INFECTIOUS DISEASES

Prenatal Zika virus infection has sex-specific effects on infant physical development and mother-infant social interactions

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There is enormous variation in the extent to which fetal Zika virus (fZIKV) infection affects the developing brain. Despite the neural consequences of fZIKV infection observed in people and animal models, many open questions about the relationship between infection dynamics and fetal and infant development remain. To further understand how ZIKV affects the developing nervous system and the behavioral consequences of prenatal infection, we adopted a nonhuman primate model of fZIKV infection in which we inoculated pregnant rhesus macaques and their fetuses with ZIKV in the early second trimester of fetal development. We then tracked their health across gestation and characterized infant development across the first month of life. ZIKV-infected pregnant mothers had long periods of viremia and mild changes to their hematological profiles. ZIKV RNA concentrations, an indicator of infection magnitude, were higher in mothers whose fetuses were male, and the magnitude of ZIKV RNA in the mothers' plasma or amniotic fluid predicted infant outcomes. The magnitude of ZIKV RNA was negatively associated with infant growth across the first month of life, affecting males' growth more than females' growth, although for most metrics, both males and females evidenced slower growth rates as compared with control animals whose mothers were not ZIKV inoculated. Compared with control infants, fZIKV infants also spent more time with their mothers during the first month of life, a social behavior difference that may have long-lasting consequences on psychosocial development during childhood.

INTRODUCTION

Over the course of the last few years, it has become well established that Zika virus (ZIKV) infection during pregnancy can wreak havoc on the developing fetal nervous system. The consequences of pregnant women's infections included fetal death and developmental consequences for fetuses that survived to infancy. The news headlines during the ZIKV pandemic detailed of children being born with microcephaly, and the scientific data that followed bore out the observation that maternal ZIKV infection causes this condition [for a review, see (*1*)].

Since the initial reports and observations of ZIKV affecting development, it has become clear that there is variation in the extent to which exposure to ZIKV during fetal development [herein, for brevity referred to as fetal ZIKV (fZIKV)] affects the developing brain and that children born with normal-sized heads and brains may also be affected. Children with congenital ZIKV syndrome (CZS), which presents both with and without microcephaly, have a myriad of neurological symptoms, including ventriculomegaly, lissencephaly, areas of calcification and cysts throughout the brain, and cortical malformations (*2*–*7*). Children with CZS can present smaller in size and weight (*8*–*10*) and may exhibit muscle weakness, loss of reflexes, dyspnea, hypertonia, spasticity, arthrogryposis, epilepsy, and developmental delays and may have severe learning disabilities [for a review, see (*11*)]. An increasing number of studies have documented developmental outcomes of children born to mothers who had confirmed ZIKV infection during gestation but who were born asymptomatic at birth. In some cases, these children have gone on to develop cognitive, motor, and language impairments (*5*, *12*). These observations suggest that the reach and impact of the ZIKV pandemic may be even greater than originally appreciated, although the long-term developmental consequences of fZIKV infection are not clear. Further, there are major outstanding questions about the timing of fZIKV infection relative to the magnitude of neural abnormalities and the presence of CZS in children (*13*).

To understand how ZIKV affects the developing nervous system and the behavioral consequences of prenatal viral infection across infant and childhood development, we used a nonhuman primate (NHP) model of fZIKV infection, inoculating pregnant rhesus macaques and their fetuses in the early second trimester of development. We previously determined that this experimental approach ensures fetal infection (*14*). In this initial report from the project, we detail the mothers' viral and infection dynamics during gestation and the behavioral consequences of fZIKV infection on infants immediately after birth.

Although multiple rodent models of ZIKV exist (*15*–*19*), NHP models of ZIKV and fZIKV remain best suited for modeling human neurological and psychological development because of both limitations of the rodent models and strengths of the NHP model. NHPs, such as monkeys from the genus *Macaca,* demonstrate transplacental transmission of the ZIKV to the fetus (*20*–*23*) and also share many homologies in terms of cortical structure and function with humans (*24*). In addition, NHPs share many features of fetal development with humans (*25*), including the trajectories and timing of brain development (*26*). Monkeys have also been extensively studied across as models for human behavioral

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and psychological development [for reviews, see (*27*–*32*)], and standardized behavioral evaluations of their development exist, which mirror those used in humans (for example, a monkey version of the Brazelton evaluation) (*33*–*35*). Thus, NHP models of ZIKV (*14*, *21*, *23*, *36*–*40*) bridge the translational divide between bench and bedside for understanding the human condition.

Monkey models of fZIKV share many features of human fZIKV, including prolonged viremia, or virus in the blood stream, in pregnant females compared with nonpregnant animals, decreased fetal growth, and fetal brain lesions and calcifications (*14*, *21*, *41*–*44*). We have recently demonstrated that fZIKV causes brain-wide abnormalities that track with the pattern of neural development along the rostral-caudal brain axis; these abnormalities seem to occur as a result of direct infection of neurons and inflammatory processes that activate glia (*45*). How these fetal consequences play out across infancy is largely unknown because so few studies follow fZIKV-infected infants across development. What little evidence exists demonstrates that infant rhesus macaques infected with ZIKV in the first or second trimester of their fetal development exhibited some CZS-like symptoms such as cardiomyopathy, motor delay, seizures, growth abnormalities, vision dysfunction, and neuropathology (*46*–*50*). However, it is difficult to draw conclusions from these studies because of methodological limitations. In the existing studies, infants were delivered early through cesarean section [see (*51*) regarding appropriate gestational development in rhesus macaques], reared in adverse conditions [outside of a group, in a nursery either without mother or with mother only; see (*52*–*54*) regarding how adverse rearing affects development], or inoculated using methods that do not ensure that fetuses are exposed to ZIKV in utero.

