdarsurengin and Steger, 2016) for review].

Perhaps the most extreme perturbation in the environment of the preimplantation embryo occurs when the embryo is produced in vitro. In this situation, nutrients, regulatory molecules, ions, dissolved gases, the substratum, and other molecules in the reproductive tract are replaced by artificial culture media. The result can be alterations in embryonic gene expression, DNA methylation, metabolome, cell allocation into inner cell mass and trophectoderm lineages, and competence to establish pregnancy after transfer to recipient females (Thompson, 1997; Lonergan et al., 2006; Urrego et al., 2014). Even an assisted reproductive technology (**ART**) such as multiple ovulation and embryo transfer (**MOET**), where embryonic development occurs in vivo, can perturb embryonic function due to changes in the reproductive tract or characteristics of the oocytes from which embryos are derived (Market-Velker et al., 2010; Gad et al., 2011; Mainigi et al., 2014).

Conceptuses generated from MOET or in vitro production of embryos (**IVP**) have altered placental growth and function (Miles, 2004; de Waal et al., 2014; Mainigi et al., 2014). In addition, IVP has been associated with abnormal fetal development in sheep, cattle, mice, and humans (Young et al., 1998; Farin et al., 2010; Bloise

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et al., 2014). The result can be increased incidence of pregnancy loss (Kruip and den Daas, 1997; Farin et al., 2006), preterm delivery (Ceelen et al., 2008), perinatal morbidity and mortality (Jackson et al., 2004; Hansen and Bower, 2014), and a phenomenon known as large offspring syndrome in which offspring are of unusually large size (Farin et al., 2006, 2010). Offspring born as a result of IVP can have altered phenotypes that extend into juvenile and adult periods as shown in humans (Ceelen et al., 2008; Valenzuela-Alcaraz et al., 2013; Rexhaj et al., 2015) and mice (Calle et al., 2012; Rexhaj et al., 2013; Donjacour et al., 2014). Some abnormalities in mice occurred primarily in one sex (Donjacour et al., 2014) and some were transmitted transgenerationally (Calle et al., 2012; Rexhaj et al., 2013).

The question of whether alterations in developmental programing associated with ART affect postnatal function is becoming an increasingly important question because of the growing effect of these technologies on human and livestock reproduction. In cattle, the number of reported embryos transferred reached over 900,000 in 2015 (Perry, 2016) including, for North America, 271,045 transfers of embryos derived by MOET and 97,871 IVP embryos. Little is known about whether techniques like MOET or IVP are associated with alterations in economically important phenotypes in cattle. It is not even known whether a widely used procedure like sex-sorted semen can alter adult phenotypes. It might because sex-sorted semen has been reported to delay the first cleavage division of the embryo (Bermejo-Álvarez et al., 2010) and to be associated with increased frequency of stillbirths, decreased birth weights, and reduced calf viability (Healy et al., 2013; Djedović et al., 2016). In other studies, however, there were few consequences of sex-sorted semen on the resultant calf (DeJarnette et al., 2009; Norman et al., 2010).

Here we hypothesized that IVP and MOET can modify embryonic programming and lead to changes in postnatal phenotype. We also took advantage of one characteristic of assisted reproduction, namely that the embryo typically develops in a female distinct from the female that provided the oocyte from which the embryo arose, to test whether parity of the female gestating the embryo would also change the developmental program of the embryo to alter adult function.

MATERIALS AND METHODS

Experimental Design

A retrospective cohort observational study was performed to determine the fate of a female calf born following 4 methods of pregnancy establishment: AI (in vivo development), IVP with conventional semen (**IVP-conv**; embryo produced in vitro), IVP with reverse sorted sexed semen (**IVP-sexed**, embryo produced in vitro with the use of X-bearing sperm sorted after thawing), and MOET (an embryo produced in vivo with the aid of superstimulatory hormones). Data were collected from a registered Holstein dairy farm located in Bell, Florida (29.7266° N, 82.8533° W) milking ~4,000 cows/d. Animals were not research animals and institutional approval was not relevant. Calves in the AI group were conceived after estrus detection and AI. Dams were inseminated with a single dose of nonsorted frozen-thawed semen. After AI, no other intervention was performed to the dam, except for ultrasonography at d ~30 and ~60 for pregnancy diagnosis. For MOET, donors were selected within the farm herd and embryos were produced by superovulation with FSH. Donors were artificially inseminated at the end of the FSH treatment with nonsorted semen. Seven days after AI, the uterus was flushed transcervically for embryo collection. For IVP, embryos were produced in vitro by the laboratory of Trans Ova in Boonsboro, Maryland. Cumulus-oocytes complexes were collected by ultrasound-guided ovum-pick up from FSH-treated donors belonging to the farm. Fertilization in vitro was performed with conventional or reverse X-sorted semen, and embryo culture was performed using the laboratory's procedure for IVP. The reverse sorting procedure consisted of X- and Y-chromosome-bearing sperm separation by flow cytometry performed on frozen-thawed semen straws. Donors were maintained at the Trans Ova facility in Maryland and embryos were shipped to the farm for transfer on d 7 of development, typically at the blastocyst stage. Embryos were transferred either fresh (IVP-conv, IVP-sexed, and MOET) or frozen-thawed after conventional slow freezing with ethylene-glycol (MOET) to recipient heifers and cows (a single embryo per recipient) that were at d 6 to 8 of the estrous cycle. No differences were observed between calves produced by MOET and transferred fresh or frozen, and data were combined for these groups. Pregnancy diagnosis was performed at ~30 and ~60 d after embryo transfer.

Study Subjects

Farm records were screened to identify females for inclusion in the study. Inclusion criteria for subjects in the MOET and IVP groups were as follows: conceived by embryo transfer (MOET or IVP), female sex, born alive, entered into the dairy herd, and for lactation data, possessed lactation records through at least the first 50 d after calving as of January 2016. The final

data set included 1,252 female calves born alive (249 MOET, 345 IVP-conv, and 658 IVP-sexed) and 831 adult cows (183 MOET, 218 IVP-conv, and 430 IVP-sexed) that had the requisite lactation records. Similar criteria were used to include subjects for the AI group: conceived by AI, female sex, born alive, entered into the dairy herd, and lactation records through at least the first 50 d after calving as of January 2016. Furthermore, only animals born within the period of births of MOET and IVP calves (June 2012 to April 2014) were included. The number of AI subjects matching these criteria that were born alive was 3,465. Among these, 2,037 had requisite lactation data.

