

1 Developmental origins of pregnancy loss in the adult female common marmoset
2 monkey (*Callithrix jacchus*)

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25 **Key Words**

26 Developmental programming, litter size, life history, stillbirth, prenatal androgens,

27 “brother effect”

28

29 **Abstract**

30 Background

31 The impact of the intrauterine environment on the developmental programming of adult
32 female reproductive success is still poorly understood and potentially underestimated.
33 Litter size variation in a nonhuman primate, the common marmoset monkey (*Callithrix*
34 *jacchus*), allows us to model the effects of varying intrauterine environments (e.g.
35 nutrient restriction, exposure to male womb-mates) on the risk of losing fetuses in
36 adulthood. Our previous work has characterized the fetuses of triplet pregnancies as
37 experiencing intrauterine nutritional restriction.

38 Methodology/Principal Findings

39 We used over a decade of demographic data from the Southwest National Primate
40 Research Center common marmoset colony. We evaluated differences between twin
41 and triplet females in the number of pregnancies they produce and the proportion of
42 those pregnancies that ended in fetal loss. We found that triplet females produced the
43 same number of total offspring as twin females, but lost offspring during pregnancy at a
44 significantly higher rate than did twins (38% vs. 13%, $p=0.02$). Regardless of their own
45 birth weight or the sex ratio of the litter the experienced as fetuses, triplet females lost
46 more fetuses than did twins. Females with a male littermate experienced a significant
47 increase in the proportion of stillbirths.

48 Conclusions/Significance

49 These striking findings anchor pregnancy loss in the mother's own fetal environment
50 and development, underscoring a "Womb to Womb" view of the lifecourse and the
51 intergenerational consequences of development. This has important translational

52 implications for understanding the large proportion of human stillbirths that are
53 unexplained. Our findings provide strong evidence that a full understanding of
54 mammalian life history and reproductive biology requires a developmental foundation.

55

56 **Introduction**

57 Developmental programming theory suggests that the intrauterine environment –
58 the intersection of maternal ecology and fetal development [1] – can have a lasting
59 impact on adult health and function. A consistent link has been found in humans and
60 animal models between maternal energy status during gestation or low birth weight (a
61 proxy of a stressed developmental milieu), and obesity, diabetes, chronic cardiovascular
62 disease, and reduced immunocompetence in adolescent and adult offspring [2-4]. Birth
63 weight is the most common proxy measure of the quality of the intrauterine environment
64 in mammals; it is easily measured and is strongly linked to postnatal and adult
65 outcomes in a wide variety of species, including humans [2,5]. However, birth weight
66 itself is a product of dynamic processes experienced by the mother prior to conception
67 and by both mother and fetus throughout gestation, and cannot be taken to reflect the
68 entirety of these intrauterine processes [1]. While low birth weight has associations with
69 later life outcomes, the intrauterine environment may be altering development of
70 physiological function in ways that are not reflected by birth weight. Developmental
71 programming occurs across the range of birth weights, not just at the low end [6,7]. For
72 example, maternal dietary composition may have differential impact on fetal endocrine
73 pancreas development leading to diabetes in later life, without producing reductions in
74 fetal body weight [8].

75 Fetal number is another source of variation in the quality of the intrauterine
76 environment that may have intergenerational effects. One of the classic life history
77 tradeoffs is the balance between number and quality of offspring [9,10]. As the number
78 of offspring increases, individual weights decrease, sometimes with impact on mortality
79 risk [10]. Increased fetal number is associated with reduced birth weight and greater
80 perinatal mortality in many taxa (sheep [11]; wood rats [12]; red squirrels [13]; common
81 marmosets [14,15]; humans [16]). Litter size does not account entirely for variation in
82 birth weight [17], suggesting that these phenomena and their downstream effects may
83 be decoupled under certain circumstances. Little is known about the long-term life
84 history and reproductive impact of litter size at birth when controlled for birth weight.

85 The common marmoset monkey (*Callithrix jacchus*), like all marmosets and
86 tamarins (Order, Primates; Suborder, Anthropeidea; Family, Cebidae; Subfamily,
87 Callitrichinae [18]), expresses a highly plastic reproductive phenotype, regularly
88 producing litters ranging from one to five multizygotic fetuses in captivity. Twins and
89 triplets are the most common litter sizes [19] and mixed sex litters occur frequently. This
90 variability is tied to maternal ecology; elevated maternal mass is the best predictor of
91 greater ovulation number and litter size [20]. Individual repeatability of litter size is low,
92 and the litter size a female experiences as a fetus does not predict the litter size she will
93 produce as an adult, together suggesting that litter size is not genetically constrained
94 but ecologically responsive [16,20,21]. Importantly, several occurrences of triplets have
95 been observed in wild callitrichine species as well (cotton-top tamarins [22]; common
96 marmosets [23]; golden lion tamarins [24]). This suggests that conceiving and gestating
97 (although not rearing [19,25]) more than two fetuses may be a common a feature of

98 callitrichine reproductive biology both in the wild and in captivity. Variation in fetal
99 number presents the opportunity to model varying intrauterine environments and their
100 long-term effects.