Across both the existing monkey model and human literatures, studies of fZIKV leave many questions unanswered. First, published reports provide limited information about the health of the pregnant mothers. Second, the evaluation of behavioral and psychosocial consequences of fZIKV on infants is limited. As a result, there is little information about how the dynamics of fZIKV infection relate to health and other infant developmental outcomes. The current study addresses these gaps by tracking pregnant females and their fetuses, half of whom were inoculated with ZIKV, across pregnancy and the first month of their infants' lives with frequent sampling of health metrics and behavior. Pregnant females and their fetuses were exposed to ZIKV at approximately gestational day (GD) 64, which is early in the second trimester for rhesus macaques (*Macaca mulatta*), where 55 days is approximately one "trimester." We based this timing on our previous demonstration that fetal demise occurs at much higher rates with ZIKV exposure that occurs before GD55 (*42*). We carried out this work with group-living rhesus macaques to provide the most normative social rearing environment possible [and in contrast to how infectious disease studies are typically carried out; (*55*)]. We evaluated prenatal health of dams, viremia, prenatal fetal physical development, and infant postnatal physical, as well as sensorimotor and behavioral, development during the first 30 days of life.

RESULTS

Overview of the experimental design

Results below were generated from a study of socially housed rhesus macaques living in small groups composed of an adult male and multiple adult females who were pregnant and gave birth during the study; their infants were maintained in groups with them after birth. Pregnant females and their fetuses were inoculated with ZIKV through the intravenous route to the mother and intraamniotic route to the fetus at about 64 days of gestation and followed through frequent assessments over the course of fetal development (regular sampling during first week postinoculation, then weekly blood and amniotic fluid sample collections and ultrasound assessments) and the first month of the infants' lives (growth and behavioral evaluations) to evaluate how fZIKV infection affects infant development. Because vertical transmission rates are variable with peripheral inoculation (e.g., there was no vertical transmission in one recent study using macaques) (*56*) and the goal of the study was to model the outcomes of fetal infection on prenatal and postnatal development, inoculation of both the dam (intravenous) and the fetus (intraamniotic) was critically important to this design. Monkeys were inoculated with a combination of the 2015 Puerto Rican isolate (PRVABC-59; GenBank KU501215) and a 2015 Brazilian isolate (strain Zika virus/H.sapiens-tc/BRA/2015/Brazil_SPH2015; GenBank KU321639.1), which were used in previous studies to mimic a hyperendemic area (*14*, *23*, *44*, *45*, *50*). Details about enrolled females, treatment groups, and pregnancy outcomes can be found in Table 1.

No obvious clinical signs of ZIKV infection were observed in pregnant mothers

There were no obvious clinical signs of ZIKV infection of mothers. Two of the ZIKV pregnant females evidenced mild anemia during pregnancy [detected at day postinoculation (DPI) 28 for one and DPI 77 for the other] that was successfully treated with iron dextran. One of the ZIKV pregnant females was noted to have reduced amniotic fluid. All control (CON) animals were normal during pregnancy. After pregnancy, one CON and four ZIKV-infected adult females were mildly anemic, one CON and two ZIKV-infected females were noted to have metritis, and one ZIKV-infected female had low milk production, all of which resolved and none of which were atypical for a sample of this size. There were no other clinical symptoms noted throughout pregnancy or during the first month postpartum (for the mothers) or first month of life (for the infants). No signs of RNAemia were observed in any of the animals (dams or infants) after birth.

Viral RNA kinetics in amniotic fluid and maternal plasma revealed prolonged infection whose course depended on fetal sex

We measured ZIKV RNA to evaluate RNAemia throughout this study (Fig. 1A). ZIKV RNA was measured in plasma and amniotic fluid immediately before inoculation and then every 7 days until birth in $N = 10$ ZIKV-infected females. ZIKV RNA was additionally measured in maternal plasma at DPI 3 and 5. ZIKV RNA was detected in at least one plasma and amniotic fluid sample from all pregnant females that were ZIKV inoculated. The average RNA concentration in maternal plasma was 2.93 $\log_{10}$/ml [standard error (SE) = 0.09] with a peak value of 5.53 $\log_{10}$/ml at DPI 3. The average RNA concentration in amniotic fluid was 3.20 $\log_{10}$/ml (SE = 0.12) with a peak value of 6.33 $\log_{10}$/ml at DPI 7 (Fig. 1). The average duration of infection [DPI when viral RNA (vRNA) was last detected over the limit of detection (LOD)] was