Genomic Testing and Donor Selection

All animals underwent genomic testing to determine genetic merit. The test was performed using a microchip containing 19,000 selection markers (Clarifide ZL2 19K, Zoetis Genetics, Kalamazoo, MI). Genomic estimates included those for predicted transmitting ability for milk (**GPTAM**), fat (**GPTAFat**), and protein (**GPTAPro**), net merit dollars (**GNM$**), and daughter pregnancy rate (**GDPR**). Donor cows chosen for embryo production by MOET or IVP were chosen primarily based on a custom genetic selection index developed by the farm based on production and health traits. Oocyte donor cows for IVP were selected from the top 1% of females ranked for this index, whereas donors used for superovulation and production of MOET embryos were selected from the top 5% of the same index. Sires were selected based on their USDA genetic proofs, and a combination of health and production traits were taken into account. Sire and dam predicted transmitting abilities for milk (sire and dam **PTAM**) were also recorded for each offspring.

Statistical analysis of genomic traits of the offspring (GPTAM, GPTAFat, GPTAPro, GNM$, and GDPR) and their parents (sire PTAM and dam PTAM) were performed by ANOVA using the general linear models procedure of SAS version 9.4 (SAS Inst. Inc., Cary, NC) with main effects of group (AI, IVP-conv, IVP-sexed, MOET). Given that breeding females used to produce subject animals were assigned to a particular reproductive technique based on genetic merit, differences were present in specific genetic traits between groups (Table 1). In particular, GPTAM, GPTAFat, GPTAPro, GNM$, GDPR of the enrolled subjects, and sire and dam PTAM were affected by group ($P < 0.0001$). In all cases, AI offspring had the lowest values. In general, IVP-conv and IVP-sexed were the highest and not different from each other and MOET offspring were intermediate.

On-Farm Management of Offspring

Cattle enrolled in the study were raised together under the same management conditions without dis-

Table 1. Effects of technique used to produce a pregnancy on characteristics of the resultant offspring[1]

Endpoint	AI	IVP-conv	IVP-sexed	MOET	*P*-value[2]
Growth trait					
Gestation length (d)	276.3 ± 0.1	276.5 ± 0.4	276.2 ± 0.3	275.5 ± 0.5	0.7222
Birth weight (kg)	38.5 ± 0.1^{a}	39.4 ± 0.3^{b}	39.0 ± 0.2^{b}	38.7 ± 0.4^{ab}	0.0280
Weaning weight (kg)	88.2 ± 0.6	88.3 ± 1.7	89.4 ± 1.2	87.0 ± 2.4	0.7718
Weight at first breeding (kg)	344.3 ± 0.8^{a}	351.0 ± 2.8^{bc}	355.3 ± 2.0^{c}	346.5 ± 3.2^{ab}	<0.0001
ADG, birth to weaning (kg/d)	0.69 ± 0.01	0.68 ± 0.03	0.70 ± 0.03	0.66 ± 0.05	0.8925
ADG, weaning to breeding (kg/d)	0.85 ± 0.004^{a}	0.87 ± 0.01^{ab}	0.89 ± 0.01^{b}	0.87 ± 0.02^{ab}	0.0015
Reproduction trait					
Age at first calving (mo)	23.5 ± 0.1	23.8 ± 0.3	23.2 ± 0.2	23.3 ± 0.3	0.4520
Days open, first lactation (d)	100.0 ± 2.1	108.3 ± 5.5	102.7 ± 3.9	87.5 ± 7.6	0.1479
Production trait					
Projected actual milk yield, 305 d (kg)	11,038 ± 31^{a}	10,946 ± 100^{ab}	10,717 ± 76^{b}	10,891 ± 149^{ab}	0.0014
Projected actual fat yield, 305 d (kg)	388.3 ± 1.2^{a}	385.6 ± 3.9^{ab}	377.1 ± 3.0^{b}	384.7 ± 5.8^{ab}	0.0072
Projected actual protein yield, 305 d (kg)	334.6 ± 1.0^{a}	336.5 ± 3.3^{a}	327.1 ± 2.5^{b}	331.2 ± 4.8^{ab}	0.0318
Genetic trait					
Genomic PTA for milk (kg)	203.2 ± 5.4^{a}	290.2 ± 16.6^{b}	284.2 ± 12.0^{b}	234.7 ± 18.3^{ab}	<0.0001
Genomic PTA for fat (kg)	8.9 ± 0.2^{a}	14.8 ± 0.7^{b}	14.3 ± 0.5^{b}	14.6 ± 0.7^{b}	<0.0001
Genomic PTA for protein (kg)	7.3 ± 0.1^{a}	11.1 ± 0.4^{b}	10.6 ± 0.3^{b}	9.6 ± 0.4^{b}	<0.0001
Dam PTA for milk (kg)	68.9 ± 6.1^{a}	216.9 ± 33.2^{b}	182.2 ± 27.9^{b}	20.6 ± 30.2^{a}	<0.0001
Sire PTA for milk (kg)	330.4 ± 6.2^{a}	346.5 ± 18.8^{a}	461.3 ± 13.3^{b}	366.7 ± 20.8^{a}	<0.0001
Net merit ($)	321.0 ± 2.9^{a}	455.7 ± 9.0^{b}	463.6 ± 6.5^{b}	420.2 ± 9.9^{c}	<0.0001
Genomic PTA for DPR	1.9 ± 0.03^{a}	2.0 ± 0.09^{a}	2.4 ± 0.06^{b}	2.1 ± 0.1^{ab}	<0.0001

$^{a–c}$Within a row, means without a common superscript differ at $P = 0.059$ (birth weight, IVP-sexed vs. AI) or $P < 0.05$ (all other comparisons).