101 We have shown previously that triplet marmosets experience an intrauterine
102 environment that is qualitatively poorer than that experienced by twins, based on
103 differences in maternal:neonatal weight ratios and placental efficiency [1,26] and
104 microscopic characteristics of the placental interface [27, 28] . Further, while both twins
105 and triplet marmosets born at high birth weights tend to grow into high-weight adults,
106 low birth weight triplets are much more likely to grow into large adults than are low birth
107 weight twins [29,30]. This pattern of “centile crossing” over the lifecourse has been
108 implicated in the developmental programming literature as the phenotype carrying the
109 greatest risk of adult disease [31-34]. For these reasons, twin marmosets can be viewed
110 as the “control” developmental phenotype, with triplets exhibiting the “restricted”
111 developmental phenotype. Triplet females also carry the potential burden of greater
112 exposure to prenatal androgens from their male littermates.

113 This paper first characterizes reproductive parameters in a colony of captive
114 common marmoset monkeys (*Callithrix jacchus*) overall and according to litter size and
115 birth weight, and then explores the relationship between a female marmoset’s birth
116 condition (her litter size, intralitter sex ratio, and birth weight) and her risk of pregnancy
117 loss in adulthood.

118

119 **Methods**

120 *Ethics statement*

121 All animal procedures, husbandry, and housing were conducted according to
122 Southwest National Primate Research Center Institutional Animal Care and Use
123 Committee requirements.

124 *Colony and housing*

125 Demographic records from the Southwest National Primate Research Center in
126 San Antonio, Texas dating from 1994 to 2012 were available for a total of 1395 animals
127 of both sexes and all birth conditions. Because the intent was to focus on the
128 intrauterine contribution to life history and reproductive output, analyses were restricted
129 to females for whom a full complement of birth condition (weight and litter size) and
130 adult reproductive parameters were known. Analyses were conducted on subsets of this
131 group (i.e. twins and triplets). Adult females were housed with at least their adult male
132 mate, but often with older offspring. It is not uncommon for the family group to contain
133 adolescent, juvenile, and infant offspring at the same time. Family structure at the time
134 of each pregnancy studied was not recorded and thus could not be considered in this
135 study. Groups were housed according to Institutional Animal Care and Use Committee
136 standards for marmosets.

137

138 *Coding pregnancy loss*

139 In the original database birth status was coded as follows: STILL, meaning fully
140 developed fetus delivered at term with no sign of earlier death *in utero* and/or no lung
141 flotation; DIU (“dead *in utero*”), meaning a mostly or fully developed fetus either aborted
142 or discovered at term delivery but showing clear evidence of *in utero* death preceding
143 labor (macerated flesh, skin slippage, “mushiness”); and ABORT, meaning found or

144 delivered before due date (gestation in common marmosets is ~143 days, [35]). All of
145 these categories were combined to generate total loss. Thus, early loss is likely
146 conflated with the equivalent of antepartum stillbirth, i.e. fetal loss occurring in the last
147 trimester but prior to labor and delivery. Related to this limitation, we do not have any
148 record of offspring that were lost so early that there was no visible evidence of loss so
149 we cannot extrapolate our findings to very early pregnancy loss. Further, given that
150 placentophagy and even fetophagy are not uncommon practices and that parturition is
151 typically nocturnal, our estimates of loss of even late gestation fetuses may be
152 underestimated. Finally, we did not regularly conduct lung flotation, a highly accurate
153 method of determining stillbirth in humans [36].

154

155 *Predictor and outcome variables*

156 Litter size at birth was the most consistently used predictor variable. We
157 evaluated the impact of litter size on total number of offspring produced, total litters
158 produced, total lost fetuses, total affected litters (litters in which at least one fetus was
159 lost), and total lost litters (litters wherein all fetuses were lost), all fitted as continuous
160 variables. We controlled all regression models for birth weight, birth year (to control
161 cohort effects), and early adult weight. As described by Tardif and Bales [29], early adult
162 weight was measured between 17-22 months. This is a few months later than the
163 average age of puberty (11-13 months) and precedes the average age of first
164 conception (2.49 years) [19], but reflects the achievement of adult weight [37, 38].

165

166 *Statistics*

167 Although litter size ranges from 1-5 in the captive common marmoset, analyses
168 were restricted to twins and triplets because these are by far the two most
169 representative litter sizes of origin of those females who survived to the age of maturity.
170 All analyses were conducted using Stata for Windows, version 10 IC (StataCorp,
171 College Station, TX). Two-tailed T-tests were used to compare twins and triplets to each
172 other in terms of birth condition and other life history characteristics. Z-tests were used
173 to test the significance of the difference in proportion of fetal losses between twin and
174 triplet females. There were nine pairs of females (total n=18 out of 62) in the sample
175 who were born into the same litter. We used two-tailed T-tests to compare females with
176 and without littermates in the study in terms of birth condition and the other life history
177 characteristics.