Table 1. Overview of animal and group demographics. Enrolled subjects were assigned an identifier code (subject code), with letters identifying ZIKV-infected monkeys and numbers identifying control monkeys. Codes for the ZIKV-infected monkeys match Fig. 1 (which is why lettering is purposefully out of order in this table). Groups are identified as either ZIKV groups (1 to 3) or CON groups (1 to 3). Subjects were born over two birth cohorts (year). Birth information details how subject infants were born, whether fetal demise occurred, or other issues. Fetus/infant sex was determined at only birth; M = male, F = female; U = unknown. Inoculation day is specified as the gestational day (GD) of either the ZIKV or sham inculcation, with the three replacement infants indicated as not applicable (NA). GD was determined by the timed-mating program at the California National Primate Research Center (CNPRC) and confirmed by ultrasounds in all animals other than two replacement animals noted with * (GDs for these animals were determined solely by ultrasound). ZIKV viral load information is presented as AUC_{plasma} and AUC_{amnio}. Whether subjects are included in the prenatal and postnatal datasets is indicated by a ✓. Attrition of multiple subjects in the CON group was unexpected, although within range of pregnancy loss in the time-mating program at the CNPRC for the years of study (19% loss in year 1 and 15% in year 2).

Subject code	Group	Year	Birth information	Notes	Fetus/ infant sex	Inoculation day (GD)	AUC_{plasma}	AUC_{amnio}	Prenatal dataset	Postnatal dataset
A	ZIKV1	1	Live vaginal		M	69	102.03	175.7	✓	✓
B	ZIKV1	1	Live vaginal		M	66	9.8	311.78	✓	✓
C	ZIKV1	1	Live vaginal		M	64	62.303	345.275	✓	✓
D	ZIKV1	1	Live vaginal		M	65	65.905	171.43	✓	✓
F	ZIKV2	1	Live vaginal		F	67	62.86	153.355	✓	✓
G	ZIKV2	1	Live vaginal		F	67	177.305	114.695	✓	✓
K	ZIKV2	1	Stillbirth at GD166	Remained in group with no infant	M	61	–	–		
E	ZIKV3	2	Live vaginal		M	63	176.05	252.84	✓	✓
H	ZIKV3	2	Live vaginal		F	68	180.385	107.73	✓	✓
I	ZIKV3	2	Live vaginal		F	64	102.255	108.5	✓	✓
J	ZIKV3	2	Live vaginal		F	58	340.38	217.14	✓	✓
1	CON1	1	Fetal demise at GD97	Replaced with subject 9	U	66	–	–		
2	CON1	1	Fetal demise at GD127	Replaced with subject 6	U	64	–	–		
3	CON1	1	Live vaginal		M	68	–	–	✓	✓
4	CON1	1	Live vaginal		M	67	–	–	✓	✓
5	CON1	1	Live vaginal, infant died at 9 days	*Replacement animal; joined at GD123	M	NA	–	–	✓	
6	CON1	1	Live cesarean	*Replacement animal; joined at GD138	M	NA	–	–	✓	✓
7	CON2	2	Live vaginal		M	63	–	–	✓	✓
8	CON2	2	Live vaginal		F	61	–	–	✓	✓
9	CON2	2	Live vaginal		M	63	–	–	✓	✓
10	CON2	2	Live vaginal–induced		F	66	–	–	✓	✓
11	CON2	2	Fetal demise at GD76	Removed from group; not replaced	U	69	–	–		
12	CON3	2	Live vaginal		F	67	–	–	✓	✓
13	CON3	2	Live vaginal	Replacement animal; joined at GD95	F	NA	–	–	✓	✓
14	CON3	2	Stillbirth at GD171	Remained in group with no infant	F	64	–	–		
15	CON3	2	Removed from group for medical reasons at GD94	Replaced with subject 13	U	64	–	–		