[1]IVP-conv = in vitro embryo production with conventional semen; IVP-sexed = in vitro embryo production with reverse X-sorted semen; MOET = multiple ovulation and embryo transfer; PTA = predicted transmitting ability; DPR = daughter pregnancy rate. Data are LSM ± SEM.

[2]*P*-values for the main effect of reproductive technique (AI, IVP-conv, IVP-sexed, MOET).

tinction of origin (AI, IVP-conv, IVP-sexed, or MOET) and following standard operational procedures of the farm. Parturition was induced with dexamethasone and $PGF_{2\alpha}$ for dams of some calves in each group. Induction of parturition was not recorded. Calves were raised without distinction of origin either individually in hutches or, beginning in 2014, in groups in indoor pens using automatic liquid feeders. Weaning occurred at ~60 d of age, after which heifers were maintained in outdoor pens. After puberty, heifers entered into the breeding program and were bred by AI following estrus detection. Lactating cows were kept in an indoor confinement system in free-stall barns equipped with cooling devices (fans and sprinkles) or in tunnel ventilation barns. Cows were fed according to the NRC requirements (NRC, 2001) and were milked 3 times daily. All data from birth to puberty were collected by farm personnel and recorded in a management software (PCDART, Dairy Records Management Systems, Raleigh, NC). Lactation data were collected once a month by DHIA personnel and also entered into the PCDART software.

Outcome Variables and Database Assessment

Data were recovered using built-in tools in the herd management software (PCDART). Reports were created for 2 main categories of outcome variables: (1) growth, health, and reproduction traits and (2) production traits. The first category included birth month; risk of death at 30, 60, 90, 120, 150, and 180 d of age for calves born alive; weight at birth for calves born alive; weight at weaning; weight at breeding; ADG from birth to weaning; ADG from weaning to breeding; age at first calving; and interval from parturition to conception (days open) in first lactation. Death rates at birth were not examined due to the lack of accuracy in records. The second category included projected 305-d actual milk, actual fat, and actual protein yields, and average SCS in the first lactation. Projected 305-d actual yields are an estimate of what a cow with a minimum of 50 d of lactation records would produce in the first 305 d of the current lactation, based upon current lactation records to date. For cows with lactation records for 305 d, projected milk yield equals actual milk yield.

Statistical Analysis

Data were examined for normality and homogeneity of variances. Average SCS was not normally distributed and therefore transformed into natural logarithms before analysis. Statistical analysis of discrete variables (death rates) was performed using the chi-squared test with Yates' correction to determine differences in mortality among calves derived by the 4 types of reproductive techniques. Risk of death was calculated by dividing the cumulative number of dead animals at each time point (30, 60, 90, 120, 150, and 180 d of age) by the number of born alive (live calves at 48 h after birth). Continuous variables were analyzed by ANOVA using the general linear models procedure of SAS. If a main effect of group was detected at $P < 0.10$, pairwise comparisons were performed using the PDIFF statement of SAS to determine differences among techniques. For growth, reproduction, and production traits, the statistical model tested the effects of technique, dam parity while pregnant, their interaction, and birth month. The GPTAM, GPTAFat, and GPTAPro for the offspring were used as covariates for production data (GPTAM for milk, GPTAFat for fat, and GPTAPro for protein yields). The same variables were analyzed for the effect of dam parity (nulliparous, parity = 0 vs. parous, parity $\geq$1). Results are presented as least squares means $\pm$ SEM. In addition, a subset of data from IVP-conv, IVP-sexed, and MOET was analyzed to determine effects of dam parity independent of AI (the only group in which parity affects oocyte donor as well as reproductive tract).

RESULTS

Gestation Lengths, Birth Weights, and Neonatal Survival Until 180 d of Age

Gestation length did not differ between groups (Table 1), but birth weight was higher for IVP-conv calves compared with calves produced by AI ($P = 0.01$) and tended to be higher for IVP-sexed compared with AI ($P = 0.059$). Birth weights of MOET calves were intermediate and not different from other groups (Table 1). Mortality rates through 180 d of age for offspring derived by AI, IVP-conv, IVP-sexed, or MOET that were born alive are shown in Figure 1. No differences in death rates were observed at 30 and 60 d. Thereafter, however, calves in the IVP-sexed group were at a higher risk of being dead by 90 ($P = 0.051$), 120 ($P = 0.008$), 150 ($P = 0.018$), and 180 d of age ($P = 0.008$) compared with AI calves. Calves from IVP-conv and MOET had intermediate mortality rates which did not differ significantly from IVP-sexed or AI. Note that the gestation lengths of calves that died were not different from gestation lengths of calves that survived (275.9 $\pm$ 0.1 vs. 276.0 $\pm$ 0.1 d).

Postnatal Growth from Birth to Breeding

The results are shown in Table 1. Weaning weight did not differ among groups, but weight at first breed-

ing was lower for AI-derived heifers than IVP-conv ($P = 0.02$) and IVP-sexed ($P < 0.0001$). Weight at first breeding was also lower for heifers in the MOET group than for IVP-sexed heifers ($P = 0.02$), but MOET was not different from AI or IVP-conv. The ADG from birth to weaning did not differ among groups, whereas ADG from weaning to breeding was highest for IVP-sexed heifers, lowest for AI (AI vs. IVP-sexed; $P = 0.0002$), and intermediate for IVP-conv and MOET heifers.

Reproductive Function and Lactational Performance

Neither age at calving nor days open after first calving differed among heifers derived by the different reproductive techniques (Table 1).

The results for lactational performance are shown in Table 1. Adjusted for GPTAM, projected 305-d milk yield for first lactation was affected by the main effect of group ($P = 0.0014$), with milk yield lower for IVP-sexed offspring compared with AI ($P = 0.0001$) and with IVP-conv and MOET-derived cows having intermediate milk yields not different from either AI or IVP-sexed. Adjusted for GPTAFat, projected 305-d fat yield in first lactation also varied between groups ($P = 0.0072$). Fat yield was lower for IVP-sexed offspring compared with cows derived by AI ($P = 0.0005$), whereas IVP-conv and MOET-derived cows had intermediate fat yield. Projected 305-d protein yield after adjustment for GPTAPro was affected by group ($P = 0.0318$) and lower for IVP-sexed compared with AI ($P = 0.0056$) or with IVP-conv ($P = 0.0210$), but was not different from MOET.