178 In some cases we wanted to evaluate the predictive relationships between litter
179 size and pregnancy loss variables; therefore, we used simple and multiple linear
180 regression modeling. We assigned all females a litter ID (to control for those females
181 born into the same litters as described above) and ran that ID as a random effect in
182 regression models. We assumed that many of our predictor and outcome variables in
183 these models would exhibit collinearity. Therefore, all models were evaluated for
184 collinearity using the *estat VIF* command in Stata to measure the variance inflation
185 factor. Models returning a VIF of >5 (indicative of high collinearity) were subject to
186 rejection; none of our models returned a rejectable VIF.

187

188 *Data availability*

189 Raw data are stored in databases at the SNPRC and the University of Illinois at
 190 Chicago. Requests for data can be directed to the primary author.

191

192 **Results**

193 *Birth condition and reproductive demographics of the Southwest National Primate*
 194 *Research Center female marmosets*

195 At the time of these analyses, there were 1395 animals in the Southwest National
 196 Primate Research Center marmoset colony database; a large proportion of this number
 197 includes animals that died before juvenility. Of adult animals, 113 were reproducing
 198 females. Not all females were born in the colony or entered the colony with birth data,
 199 so litter size at birth was known for 79 of these females, of which 75 were either twins
 200 (n=37) or triplets (n=38). These 75 females accounted for 94.95% of the reproducing
 201 females of known litter size at birth; remaining analyses are thus restricted to these twin
 202 and triplet females. When restricted to twin and triplet females of known birth weight and
 203 early adult weight, the sample size for analysis was 62 (twins=30, triplets=32; Table 1).
 204 Sex composition of a female’s birth litter was known for 27 twins and 29 triplets.

205 Table 1: Sample characteristics, stratified by litter size

	All (n=62) Mean (±SD)	Twins* (n=30) Mean (±SD)	Triplets* (n=32) Mean (±SD)	P value
Number of male littermates	0.84 (0.71)	0.48 (0.51)	1.22 (0.70)	<0.00001
Birth weight (bw), g	29.90 (3.19)	31.17 (3.29)	28.72 (2.62)	0.002
Early adult weight (eadwt), g	414.09 (83.07)	403.76 (75.87)	423.78 (89.41)	0.35
Low bw & high eadwt** , @	28%	13.79%	40.74%	0.01
Age at first reproduction, years	2.94 (0.62)	3.00 (0.11)	2.88 (0.11)	0.43
Total number of litters	3.92 (3.28)	3.87 (2.67)	3.97 (3.80)	0.90
Triplet litters, out of total litters	40.58%	47.63%	33.97%	0.14
Total number of offspring	9.81 (8.68)	9.90 (7.31)	9.72 (9.92)	0.97
% Offspring lost **	26.03%	12.97%	38.27%	0.02
Affected litters***, out of total litters**	35.85%	26.70%	44.42%	0.14
Entire litter lost, out of total litters**	22.15%	12.55%	31.15%	0.07

206 @ Median split: low birth weight ≤27.86g, high adult weight ≥479.20

207 * Unpaired two-tailed T-test

208 ** Out of total number of offspring; Difference in proportion, unpaired two-tailed Z-test

209 ***Litter affected by loss of at least one fetus

210

211 Females who had littermates in the study (n=20) did not differ from the rest of the

212 sample (n=42) in litter size, birth weight, or adult weight; they had significantly fewer

213 male littermates than the rest of the sample (Supporting Table 1). Triplets were born at

214 significantly lower birth weights than twins, but did not differ significantly in weight at the

215 early adulthood mark (Table 1). Birth weight and adult weight were divided into high

216 and low categories via median splits. Low birth weight triplets were significantly more

217 likely to grow into high weight adults than were low birth weight twins.

218 Table S1: Sample characteristics, stratified by females with and without littermates in the study

	All (n=62) Mean (\pm SD)	No littermates in study* (n=42) Mean (\pm SD)	Littermates in study* (n=20) Mean (\pm SD)	P value
Litter size	2.52 (0.50)	2.52 (0.51)	2.50 (0.51)	0.86
Number of male littermates	N=56: 0.84 (0.71)	N=38: 1.13 (0.62)	N=18: 0.22 (0.43)	<0.0000 1
Birth weight (bw), g	29.90 (3.19)	29.50 (3.29)	30.75 (2.85)	0.15
Early adult weight, g	414.09 (83.07)	410.02 (87.38)	422.65 (74.62)	0.58
Age at first reproduction, years	2.94 (0.62)	3.02 (0.60)	2.77 (0.64)	0.15
Total number of litters	3.92 (3.28)	3.62 (2.64)	4.55 (4.33)	0.30
Triplet litters, out of total litters**	40.58%	39.67%	42.51%	0.77
Total number of offspring	9.81 (8.68)	8.98 (7.11)	11.55 (11.33)	0.28
% Offspring lost**	26.03%	32.13%	13.21%	0.02
Affected litters***, out of total litters**	35.85%	41.35%	24.28%	0.09
Entire litter lost, out of total litters**	22.15%	26.35%	13.32%	0.10

219 * Unpaired two-tailed T-test

220 ** Out of total number of offspring; Difference in proportion, unpaired two-tailed Z-test

221 ***Litter affected by loss of at least one fetus

222

223

224 *Impact of birth weight and litter size on reproductive parameters in adulthood*

225 Twins and triplets did not differ in their age at first reproduction, nor did they differ

226 in the number of litters produced or the total number of offspring gestated (Table 1).