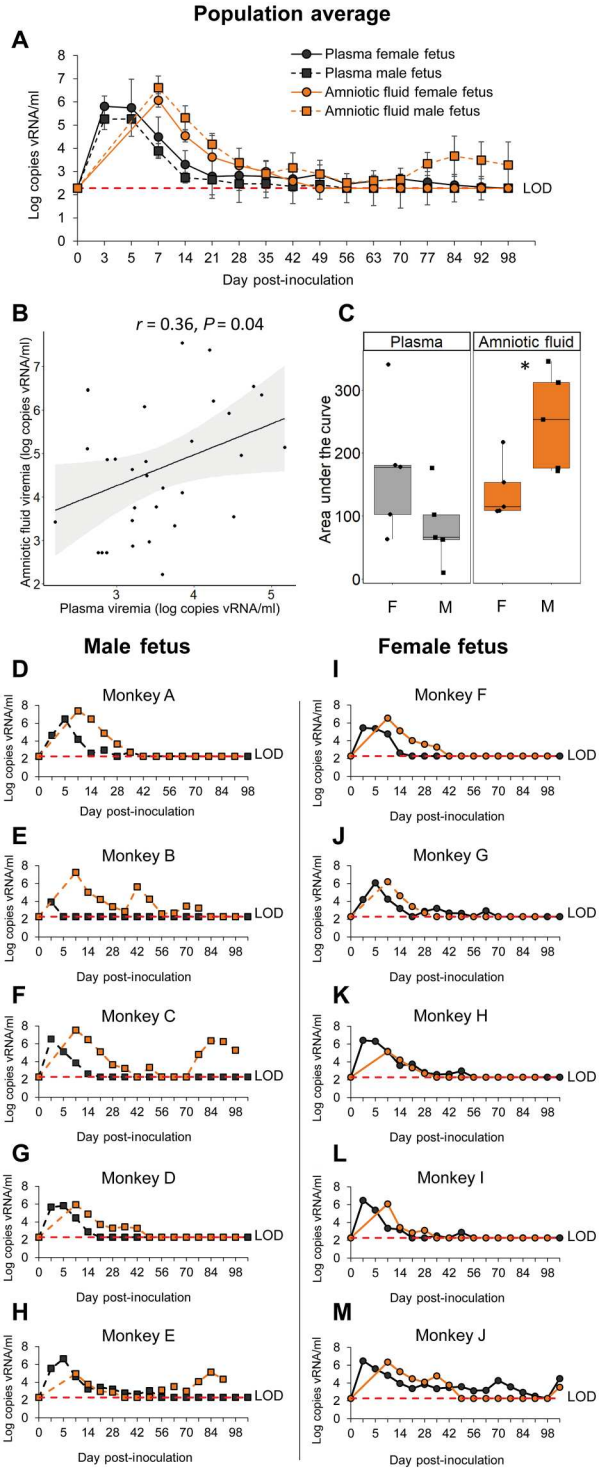

Fig. 1. Viral RNA quantity after inoculation depends on fetal sex. (**A**) Average viral RNA (vRNA) concentrations per DPI is shown according to fetal sex and sampled fluid (plasma or amniotic fluid). Data are presented as mean ± SE. (**B**) Correlation is illustrated between vRNA quantities in plasma and amniotic fluid when infection was found in both fluids. (**C**) Average infection magnitude (cumulated area under the curve) is shown according to fetal sex and sampled fluid. Data are presented as mean ± SE, and dots represent individual animals. Mann-Whitney, *$P < 0.05$. (**D** to **M**) Individual viremia values are shown over time according to sampled fluid. vRNA quantity is shown for ZIKV-inoculated pregnant rhesus macaques (mean and SE) in plasma (black) and amniotic fluid (orange). Three pregnant females (monkeys B, E, and J) had one ZIKV RNA rebound (reoccurrence of viremia after disappearance of first episode), and one pregnant female had two ZIKV RNA rebounds (monkey C). In the amniotic fluid, two females had one ZIKV RNA rebound, and one female had two ZIKV RNA infection rebounds. Samples for which ZIKV RNA was not detected are graphed at the limit of detection (LOD). Individuals are identified according to their subject codes (Table 1). Data can be found in data file S1.

54.7 days (SE = 6.54) for the amniotic fluid and 44.6 days (SE = 9.32) in maternal plasma.

ZIKV RNA was more frequently detected in only one of the fluids than in both simultaneously (co-occurrence: 37.65%, binomial test: $Z = -2.17$, $P = 0.01$). When evidence of infection was found simultaneously in both fluids, ZIKV RNA quantities in maternal plasma and amniotic fluid were correlated ($r = 0.36$, $P = 0.04$; Fig. 1B). We quantified the "magnitude of infection" by computing the area under the curve (AUC); this magnitude was higher in the amniotic fluid when the fetus was a male (mean = 251.41, SE = 35.06) than when the fetus was a female (mean = 140.28, SE = 20.98; Mann-Whitney, $P = 0.03$; Fig. 1C). The magnitude of ZIKV RNA in maternal plasma was not different whether the fetus was a male (mean = 83.22, SE = 27.45) or a female (mean =

172.64, SE = 47.55; Mann-Whitney, $P = 0.09$; Fig. 1B). Individual vRNA concentrations for each mother-fetus pair are shown in Fig. 1 (D to M).

ZIKV infection resulted in less and slower weight gain during pregnancy

We explored the influence of exposure to ZIKV on maternal weight gain during gestation. ZIKV-infected ($N = 10$) and CON ($N = 10$) macaques were weighted weekly from inoculation to parturition. On inoculation day, pregnant ZIKV-infected and CON females did not differ in their weight, $t = 1.56$, $P = 0.15$ ($\text{mean}_{ZIKV} = 8.04$, $\text{SE}_{ZIKV} = 0.44$ kg, $\text{mean}_{CON} = 9.40$, $\text{SE}_{CON} = 0.76$ kg).