An additional analysis was performed to determine whether shortened gestation lengths caused by induced parturition could be responsible for differences between groups. Differences in lactation between groups remained when analyses were restricted to calves born following gestation lengths of 273 d or more (n = 2365). For example, projected 305-d milk yield for first lactation adjusted for GPTAM was affected by the main effect of group ($P = 0.0003$), with milk yield for IVP-sexed offspring (10,681 ± 83 kg) lower than AI (11,050 ± 32 kg; $P < 0.0001$) and MOET (11,059 ± 206 kg; $P < 0.088$), and IVP-conv (10,836 ± 113 kg) tending to be different ($P = 0.065$) from AI but not other groups.

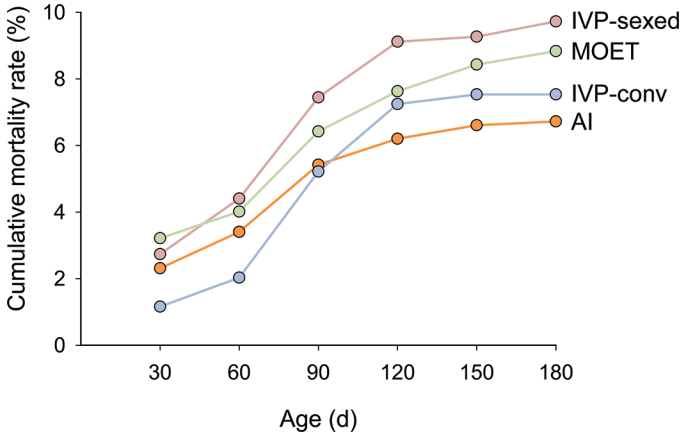

Figure 1. Technique used to produce a pregnancy affects the risk of death in the first 6 mo of age. Cumulative risk of death (percent of animals alive at birth that were dead at subsequent time points) is shown for heifers produced by AI (in vivo development), in vitro embryo production with conventional semen (IVP-conv; in vitro development), in vitro embryo production with reverse X-sorted semen (IVP-sexed; in vitro development), and multiple ovulation and embryo transfer (MOET; in vivo development) from 30 to 180 d of age. The IVP-sexed calves were at a higher risk of being dead by 90 ($P = 0.051$), 120 ($P = 0.008$), 150 ($P = 0.018$), and 180 d of age ($P = 0.008$) compared with AI calves. The IVP-conv and MOET-derived calves had intermediate mortality rates that did not differ significantly from AI or IVP-sexed. Number of live births were as follows: AI, n = 3,465; IVP-conv, n = 345; IVP-sexed, n = 658; and MOET, n = 249. Color version available online.

Developmental Programming Associated with Dam/Recipient Parity

The effect of parity of the female gestating the calf (i.e., embryo transfer recipient for IVP and MOET and the inseminated female for AI) was examined as an additional determination of developmental programming (Table 2). Calves born from nulliparous heifers (parity = 0 while pregnant) had lower weights at birth ($P < 0.0001$), weaning ($P < 0.0001$), and breeding ($P = 0.0025$) compared with parous dams (parity ≥ 1 while pregnant). Dam parity while pregnant did not affect ADG, age at first calving, or days open in first lactation. Offspring born from nulliparous dams produced less milk ($P = 0.0019$), fat ($P < 0.0001$), and protein ($P = 0.0007$) in their first lactation. No significant interactions were observed between dam parity × reproductive group, except for weaning weight ($P = 0.028$). This interaction occurred because groups differed only for offspring born from nulliparous dams. For these offspring, IVP-sexed offspring were heavier ($P = 0.0051$) than IVP-conv calves (86.7 ± 1.4 vs. 79.7 ± 2.1 kg, respectively) and tended ($P = 0.096$) to be heavier than AI (84.0 ± 0.9 kg). Moreover, IVP-conv were lighter ($P = 0.055$) than AI; MOET (81.3 ± 4.4 kg) did not differ from any other groups. For dam parity ≥1, no differences between groups were observed. Differences due to parity were also observed when data were restricted to animals born with gestation lengths ≥273 d (results not shown).

To remove possible effects of parity of the dam on the ovarian follicle and oocyte, parity effects were also

Table 2. Effects of parity of the dam carrying the pregnancy on characteristics of the resultant offspring (LSM ± SEM)

Endpoint	Dam parity		*P*-value	
	Nulliparous (parity = 0)	Parous (parity ≥1)	Dam parity	Dam parity by technique interaction
Growth trait				
Gestation length (d)	274.8 ± 0.2	277.5 ± 0.21	<0.0001	0.154
Birth weight (kg)	36.9 ± 0.2	40.9 ± 0.2	<0.0001	0.13
Weaning weight (kg)	82.9 ± 1.3	93.6 ± 1.0	<0.0001	0.028[1]
Weight at first breeding (kg)	345.7 ± 1.7	352.8 ± 1.6	0.0025	0.87
ADG, birth to weaning (kg/d)	0.66 ± 0.03	0.70 ± 0.02	0.22	0.49
ADG, weaning to breeding (kg/d)	0.86 ± 0.009	0.87 ± 0.007	0.39	0.60
Reproduction trait				
Age at first calving (mo)	23.3 ± 0.2	23.6 ± 0.2	0.29	0.16
Days open, first lactation	96.0 ± 4.2	103.3 ± 3.2	0.15	0.36
Production trait				
Projected actual milk yield, 305 d (kg)	10,761 ± 70	11,035 ± 62	0.0019	0.37
Projected actual fat yield, 305 d (kg)	377.0 ± 2.7	390.7 ± 2.4	<0.0001	0.17
Projected actual protein yield, 305 d (kg)	327.5 ± 2.3	337.2 ± 2.0	0.0007	0.22

[1]The significant interaction between dam parity and technique occurred because for dam parity = 0, IVP-sexed offspring were heavier than IVP-conv (86.7 ± 1.4 vs. 79.7 ± 2.1, respectively) and tended to be heavier than AI (84.0 ± 0.9), whereas IVP-conv calves were lighter than AI and MOET (81.3 ± 4.4) did not differ from any other groups. For dam parity ≥1, no differences were observed. IVP-conv = in vitro embryo production with conventional semen; IVP-sexed = in vitro embryo production with reverse X-sorted semen; MOET = multiple ovulation and embryo transfer.

examined in a subset of data from offspring derived by surrogate recipients (i.e., IVP-conv, IVP-sexed, and MOET). Dam parity had a significant effect on weights at birth, weaning, and breeding as well as milk, fat, and protein yields in first lactation (Table 3). Effects were in the same direction as for the larger data set.