227 Triplet females were not more likely than twin females to produce triplet litters. None of

228 these outcomes differed when females were stratified on birth weight (Supporting Table

229 2). Females with littermates in the study did not differ from the rest of the sample in age
 230 at first reproduction or in the total number of offspring produced (Supplemental Table 1).

231 Despite the lack of difference in total offspring gestated, triplets lost three times
 232 as many offspring during pregnancy (Table 1). Triplet females tended to experience
 233 these losses across more pregnancies than did twins, with triplets losing entire litters
 234 2.48 times more than twins, though this difference was not significant ($p= 0.07$; Table 1).
 235 Similar analyses of adult females stratified by birth weight showed no differences in
 236 rates of loss (Supporting Table 2). Triplet females lost more fetuses in each of three
 237 categories of birth weight (Figure 1), with the difference being significant in low and
 238 medium weight categories. Triplet females who were born in the lowest birth weight
 239 tertile experienced the highest proportion of fetal loss in adulthood.

240 Table S2: Sample characteristics, stratified by birth weight (median split)

	All (n=62) Mean (\pm SD)	Lower birth weight* (n=37) Mean (\pm SD)	Higher birth weight* (n=25) Mean (\pm SD)	P value
Litter size	2.52 (0.50)	2.68 (0.47)	2.28 (0.46)	0.002
Number of male littermates[@]	0.84 (0.71)	1.03 (0.75)	0.60 (0.58)	0.02
Birth weight (bw), g	29.90 (3.19)	27.79 (2.01)	33.09 (1.93)	<0.00001
Early adult weight, g	414.09 (83.07)	401.06 (82.27)	433.38 (82.09)	0.13
Age at first reproduction, years	2.94 (0.62)	2.95 (0.11)	2.92 (0.12)	0.86
Total number of litters	3.92 (3.28)	4.11 (3.75)	3.64 (2.45)	0.59
Triplet litters, out of total litters	40.58%	35.51%	48.09%	0.18
Total number of offspring	9.81 (8.68)	9.14 (9.37)	9.60 (7.77)	0.84
% Offspring lost**	26.03%	29.44%	20.98%	0.45
Affected litters***, out of total litters**	35.85%	37.97%	32.71%	0.68
Entire litter lost, out of total litters**	22.15%	26.42%	15.82%	0.31

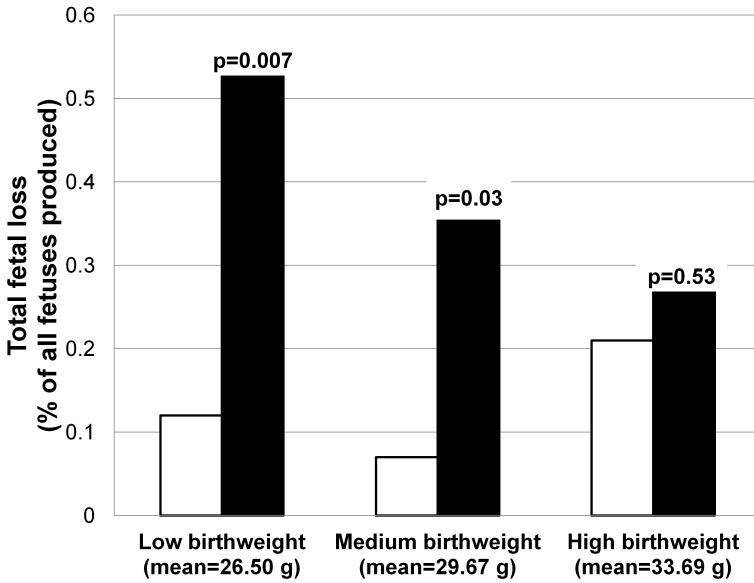
241 [@] Sex ratio known for n=56: Lower birth weight, n=38; Higher birth weight, n=18

242 * Unpaired two-tailed T-test

243 ** Out of total number of offspring; Difference in proportion, unpaired two-tailed Z-test

244 ***Litter affected by loss of at least one fetus

245



246

247 Figure 1: Pregnancy loss in twin and triplet adult females across three tertiles of the
 248 females' own birth weights.
 249

250 A multiple regression model including a female's own litter size, birth weight,
 251 early adult weight, and birth year significantly predicted her rate of total loss, explaining
 252 19% of the variance (Table 2: model 1). In this model, litter size was the sole significant
 253 independent predictor of a female's rate of total stillbirth rate: the larger the litter at birth,
 254 the greater the rate of loss in adulthood. A multiple regression model excluding birth
 255 weight remained predictive of total loss, with litter size being the sole predictor of loss
 256 (Table 2: model 2). When litter size was excluded, none of the models were significant
 257 overall (Table 2: model 3). Models containing litter size tended to explain a greater
 258 proportion of the variance in the outcomes than those without. Litter size alone
 259 explained 18% of the variance in total loss, compared to only 7% for birth weight alone
 260 (Table 3: models 1 and 2).