There was a general effect of ZIKV inoculation such that pregnant ZIKV-infected females were lighter than CON pregnant females ($\beta = -3.20$, SE = 1.11 kg, $\chi^2 = 4.93$, $P = 0.03$). Between inoculation and birth, all pregnant females gained weight in a nonlinear fashion, although the patterns were different for ZIKV-infected and CON females. An interaction between ZIKV inoculation and DPI, and between ZIKV inoculation and DPI^2 (the quadratic term to account for nonlinear growth), showed that, compared with CON females, pregnant ZIKV-infected females gained less weight after ZIKV inoculation and that they gained weight more slowly during the early postinoculation phase but faster around the end of gestation ($\chi^2 = 41.09$, $P < 0.001$; ZIKV inoculation × DPI^2: $\chi^2 = 31.69$, $P < 0.001$; Fig. 2). Additionally, a three-way interaction between ZIKV inoculation, fetal sex, and DPI^2 ($\chi^2 = 5.44$, $P = 0.02$) revealed that this pattern of slower weight gain in the early postinoculation phase and faster weight gain at the end of gestation for ZIKV-infected pregnant females was stronger when the fetus was a male than when the fetus was a female (Fig. 2). For pregnant ZIKV-infected females, females with greater ZIKV RNA in their plasma and amniotic fluid gained weight more slowly (plasma: $\beta = -9.03$, SE = 1.95 g/day, $\chi^2 = 21.57$, $P < 0.001$; amniotic fluid: $\beta = -4.76$, SE = 1.77 g/day, $\chi^2 = 7.26$, $P = 0.007$).

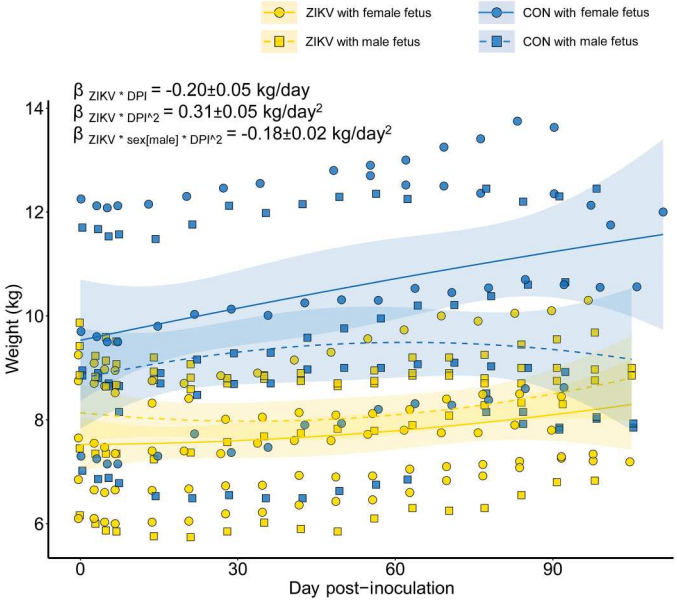

Fig. 2. The effect of ZIKV inoculation on pregnant females' weight gain depends on fetal sex. Pregnant females' weights are shown on the basis of experimental condition and time. ZIKV-infected pregnant females ($N = 10$) are plotted in yellow; CON pregnant females ($N = 10$) are plotted in blue. Mothers of female fetuses are plotted with circles, and mothers of male fetuses are plotted with squares. Fitted curves are plotted in yellow for ZIKV animals and in blue for CON animals, with solid lines when fetuses were females and dashed lines when fetuses were males. Shaded areas represent 95% confidence intervals. Data can be found in data file S2.

Maternal hematology was clinically normal

Maternal hematological parameters were measured from blood collected weekly from inoculation until parturition in ZIKV-infected ($N = 10$) and CON ($N = 10$) pregnant females (fig. S1). At the time point immediately before inoculation, there were no significant differences in any of the hematological measures between pregnant ZIKV-infected and CON females (all t tests, $P > 0.05$; table S1). Compared with CON, inoculation with ZIKV affected several hematological parameters during gestation, including reducing the concentration of white blood cells ($P = 0.02$), reducing hemoglobin ($P = 0.02$) and platelets ($P = 0.03$), and lowering the hematocrit ($P = 0.02$); in some cases, there were interactions over time. Despite these effects, values largely remained within normal limits (*57*), suggesting that they may not have been clinically meaningful. As such, these data are reported in table S2. The magnitude of viral infection, as indexed by the log number of vRNA copies of ZIKV, had a negative effect on most of those hematological indices for pregnant ZIKV animals. Those results are reported in table S3.

Pre- and postnatal offspring physical development was affected by ZIKV infection

Measurements of head growth by ultrasound are one of the primary means by which to determine fetal age and growth curves and to detect nervous system pathology during fetal development (*58*–*61*). We used biparietal diameter (BPD) as our index of head development across the fetal period because it is a widely used measure in humans (*62*–*64*). Fetuses exposed to ZIKV had smaller—although not significantly smaller (P value did not reach 0.05)—BPDs in utero ($\beta = -0.16$, SE = 0.07 cm, $\chi^2 = 3.07$, $P = 0.08$) and significantly slowed BPD growth across gestation (ZIKV inoculation × DPI: $\beta = -0.08$, SE = 0.02 cm/day, $\chi^2 = 23.20$, $P < 0.001$; Fig. 3). The rate of growth slowed toward the end of gestation for both fZIKV and CON fetuses, but the day at which growth slowed was later for fZIKV than for CON fetuses (ZIKV inoculation × DPI^2: $\beta = 0.05$, SE = 0.02 cm/day^2, $\chi^2 = 6.46$, $P = 0.01$). There were no sex differences in any of these effects (all P values >0 .05). Although smaller, most BPD measurements were within 2 SDs of our colony's average (fig. S2 shows BPD measurements relative to the colony average by gestational day). Fetal BPD (fBPD) daily growth of ZIKV-infected fetuses was negatively influenced by the magnitude of viremia in the amniotic fluid ($\beta = -0.06$, SE = 0.03 cm/day, $\chi^2 = 4.00$, $P = 0.05$) such that growth was slower when the quantity of ZIKV RNA was higher. Viremia in maternal plasma had no influence on fBPD daily growth ($P = 0.92$).