DISCUSSION

Here we show for the first time that use of in vitro fertilization with reverse X-sorted semen in dairy cattle has consequences that extend to adult life and reduces biological efficiency of food production. Compared with calves born following AI, IVP-sexed calves had higher birth weight, accelerated postnatal growth from weaning to breeding, and increased risk of mortality up to 180 d of age. Moreover, milk production was reduced compared with cows conceived by AI. These effects were specific to reverse-sorted semen because, in general, calves born as a result of other ART were not significantly different from calves born from AI. An additional finding was that parity of the recipient affects

Table 3. Effects of parity of the dam carrying the pregnancy on characteristics of the resultant offspring in a subset of data comprising only offspring in the in vitro-production and multiple ovulation and embryo transfer groups (i.e., born from surrogate recipients; LSM ± SEM)

Endpoint	Dam parity while pregnant		*P*-value[1]
	Nulliparous	Parous	
Growth trait			
Gestation length (d)	274.8 ± 0.3	277.3 ± 0.3	<0.0001
Birth weight (kg)	37.2 ± 0.3	41.1 ± 0.3	<0.0001
Weaning weight (kg)	82.6 ± 1.3	93.6 ± 1.1	<0.0001
Weight at first breeding (kg)	347.7 ± 2.2	354.3 ± 2.2	0.0319
ADG, birth to weaning (kg/d)	0.66 ± 0.02	0.71 ± 0.02	0.0849
ADG, weaning to breeding (kg/d)	0.86 ± 0.01	0.87 ± 0.01	0.4452
Reproduction trait			
Age at first calving (mo)	23.4 ± 0.3	23.6 ± 0.3	0.4919
Days open, first lactation	95.6 ± 5.2	104.0 ± 4.2	0.1868
Production trait			
Projected actual milk yield, 305 d (kg)	10,860 ± 88	11,113 ± 79	0.0235
Projected actual fat yield, 305 d (kg)	387.9 ± 3.4	400.1 ± 3.1	0.0047
Projected actual protein yield, 305 d (kg)	332.7 ± 2.8	341.9 ± 2.5	0.0103

[1]*P*-values for parity effects.

postnatal phenotype. This result strengthens the idea that variation in maternal environment can program development in a way that changes adult phenotype.

The nature of the methods used to produce calves does not allow determination of whether differences in postnatal phenotype between offspring born using IVP-sexed as compared with other groups is a consequence of an embryo being derived from sperm subjected to sex sorting or is rather the result of interactions of sperm and oocyte in the specific environment used for in vitro fertilization. Put differently, it cannot be resolved here whether one would expect that use of sexed semen would change adult phenotype even when used for AI or whether differences depend upon use of the reverse-sorting process and in vitro fertilization. Some indications in the literature show that sexed semen is associated with alterations in the characteristics of the resultant offspring. A tendency was observed for greater pregnancy loss for pregnancies established by embryos produced in vitro with X-sorted semen than for embryos produced in vitro with conventional semen (Rasmussen et al., 2013). Also, a slight increase occurred in the incidence of stillbirths in females born from sexed semen (Healy et al., 2013) and in male calves born after X-sorted sperm insemination (DeJarnette et al., 2009). Moreover, a recent study demonstrated an effect of semen type (nonsexed vs. sexed) on conception rate, birth weight, stillbirths, and calf viability within 48 h of birth. In this study, use of sexed semen reduced conception rate, increased percentage of stillbirths, decreased birth weight, and reduced calf viability (Djedović et al., 2016). In contrast, no apparent adverse effects were observed of sexed semen on birth characteristics and perinatal morbidity in other studies (Seidel et al., 1999; Tubman et al., 2004; DeJarnette et al., 2009). Adult performance of offspring born from sexed-sorted semen has not been investigated to date, but there is a compelling need to do so.

Paternal programming of development is often related to epigenetic changes in male gametes during spermatogenesis (Rando and Simmons, 2015; Schagdarsurengin and Steger, 2016). However, this mechanism would not be relevant for sexed semen, where sperm are subjected to sorting after the completion of spermatogenesis unless the sorting process inadvertently selected sperm based on epigenetic character. It might be that the sorting process induces other changes in the spermatozoa that has consequences for fertilization and development. Sperm sexing by flow cytometry involves DNA labeling, exposure to a laser beam, and intensive manipulation (Morrell, 1991; Johnson, 1995; Cran, 2007) that could potentially be detrimental for integrity of sperm cells. Specific sperm-borne miRNA are involved in the regulation of the first cleavage divisions in embryo and the absence of these miRNA can negatively affect embryonic development in mice (Liu et al., 2012). Perhaps, the sorting process results in a loss of miRNA or other molecules important for the embryo. The use of sex-sorted semen in vitro has been reported to alter kinetics of embryonic development by delaying the first cleavage division (Bermejo-Álvarez et al., 2010), and such an effect could conceivably change the developmental program of the embryo.

Although calves born following IVP-sexed experienced alterations in postnatal phenotype compared with those born by AI, there was little consequence of being born following IVP-conv or MOET. The exception was for a slight increase in birth weight and weight at breeding for calves born as a result of IVP-conv. Thus, there is little evidence for deleterious consequences of either MOET or IVP using conventional semen on the resultant calves. This result is in contrast to the negative effects of IVP on juvenile and adult animals derived from the procedure in humans (Ceelen et al., 2008; Rexhaj et al., 2015; Scherrer et al., 2015) and mice (Valenzuela-Alcaraz et al., 2013; Bloise et al., 2014; Feuer et al., 2014). Perhaps the discrepancy in results is related to the endpoints measured rather than to inherent differences in the nature of developmental programming.