261
 262
 263
 264

265 Table 2: Multiple regression models predicting pregnancy loss in adult female marmosets (n=62)

	Model 1: All predictor variables β (95% C.I.) Total*	Model 2: Birth weight excluded β (95% C.I.) Total**	Model 3: Litter size excluded β (95% C.I.) Total
Litter size at birth	0.23** (0.07, 0.39)	0.26 *** (0.11, 0.40)	---
Birth weight (g)	-0.01 (-0.04, 0.02)	---	-0.03* (-0.05, -0.003)
Birth year	0.0007 (-0.02, 0.02)	-0.001 (-0.02, 0.02)	0.004 (-0.02, 0.02)
Adult weight (g)	-0.0002 (-0.001, 0.001)	-0.0003 (-0.001, 0.001)	0.0001 (-0.001, 0.001)
Model R ²	0.19	0.18	0.08

266 ****p<=0.0001, ***p<=0.001, **p<=0.01, *p<=0.05

267

268

269

270 Table 3: Simple regression models predicting pregnancy loss in adult female marmosets (n=62)

	Model 1: Birth weight only β (95% C.I.)	Model 2: Litter size only β (95% C.I.)
Litter size at birth	---	0.25*** (0.11, 0.39)
Birth weight (g)	-0.03* (-0.05, 0.002)	---
Model R ²	0.07	0.18

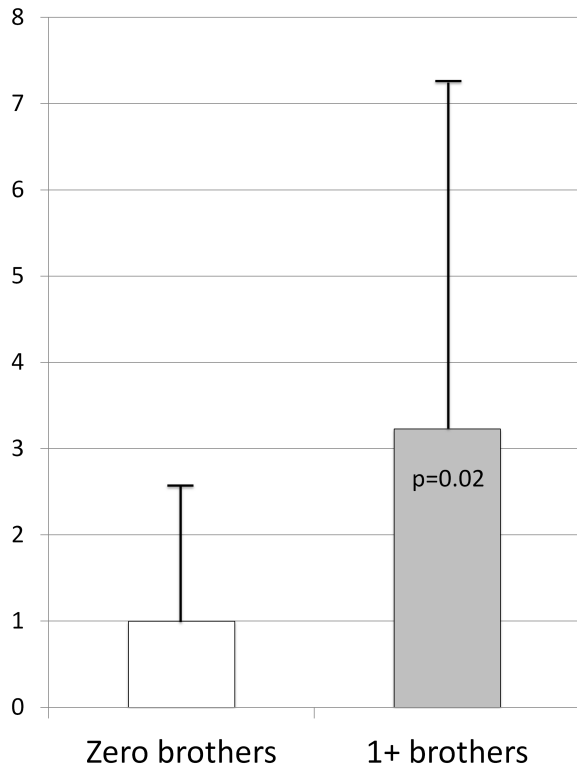
271 ****p<=0.0001, ***p<=0.001, **p<=0.01, *p<=0.05

272

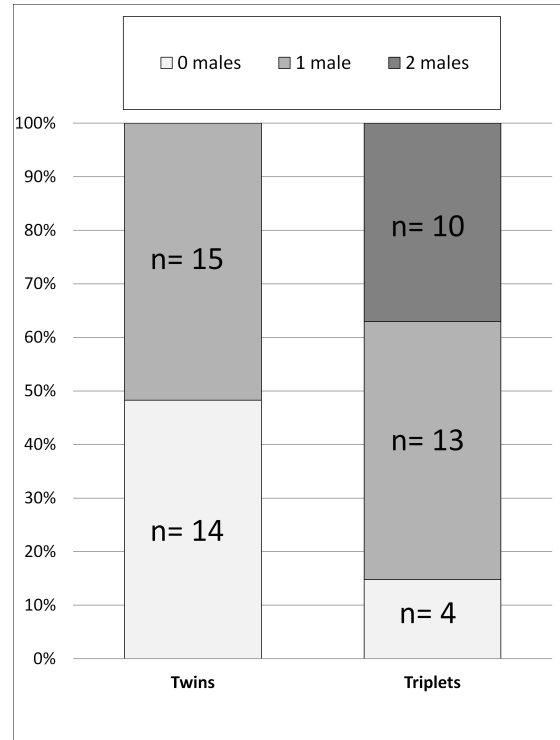
273

274 The impact of exposure to male littermates on pregnancy success in the adult
 275 females was assessed in three ways. First, females with littermates in the study differed
 276 from the rest of the sample in being less likely to have had a male littermate compared
 277 to the rest of the sample. These females did not differ in the number of total offspring
 278 produced, but they had significantly lower loss rates compared to the rest of the sample
 279 (Supplementary Table 1). Second, females with one or more brother regardless of litter
 280 size experienced a significant increase in fetal loss compared to females from female-
 281 only litters (Figure 2). Third, male exposure as a function of litter size was assessed.
 282 Twin females had an average of 0.48 brothers *in utero*, compared to triplets who had
 283 1.22 brothers (p<0.00001; Table 1). Twin females were equally likely to have had either
 284 a male or a female littermate (Figure 3). In contrast, 85% of triplet females had either
 285 one or two male littermates; only 4 of the 27 triplet females for whom birth sex ratio was
 286 known were born into all-female litters (Figure 3). The triplets did not exhibit a significant