We monitored the morphological parameters of infants across the first month of life to detect the influence of fZIKV exposure on physical development. Measured parameters included weight, stature, head size, chest and biceps circumferences, limbs lengths, and arm and foot lengths. Morphological measures were taken when the fZIKV ($N = 10$) and CON ($N = 9$) infants were 3, 7, 14, 21, and 28 days old.

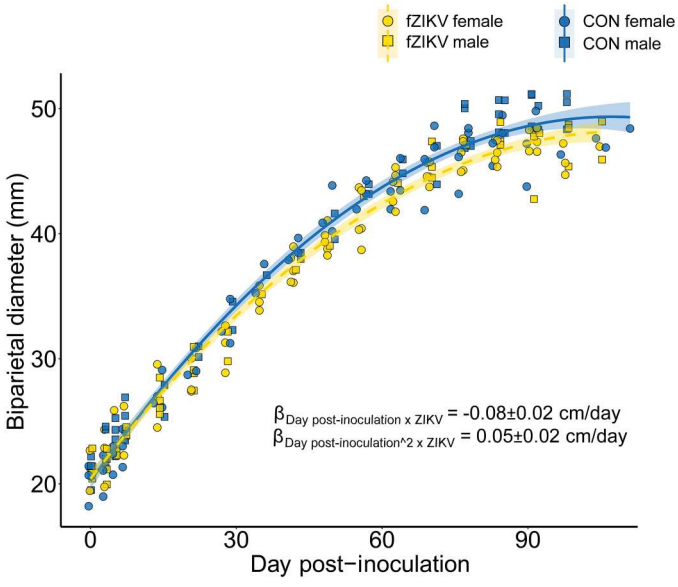

Fig. 3. ZIKV inoculation slows fBPD growth across gestation. fBPD is shown according to fZIKV exposure and time. fZIKV fetuses (N = 10) are plotted in yellow, and CON fetuses (N = 10) are plotted in blue. Females are plotted with circles, and males are plotted with squares. Fitted curves are plotted in yellow for fZIKV animals and in blue for CON animals. Shaded areas represent 95% confidence intervals. See fig. S2 for plot relative to GD, rather than DPI, in comparison with colony norms. Data can be found in data file S2.

Infant weight at birth was affected by prenatal exposure to ZIKV; regardless of sex, CON infants were born heavier than fZIKV infants, t = 2.19, P = 0.05 ($mean_{fZIKV}$ = 435.00 g, SE_{fZIKV} = 14.62 g, $mean_{CON}$ = 507.78 g, SE_{CON} = 29.85 g). After birth, infant weight was predicted by an interaction between ZIKV inoculation, age, and sex (χ^2 = 16.57, P < 0.001). fZIKV males gained less weight across the first month of life compared with CON males, but fZIKV females gained more weight across the first month of life than CON females (Fig. 4A). The magnitude of ZIKV infection and sex predicted infant weight gain. The growth of fZIKV males was more greatly negatively affected by the magnitude of infection in the amniotic fluid ($\beta = -3.07 \times 10^{-5}$, SE = 1.50×10^{-5}, χ^2 = 4.18, P = 0.04) and in maternal plasma ($\beta = -3.96 \times 10^{-5}$, SE = 1.03×10^{-5}, χ^2 = 14.90, P < 0.001) than the growth of fZIKV-infected females. Infant crown-rump length was not affected by the ZIKV inoculation either alone (χ^2 = 2.31, P = 0.12) or in any interaction term (ZIKV inoculation × age: χ^2 = 1.35, P = 0.24; ZIKV inoculation × sex: χ^2 = 1.04, P = 0.31). Dam weight at late gestation was retained in the model as a positive predictor of infant crown-rump length throughout the first month of life, although this effect did not reach P < 0.05 (β = 0.21, SE = 0.12 cm/kg, χ^2 = 3.10, P = 0.08).