There are several caveats to this study. The first is that the control group, calves born following AI, are themselves the result of an ART. Evidence from mice indicates that events in the reproductive tract associated with deposition of semen can program development. In particular, male mice subjected to excision of the seminal vesicle glands generated offspring with abnormal postnatal phenotypes (Bromfield et al., 2014). Further research on cohorts derived by AI compared with progeny born from natural mating would provide an understanding of whether seminal plasma can modify the developmental program of bovine embryos. The second caveat is that females used to produce the offspring studied here were not randomly assigned to group but were rather selected by the dairy based on considerations that included genetic merit. Thus, genetic merit for production traits varied between groups. We adjusted for the difference in genetic merit between groups by including genomic predicted transmitting ability as covariates in the analyses. In addition, differences in genetic merit are unlikely to explain differences in postnatal function between calves born by IVP-sexed and IVP-conv because most estimates of predicted transmitting ability were similar between these 2 groups. It should also be considered that IVP embryos were produced using a specific com-

bination of procedures. Thus, consequences or lack of consequences of IVP seen here could be different for other systems to produce embryos. Unravelling the particular components of the IVP-sexed production system that is responsible for altered phenotype in the adult offspring is likely to be difficult because of the time periods involved and the requirement for large numbers of animals to observe effects. Finally, data were only available for calves that were alive at birth and therefore it is not known whether ART contributed to an increase in stillbirths, neonatal death, or large offspring syndrome.

Calves were not produced experimentally or animals assigned at random to treatment. Thus, other biases could exist. For example, one report indicates that fetal sex can affect the existing lactation (Hinde et al., 2014), and although unlikely, it cannot be ruled out whether sex ratio of pregnancies in first lactation varied between groups. Additional epidemiological data on adult offspring generated by AI with X-sorted semen and by IVP and MOET with conventional or X-sorted semen could provide further evidence to support the potential role of semen sexing with or without IVP on programming.

An additional important finding in this study was the effect of dam parity while pregnant on adult milk production of the offspring. Heifers born from nulliparous animals were lighter at birth, remained smaller until first breeding, and eventually produced less milk in the first lactation than heifers born from mature, parous cows. These results indicate that developmental programming can occur because of differences in the maternal environment during gestation, which has been described by others (Tao and Dahl, 2013), including a study indicating that calves born to parous cows had higher birth weights, heart girth, and withers height compared with offspring of nulliparous heifers (Kamal et al., 2015). Nulliparous heifers are smaller and still partitioning nutrients toward body growth while pregnant and their reproductive tracts are also usually smaller than mature cows. Thus, some degree of intrauterine growth restriction (**IUGR**) was probably experienced by offspring gestated in uteri of nulliparous females. It is well known that IUGR can adversely affect postnatal function, as shown for rodents (reviewed by Zohdi et al., 2014), sheep (Morrison, 2008), and pigs (Foxcroft et al., 2006). A novelty of this study was that it was possible to evaluate effect of dam parity of the uterus independent of effects on the ovary by examining parity effects in the offspring born to surrogate uteri (recipients of IVP and MOET embryos). In both cases, parity effects were similar whether all data or only those data from IVP and MOET were studied. We therefore infer that IUGR was an important causal factor for the differences among offspring born to nulliparous compared with parous dams.

In conclusion, data were presented to indicate that IVP with sexed semen produced by reverse sorting has consequences for dairy cattle that extend to adult life. Compared with females born following AI, IVP-sexed calves had greater birth weights, postnatal growth, and mortality rates and reduced milk, fat, and protein production. Thus, some benefits of IVP with reverse-sorted semen for genetic improvement may be offset by adverse programming events. In contrast to IVP-sexed, minimal consequences were observed for IVP-conv or MOET for postnatal function.

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REFERENCES

Bermejo-Álvarez, P., P. Lonergan, D. Rath, A. Gutiérrez-Adan, and D. Rizos. 2010. Developmental kinetics and gene expression in male and female bovine embryos produced in vitro with sex-sorted spermatozoa. Reprod. Fertil. Dev. 22:426. https://doi.org/10.1071/RD09142.

Bloise, E., S. K. Feuer, and P. F. Rinaudo. 2014. Comparative intrauterine development and placental function of ART concepti: Implications for human reproductive medicine and animal breeding. Hum. Reprod. Update 20:822–839. https://doi.org/10.1093/humupd/dmu032.

Bromfield, J. J., J. E. Schjenken, P. Y. Chin, A. S. Care, M. J. Jasper, and S. A. Robertson. 2014. Maternal tract factors contribute to paternal seminal fluid impact on metabolic phenotype in offspring. Proc. Natl. Acad. Sci. USA 111:2200–2205. https://doi.org/10.1073/pnas.1305609111.

Calle, A., A. Miranda, R. Fernandez-Gonzalez, E. Pericuesta, R. Laguna, and A. Gutierrez-Adan. 2012. Male mice produced by in vitro culture have reduced fertility and transmit organomegaly and glucose intolerance to their male offspring. Biol. Reprod. 87:34. https://doi.org/10.1095/biolreprod.112.100743.

Ceelen, M., M. M. van Weissenbruch, J. P. W. Vermeiden, F. E. van Leeuwen, and H. A. Delemarre-van de Waal. 2008. Cardiometabolic differences in children born after in vitro fertilization: Follow-up study. J. Clin. Endocrinol. Metab. 93:1682–1688. https://doi.org/10.1210/jc.2007-2432.