287 dose response of fetal loss to having one versus two male littermates ($z=0.33$, $p=0.74$;
 288 data not shown). A categorical variable (zero versus one or more) was thus constructed
 289 for both twins and triplets. Twin females lost 2.91 more fetuses when they were
 290 exposed to a brother ($p=0.05$, Table 4) as opposed to a sister. There was not a
 291 significant effect of male exposure on fetal loss in the triplet females.



292
 293 Figure 2: Pregnancy loss across all
 294 adult females according to number of
 295 brothers with whom they shared the
 296 womb during their own fetal period.
 297



298
 299 Figure 3: Distribution of brothers with
 300 whom twin and triplet females shared
 301 the womb during their own fetal period.

302
 303 Table 4: Number of fetuses lost by females who had zero vs. one or more male littermates (unpaired T-
 304 test)

	Twin females (n=29)					Triplet females (n=27)				
	N	Mean (S.D.)	t	df	p	N	Mean (S.D.)	t	df	p
0 male littermate	15	0.87 (1.36)	-2.08	27	0.05	4	1.50 (2.38)	-0.96	25	0.34
1+ male littermate	14	2.53 (3.15)				23	3.70 (4.38)			

305 **Discussion**

306 Consistent with our previous work, triplets were born at significantly lower birth
307 weights than were twins. Further, triplets were significantly more likely than twins to
308 exhibit a lower birth weight/higher adult weight profile. This is consistent with the classic
309 growth-restricted phenotype that is typically thought to present the highest risk within
310 the developmental programming paradigm [30], strengthening the concept of the
311 marmoset monkey triplet as a model of disrupted growth and development. In further
312 support, females born as triplets lose nearly three times as many offspring before birth
313 than do females born as twins. This robust and highly striking finding speaks to the
314 importance of considering a female's own intrauterine development when considering
315 adult reproductive success and thus fitness. Triplet marmosets are born at lower birth
316 weights than twins raising the possibility that birth weight may be driving fetal loss in
317 adulthood. However, analyses of the effect of birth weight separate from litter size do
318 not support this conclusion. Litter size is independent of birth weight as a risk factor for
319 pregnancy loss in adult female marmosets, strong evidence that differential
320 developmental trajectories not reflected in birth weight have a critical impact on
321 reproductive outcomes in adulthood. That litter size in the marmoset and other species
322 is related to maternal energetic condition at multiple time points (e.g. ovulation,
323 conception, gestation) suggests that dynamic and complex physiology-ecology
324 interactions within and between generations are central to understanding the evolution
325 of life history variation within and between species.

326 Maternal birth weight influences fertility [39], offspring birth weight, preterm
327 delivery, infant and perinatal mortality [40]. However, only a few studies have

328 demonstrated a link between proxies of a female’s own growth *in utero* and fetal loss
329 experienced in adulthood. For example, women who as a fetus experienced nutrient
330 restriction during the third trimester due to the Dutch Famine during World War II had a
331 significantly higher rate of stillbirth and perinatal mortality than women who were not
332 exposed to famine as fetuses [41]. In female rhesus macaques, low maternal birth
333 weight was associated with producing more stillborn offspring as well as later age at first
334 reproduction and smaller offspring [42]. The marmoset model presented here augments
335 these previous findings while allowing us to view developmental programming across
336 the range of birth weight. Triplets had poorer reproductive outcomes than twins across
337 all categories of birth weights, even “normal” birth weight. Similar birth weights in two
338 infants may or may not predict the same outcome [1]. Restricted or redistributed nutrient
339 flow may not result in overall reductions in birth weight in order to maintain adequate
340 brain and somatic growth, while non-essential systems (e.g. reproductive organs,
341 hypothalamus-pituitary-ovarian (HPO) axis) may be affected adversely. Thus, average-
342 sized marmoset triplets may have “growth-impaired” reproductive phenotypes, which
343 would account for significant litter size differences in offspring viability regardless of birth
344 weight. That said, reproductive function in triplet females appears to be particularly poor
345 for those born at low weights.