Across the first month of life, infant head size growth as measured by biparietal diameter and orbitofrontal diameter (OFD) was affected by ZIKV exposure. At birth, fZIKV infants had smaller BPDs than CON infants ($mean_{fZIKV}$ = 50.54, SE_{fZIKV} = 0.37 mm, $mean_{CON}$ = 52.28, SE_{CON} = 0.53 mm; t = 2.68, P = 0.02), but there were no differences in OFD ($mean_{fZIKV}$ = 66.71, SE_{fZIKV} = 1.23 mm, $mean_{CON}$ = 68.29, SE_{CON} = 1.09 mm, t = 1.23, P = 0.24). After birth, BPD and OFD growth were predicted by ZIKV inoculation and sex (BPD growth: χ^2 = 3.46, P = 0.06; OFD growth: χ^2 = 4.76, P = 0.03; Table 2), although we note that although both predictors were retained in the model, BPD growth did not reach conventional significance values. The pattern was similar for these two metrics: Prenatal exposure to ZIKV slowed head growth in male infants but not in female infants (Fig. 4, B and C). The magnitude of infection in maternal plasma affected BPD such that the fZIKV infants with the greatest infection had the smallest heads and slower head growth, and this effect was greater in males than in females (BPD: χ^2 = 7.54, P = 0.006; $\beta_{Infection\ magnitude \times sex\ [males]} = -0.02$, SE = 0.01 mm; BPD growth: $\beta_{Infection\ magnitude \times age \times sex[males]} = -5.9 \times 10^{-4}$, SE = 3.00×10^{-4} mm/day, χ^2 = 3.84, P = 0.05). Comparatively, OFD and OFD growth were not influenced by the magnitude of infection in maternal plasma (OFD: χ^2 = 2.47, P = 0.12, OFD growth: χ^2 = 0.11, P = 0.74). The magnitude of infection in the amniotic fluid predicted BPD, although the P value did not reach P < 0.05 (BPD: χ^2 = 3.00, P = 0.09), but had no impact on BPD growth, OFD, or OFD growth (BPD growth: χ^2 = 0.05, P = 0.82; OFD: χ^2 = 0.01, P = 0.94; OFD growth: χ^2 = 1.54, P = 0.21).

Table 2. Linear mixed model for head metrics, including BPD and occipitofrontal diameter according to prenatal exposure to ZIKV (treatment), infant sex, and age. CON: N = 9, fZIKV: N = 10. When predictors were not retained by model selection, dashes replace the values.

	Biparietal diameter (mm)					Occipitofrontal diameter (mm)				
	χ^2	df	P	β	SE	χ^2	df	*P*	β	SE
Intercept				51.65	0.82				66.97	1.1
Dam weight (kg)	–	–	–	–	–	–	–	–	–	–
Age	188.84	1	<0.001	0.09	0.02	72.77	1	<0.001	0.1	0.03
Inoculation [fZIKV]	4.79	1	0.03	−1.87	1.09	3.12	1	0.08	−2.04	1.47
Sex [male]	4.49	1	0.03	0.84	1.1	2.73	1	0.10	2.1	1.48
Age × inoculation [fZIKV]	0.06	1	0.8	0.04	0.03	0.19	1	0.65	0.07	0.04
Age × sex [male]	2.32	1	0.13	0.07	0.03	5.37	1	0.02	0.0008	0.04
Inoculation [fZIKV] × sex [male]	0.09	1	0.76	0.51	1.5	0.3	1	0.58	0.44	2.02
Age × inoculation [fZIKV] × sex [male]	3.46	1	0.06	−0.07	0.04	4.76	1	0.03	−0.11	0.05
	R^2m = 0.45 R^2c = 0.87					R^2m = 0.34 R^2c = 0.84				