Cran, D. G. 2007. XY sperm separation and use in artificial insemination and other ARTs. Soc. Reprod. Fertil. Suppl. 65:475–491.

de Waal, E., W. Mak, S. Calhoun, P. Stein, T. Ord, C. Krapp, C. Coutifaris, R. M. Schultz, and M. S. Bartolomei. 2014. In vitro culture increases the frequency of stochastic epigenetic errors at imprinted genes in placental tissues from mouse concepti produced

through assisted reproductive technologies. Biol. Reprod. 90:22. https://doi.org/10.1095/biolreprod.113.114785.
DeJarnette, J. M., R. L. Nebel, and C. E. Marshall. 2009. Evaluating the success of sex-sorted semen in US dairy herds from on farm records. Theriogenology 71:49–58. https://doi.org/10.1016/j.theriogenology.2008.09.042.
Djedović, R., V. Bogdanović, D. Stanojević, Z. Nemes, A. Gáspárdy, and S. Cseh. 2016. Involuntary reduction in vigour of calves born from sexed semen. Acta Vet. Hung. 64:229–238. https://doi.org/10.1556/004.2016.023.
Donjacour, A., X. Liu, W. Lin, R. Simbulan, and P. F. Rinaudo. 2014. In vitro fertilization affects growth and glucose metabolism in a sex-specific manner in an outbred mouse model. Biol. Reprod. 90:80. https://doi.org/10.1095/biolreprod.113.113134.
Farin, C. E., W. T. Farmer, and P. W. Farin. 2010. Pregnancy recognition and abnormal offspring syndrome in cattle. Reprod. Fertil. Dev. 22:75–87. https://doi.org/10.1071/RD09217.
Farin, P. W., J. A. Piedrahita, and C. E. Farin. 2006. Errors in development of fetuses and placentas from in vitro-produced bovine embryos. Theriogenology 65:178–191. https://doi.org/10.1016/j.theriogenology.2005.09.022.
Feuer, S. K., X. Liu, A. Donjacour, W. Lin, R. K. Simbulan, G. Giritharan, L. D. Piane, K. Kolahi, K. Ameri, E. Maltepe, and P. F. Rinaudo. 2014. Use of a mouse in vitro fertilization model to understand the developmental origins of health and disease hypothesis. Endocrinology 155:1956–1969. https://doi.org/10.1210/en.2013-2081.
Fleming, T. P., A. J. Watkins, C. Sun, M. A. Velazquez, N. R. Smyth, and J. J. Eckert. 2015. Do little embryos make big decisions? How maternal dietary protein restriction can permanently change an embryo's potential, affecting adult health. Reprod. Fertil. Dev. 27:684. https://doi.org/10.1071/RD14455.
Foxcroft, G. R., W. T. Dixon, S. Novak, C. T. Putman, S. C. Town, and M. D. A. Vinsky. 2006. The biological basis for prenatal programming of postnatal performance in pigs. J. Anim. Sci. 84(Suppl):E105–E112.
Gad, A., U. Besenfelder, F. Rings, N. Ghanem, D. Salilew-Wondim, M. M. Hossain, D. Tesfaye, P. Lonergan, A. Becker, U. Cinar, K. Schellander, V. Havlicek, and M. Hölker. 2011. Effect of reproductive tract environment following controlled ovarian hyperstimulation treatment on embryo development and global transcriptome profile of blastocysts: Implications for animal breeding and human assisted reproduction. Hum. Reprod. 26:1693–1707. https://doi.org/10.1093/humrep/der110.
Hansen, M., and C. Bower. 2014. The impact of assisted reproductive technologies on intra-uterine growth and birth defects in singletons. Semin. Fetal Neonatal Med. 19:228–233. https://doi.org/10.1016/j.siny.2014.03.002.
Hansen, P. J., K. B. Dobbs, A. C. Denicol, and L. G. B. Siqueira. 2016. Sex and the preimplantation embryo: Implications of sexual dimorphism in the preimplantation period for maternal programming of embryonic development. Cell Tissue Res. 363:237–247. https://doi.org/10.1007/s00441-015-2287-4.
Healy, A. A., J. K. House, and P. C. Thomson. 2013. Artificial insemination field data on the use of sexed and conventional semen in nulliparous Holstein heifers. J. Dairy Sci. 96:1905–1914. https://doi.org/10.3168/jds.2012-5465.
Hinde, K., A. J. Carpenter, J. S. Clay, and B. J. Bradford. 2014. Holsteins favor heifers, not bulls: Biased milk production programmed during pregnancy as a function of fetal sex. PLoS One 9:e86169. https://doi.org/10.1371/journal.pone.0086169.
Jackson, R. A., K. A. Gibson, Y. W. Wu, and M. S. Croughan. 2004. Perinatal outcomes in singletons following in vitro fertilization: A meta-analysis. Obstet. Gynecol. 103:551–563. https://doi.org/10.1097/01.AOG.0000114989.84822.51.
Johnson, L. A. 1995. Sex preselection by flow cytometric separation of X and Y chromosome-bearing sperm based on DNA difference: A review. Reprod. Fertil. Dev. 7:893–903.
Kamal, M. M., M. Van Eetvelde, H. Bogaert, M. Hostens, L. Vandaele, M. Shamsuddin, and G. Opsomer. 2015. Environmental factors and dam characteristics associated with insulin sensitivity and insulin secretion in newborn Holstein calves. Animal 9:1490–1499. https://doi.org/10.1017/S1751731115000701.
Kruip, T. A. M., and J. H. G. den Daas. 1997. In vitro produced and cloned embryos: Effects on pregnancy, parturition and offspring. Theriogenology 47:43–52. https://doi.org/10.1016/S0093-691X(96)00338-X.
Liu, W.-M., R. T. K. Pang, P. C. N. Chiu, B. P. C. Wong, K. Lao, K.-F. Lee, and W. S. B. Yeung. 2012. Sperm-borne microRNA-34c is required for the first cleavage division in mouse. Proc. Natl. Acad. Sci. USA 109:490–494. https://doi.org/10.1073/pnas.1110368109.
Lonergan, P., T. Fair, D. Corcoran, and A. C. O. Evans. 2006. Effect of culture environment on gene expression and developmental characteristics in IVF-derived embryos. Theriogenology 65:137–152. https://doi.org/10.1016/j.theriogenology.2005.09.028.
Mainigi, M. A., D. Olalere, I. Burd, C. Sapienza, M. Bartolomei, and C. Coutifaris. 2014. Peri-implantation hormonal milieu: Elucidating mechanisms of abnormal placentation and fetal growth. Biol. Reprod. 90:26. https://doi.org/10.1095/biolreprod.113.110411.
Market-Velker, B. A., L. Zhang, L. S. Magri, A. C. Bonvissuto, and M. R. W. Mann. 2010. Dual effects of superovulation: Loss of maternal and paternal imprinted methylation in a dose-dependent manner. Hum. Mol. Genet. 19:36–51. https://doi.org/10.1093/hmg/ddp465.
Miles, J. R. 2004. Angiogenesis and morphometry of bovine placentas in late gestation from embryos produced in vivo or in vitro. Biol. Reprod. 71:1919–1926. https://doi.org/10.1095/biolreprod.104.031427.
Morrell, J. M. 1991. Applications of flow cytometry to artificial insemination: A review. Vet. Rec. 129:375–378.
Morrison, J. L. 2008. Sheep models of intrauterine growth restriction: Fetal adaptations and consequences. Clin. Exp. Pharmacol. Physiol. 35:730–743. https://doi.org/10.1111/j.1440-1681.2008.04975.x.
Norman, H. D., J. L. Hutchison, and R. H. Miller. 2010. Use of sexed semen and its effect on conception rate, calf sex, dystocia, and stillbirth of Holsteins in the United States. J. Dairy Sci. 93:3880–3890. https://doi.org/10.3168/jds.2009-2781.
NRC. 2001. Nutrient Requirements of Dairy Cattle. 7th ed. National Academy Press, Washington, DC.
Perry, G. 2016. 2015 statistics of embryo collection and transfer in domestic farm animals. Embryo Technol. Newsl. 33:10–25.
Rando, O. J., and R. A. Simmons. 2015. I'm eating for two: Parental dietary effects on offspring metabolism. Cell 161:93–105. https://doi.org/10.1016/j.cell.2015.02.021.
Rasmussen, S., J. Block, G. E. Seidel, Z. Brink, K. McSweeney, P. W. Farin, L. Bonilla, and P. J. Hansen. 2013. Pregnancy rates of lactating cows after transfer of in vitro produced embryos using X-sorted sperm. Theriogenology 79:453–461. https://doi.org/10.1016/j.theriogenology.2012.10.017.
Rexhaj, E., A. Paoloni-Giacobino, S. F. Rimoldi, D. G. Fuster, M. Anderegg, E. Somm, E. Bouillet, Y. Allemann, C. Sartori, and U. Scherrer. 2013. Mice generated by in vitro fertilization exhibit vascular dysfunction and shortened life span. J. Clin. Invest. 123:5052–5060. https://doi.org/10.1172/JCI68943.
Rexhaj, E., R. von Arx, D. Cerny, R. Soria, E. Bouillet, C. Sartori, U. Scherrer, and S. Rimoldi. 2015. Assisted reproductive technologies-induced premature vascular ageing persists and evolves into arterial hypertension in adolescents. FASEB J. 29:957.
Schagdarsurengin, U., and K. Steger. 2016. Epigenetics in male reproduction: Effect of paternal diet on sperm quality and offspring health. Nat. Rev. Urol. 13:584–595. https://doi.org/10.1038/nrurol.2016.157.
Scherrer, U., E. Rexhaj, Y. Allemann, C. Sartori, and S. F. Rimoldi. 2015. Cardiovascular dysfunction in children conceived by assisted reproductive technologies. Eur. Heart J. 36:1583–1589. https://doi.org/10.1093/eurheartj/ehv145.
Schulz, L. C. 2010. The Dutch Hunger Winter and the developmental origins of health and disease. Proc. Natl. Acad. Sci. USA 107:16757–16758. https://doi.org/10.1073/pnas.1012911107.
Seidel, G. E., J. L. Schenk, L. A. Herickhoff, S. P. Doyle, Z. Brink, R. D. Green, and D. G. Cran. 1999. Insemination of heifers with sexed sperm. Theriogenology 52:1407–1420.