346 The source of such extreme discrepancies in the triplet marmoset female’s ability
347 to successfully gestate fetuses to term is unclear, though some lines of evidence
348 suggest that differential development of the HPO axis and reproductive tract play
349 important roles. For example, small for gestational age (SGA) adolescent girls have
350 been reported to exhibit reduced ovarian dimensions [43] and reduced Follicle-

351 Stimulating Hormone (FSH) at 18 years of age [44]. Adult reproductive function thus
352 could have fetal origins in the development of the hypothalamus and pituitary, which in
353 turn would have an impact on both pituitary FSH production and ovarian organogenesis.
354 Even earlier on the developmental timeline, germ layer migration and differentiation
355 could contribute to differential HPO function. These findings suggest the possibility that
356 triplet marmosets experience altered intrauterine development affecting ovarian size,
357 the quality of the primordial follicle pool, precedents of endometrial function (with
358 implications for implantation and placentation), and even the HPO axis. Uterine size and
359 vasculature may also be altered. Limiting the physical or functional capacity of the
360 uterus in a litter-bearing primate could have direct effects on the ability to gestate live
361 offspring to term. Prospective studies are underway to track the development and
362 function of these systems from birth to first pregnancy in female marmosets in this
363 colony.

364 Transfer of prenatal testicular androgens to female fetuses as a function of mixed
365 sex litters is a key factor driving differential reproductive development in several
366 mammalian species. For example, female mice flanked by brothers *in utero* have a
367 longer anogenital distance (AGD) which is considered a masculinized phenotype [45].
368 In female swine, number of male littermates is associated with a longer AGD [46]. Some
369 physiological correlates of male littermate androgen exposure in female mice include
370 increased circulating testosterone at birth [47] and in adulthood, decreased likelihood to
371 become pregnant [48] and fewer viable litters [49]. Data are sparse for similar effects in
372 marmosets or their close litter-bearing relatives, the tamarins. Prenatal androgen levels
373 in marmosets are variable and thought to be largely of maternal or placental origin, as

374 they are apparently not related to overall litter size or presence or number of male
 375 fetuses [50, 51]. However, given current methods, it is still unknown to what extent male
 376 marmoset fetuses are producing testicular androgens [51]. Regardless of the source,
 377 marmosets are often described as escaping the virilizing effects of prenatal androgens,
 378 in part because much of genital differentiation occurs postnatally [52]. However, one
 379 colony reported a high incidence (~32%) of ambiguous or masculinized genitalia in
 380 female marmoset newborns [53]. In recent years, there have been two reports of
 381 individual female marmosets or tamarins with masculinized genitalia that express either
 382 the testis-determining *Sry* gene, the Y-linked zinc finger protein gene (ZFY), or both
 383 [54,55]. The ZFY female described in Smith et al. (2013) had a male littermate, making
 384 it “difficult to disentangle genetic from endocrine influences” (p.110, [52]). Given the
 385 current state of understanding, the organizing effects of male testicular androgens on
 386 their female siblings *in utero* cannot be ruled out. At this point it is also entirely unknown
 387 whether or the extent to which placental androgens differ in mixed sex marmoset litters.

388 The results of the current study further suggest that male exposure *in utero* may
 389 indeed have an effect on a female’s reproductive development in the marmoset
 390 monkey. Although we did not have direct measures of androgen levels, we observed
 391 that females who shared the womb with any brothers were significantly more likely to
 392 lose offspring during gestation, with loss rates threefold greater than those females from
 393 all female litters. In our sample, this effect is most apparent in twins. Twin females lost
 394 significantly more offspring in adulthood when they shared the womb with a brother
 395 instead of a sister. The existence of a “brother effect” on twin female marmosets is
 396 potential evidence for an organizing effect of testicular androgens. If this is the case,

397 then it is reasonable to expect that triplets would exhibit a higher degree of loss with
398 increasing potential exposure to brothers *in utero*. However, the effect on triplet females
399 was not significant, regardless of the number of male littermates. Our sample size may
400 be inadequate to tease out a “brother effect” in triplets. There were only four all-female
401 triplet litters, compared to the even distribution of male-female and female-female twin
402 litters, precluding appropriate statistical testing of differences.

403 The pattern thus far elucidated hints that disordered reproductive development
404 leading to pregnancy loss in triplet marmoset females is severe. It is important to note
405 that among mammals, primates are unique in having what is called the fetal zone of the
406 adrenal gland, which actively produces the androgens dehydroepiandrosterone (DHEA),
407 DHEA-sulfate (DHEA-S), and androstenedione during gestation [56]. The placenta
408 aromatizes adrenal androgens to estrogen, thus providing a buffer to female fetuses
409 [57]. However, this phenomenon is best studied in primates that produce singletons;
410 little is known about this process in the litter-bearing marmosets and tamarins. Further,
411 the pattern of placental corticotrophin releasing hormone (CRH) production differs
412 between the monkeys and apes (including humans) [58]. CRH is the hormone – usually
413 produced by the hypothalamus - that stimulates the pituitary to produce
414 adrenocorticotrophin (ACTH). In turn, ACTH stimulates the fetal zone of the adrenal
415 gland to produce androgens and glucocorticoids. In apes and humans, high levels of
416 CRH are maintained throughout pregnancy and are correlated with estrogen levels. In
417 contrast, in the marmoset monkey and the baboon CRH levels rise early in gestation,
418 peak mid-gestation, and then drop precipitously, suggesting that human patterns in this
419 regard may not be applicable to the marmoset [58]. Together, these findings raise the