Tao, S., and G. E. Dahl. 2013. Invited review: Heat stress effects during late gestation on dry cows and their calves. J. Dairy Sci. 96:4079–4093. https://doi.org/10.3168/jds.2012-6278.

Thompson, J. G. 1997. Comparison between in vivo-derived and in vitro-produced pre-elongation embryos from domestic ruminants. Reprod. Fertil. Dev. 9:341–354.

Tubman, L. M., Z. Brink, T. K. Suh, and G. E. Seidel. 2004. Characteristics of calves produced with sperm sexed by flow cytometry/cell sorting. J. Anim. Sci. 82:1029–1036.

Urrego, R., N. Rodriguez-Osorio, and H. Niemann. 2014. Epigenetic disorders and altered gene expression after use of Assisted Reproductive Technologies in domestic cattle. Epigenetics 9:803–815. https://doi.org/10.4161/epi.28711.

Valenzuela-Alcaraz, B., F. Crispi, B. Bijnens, M. Cruz-Lemini, M. Creus, M. Sitges, J. Bartrons, S. Civico, J. Balasch, and E. Gratacós. 2013. Assisted reproductive technologies are associated with cardiovascular remodeling in utero that persists postnatally. Circulation 128:1442–1450. https://doi.org/10.1161/CIRCULATIONAHA.113.002428.

Wadhwa, P. D., C. Buss, S. Entringer, and J. M. Swanson. 2009. Developmental origins of health and disease: Brief history of the approach and current focus on epigenetic mechanisms. Semin. Reprod. Med. 27:358–368. https://doi.org/10.1055/s-0029-1237424.

Young, L. E., K. D. Sinclair, and I. Wilmut. 1998. Large offspring syndrome in cattle and sheep. Rev. Reprod. 3:155–163.

Zohdi, V., K. Lim, J. Pearson, and M. Black. 2014. Developmental programming of cardiovascular disease following intrauterine growth restriction: Findings utilising a rat model of maternal protein restriction. Nutrients 7:119–152. https://doi.org/10.3390/nu7010119.