420 possibility that female fetuses may be more vulnerable to prenatal androgens in the
421 litter-bearing marmosets and tamarins than in other primates. Thus, we speculate that
422 the difference in baseline reproductive performance between twin and triplet marmoset
423 females may be due at least in part to the cumulative effect of adrenal androgen
424 production and insufficient placental buffering across more fetuses in triplet litters, which
425 could then be exacerbated by testicular androgen production by brothers. Another
426 explanation of the “brother effect” could be that males are born at larger birth weights,
427 thus monopolizing maternal nutrients and disrupting female littermate development
428 through nutrient allocation. However, males and females are born at similar birth
429 weights. Certainly more comprehensive data are needed to fully interrogate the
430 intrauterine impact of brothers versus sisters on female, specifically the role of
431 cumulative levels of adrenal androgens and related placental function.

432 Developmental processes may underlie the large proportion of unexplained
433 stillbirths in humans. Stillbirth is characterized as a multifactorial outcome with various
434 risk factors contributing in large and small ways to an overall risk profile that varies
435 widely across populations. Common maternal predictors of stillbirth in high-income
436 countries include prepregnancy obesity, diabetes, chronic hypertension, infection,
437 smoking, increasing maternal age, and lack of prenatal care [59]. In low-income
438 countries, maternal infectious disease is the major identifiable risk factor [60]. Nearly
439 45% of all stillbirths are thought to be preventable through the modification of risk
440 phenotypes (diabetes control, weight loss, smoking cessation, prenatal care, infection
441 protection, etc.) [61]. Given that this leaves the majority of stillbirths unaccounted for, it
442 is clear that not all risk factors have been identified. We argue that a consideration of

443 the developmental experience of the mother may help close this gap. In our study, litter
444 size alone explains 18% of the variance in stillbirths ($p < 0.001$), indicating that
445 considering birth history could add substantial power to current models of human
446 stillbirth.

447 There are potential limitations of our study. First, though the marmoset is an
448 anthropoid primate with many similarities to humans, the fact that it produces multiples
449 as a matter of course differentiates its reproductive physiology from that of the human,
450 typically a producer of singletons. The genetic mechanisms of litter size in marmosets
451 are beginning to be elucidated, with potential for understanding multiple births of both
452 natural and assisted origin in humans [62]. Since the etiology of multiple births is likely
453 to differ between humans and marmosets, the marmoset model is possible better
454 viewed as one of intrauterine nutrient restriction due to natural variation in litter size, as
455 opposed to an analog for human multiples. Second, since this was a retrospective study
456 of demographic records, the extant coding system did not clearly differentiate early
457 pregnancy loss from *sensu stricto* stillbirth (the loss of a fetus at a developmental stage
458 equivalent to 28 weeks of human gestation) so that losses span both the embryonic and
459 fetal periods. In our planned prospective studies, specific temporal categories of loss
460 will be employed. A third consideration is the impact of secular trends in birth weight,
461 litter size, and adult weight on reproductive parameters. Individuals are being born at
462 greater weights into larger litters and growing into larger adults than they were during
463 the early years of the colony. The differential effects of litter size and birth weight
464 reported here are independent of cohort effects, suggesting they are robust phenomena
465 unaffected by secular trends; this strengthens the observation that developmental

466 programming operates across a range of birth weights. Finally, our focus is on the
467 influence of a female’s own early life characteristics on her adult reproductive function.
468 The influence of the female’s mate was not considered in this study. Although paternal
469 contributions such as age, sperm quality, and parental care are important to
470 reproductive success and fetal outcomes, they were beyond the scope of the current
471 study. Future analyses of such contributions and their impacts are planned.

472 In summary, our data overall clearly show that fetal development has a
473 tremendous impact on adult reproductive function: triplets lost three times as many
474 fetuses as did twins. A female common marmoset monkey’s own litter size at birth – a
475 phenotype reflective of a nutritionally or otherwise stressed fetal environment – acts on
476 her ability to successfully gestate fetuses to term, perhaps via the development of the
477 HPO axis and reproductive tract. This may be due to a combination of changes in
478 nutritional allocation and prenatal androgen exposure, both of which may alter
479 developmental pathways. We suggest there are specific developmental mechanisms
480 that entrain reproductive phenotypes and life history schedules across generations,
481 providing a novel way of framing life history plasticity and evolution in litter-bearing
482 mammals. While there are obvious applications of our work to life history studies of litter
483 size and the physiology of multiple pregnancies, the broader implications of our
484 marmoset model transcend these phenomena, situated in the ability to model a
485 naturally-occurring “developmental programming” or “growth-impaired” phenotype
486 (triplets) compared to a “normal” or “control” phenotype (twins). Our findings provide
487 strong evidence that a full understanding of mammalian life history, reproductive
488 biology, and pregnancy outcomes requires a developmental foundation.

489

490

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