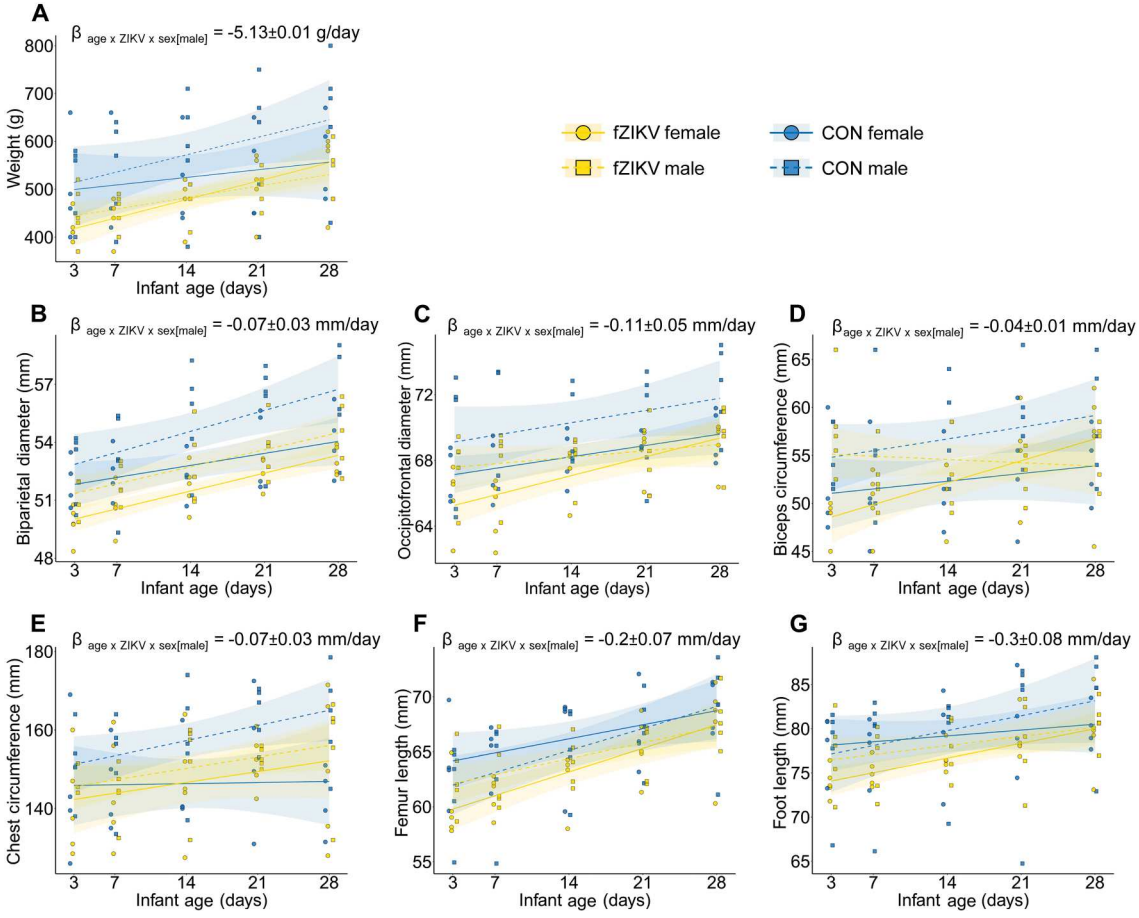

Fig. 4. Prenatal exposure to ZIKV affects postnatal growth across the first month of life with more severe effects in male infants. fZIKV infants ($N = 10$) are plotted in yellow, and CON infants ($N = 9$) are plotted in blue. Females are plotted with circles, and males are plotted with squares. Fitted lines are plotted in yellow for fZIKV animals and in blue for CON animals, with solid lines for females and dashed lines for males. Shaded areas represent 95% confidence intervals. Growth measures included (**A**) infant weight, (**B**) BPD, (**C**) occipitofrontal diameter, (**D**) biceps circumference, (**E**) chest circumference, (**F**) femur length, and (**G**) foot length according to prenatal exposure to ZIKV, treatment, and sex. Data can be found in data file S3.

Biceps and chest circumferences depended on a triple interaction between ZIKV inoculation, age, and sex of the infant (biceps: $\chi^2 = 17.13$, $P < 0.001$; chest: $\chi^2 = 6.55$, $P = 0.01$; Table 3), accounting for the positive influence of maternal weight at late pregnancy (biceps: $\chi^2 = 4.65$, $P = 0.03$; chest: $\chi^2 = 7.02$, $P = 0.008$). Biceps and chest circumferences grew slower in fZIKV males than in CON males, whereas biceps and chest circumferences grew faster for fZIKV females than for CON females (Fig. 4, D and E). fZIKV animals with the greatest magnitude of infection in plasma had the slowest rate of chest growth (β = −0.09, SE = 0.04 cm/day, $\chi^2 = 7.30$, $P = 0.007$). Comparatively, the magnitude of infection in plasma had no significant impact on biceps growth ($\chi^2 = 0.00$, $P = 0.98$). Biceps circumference depended on an interaction between the magnitude of the infection in the amniotic fluid and sex such that the negative impact of infection was stronger in males than in females, but this effect was not significant (β = −0.002, SE = 0.004 cm, $\chi^2 = 3.33$, $P = 0.07$). The magnitude of infection in the amniotic fluid did not significantly affect chest growth ($\chi^2 = 0.85$, $P = 0.36$).

Femur and foot length both depended on an interaction between ZIKV inoculation, age, and sex (femur: $\chi^2 = 7.69$, $P = 0.006$; foot: $\chi^2 = 11.03$, $P = 0.001$; Table 4). fZIKV infection negatively affected femur and foot growth but only in males (Fig. 4, F and G). fZIKV infection predicted hand growth, but, contrary to all other metrics, hands of fZIKV infants grew faster than those of CON infants regardless of sex (β = 0.10, SE = 0.04 mm/day, $\chi^2 = 5.47$, $P = 0.02$; Table 4). The greater the magnitude of infection in the plasma, the slower the femur growth (β = −0.001, SE = 0.0004 mm/day, $\chi^2 = 12.29$, $P < 0.001$). The magnitude of infection in the amniotic fluid also slowed femur growth but with a stronger effect in males than in females (β = −0.002, SE = 0.001 mm/day, $\chi^2 = 3.69$, $P = 0.05$). The greater the magnitude of the infection in the plasma, the slower the foot growth (β = −0.0007, SE = 0.0006 mm/day, $\chi^2 = 3.95$, $P = 0.05$).

fZIKV infection altered infant social behavior

During the first month of life, rhesus infants typically spend most of their time physically with and interacting with their mothers, often on their chests in ventral-ventral contact (*65*–*68*). This relationship is the arguably the most important for their development, as evidenced by the fact that infants raised without their mothers exhibit major behavioral and psychological abnormalities (*69*–*71*).

Table 3. Linear mixed model for chest and biceps circumferences (mm), according to prenatal exposure to ZIKV, infant sex, and age. fZIKV: $N = 10$, CON: $N = 9$.

	Chest circumference (mm)					Biceps circumference (mm)				
	χ^2	df	*P*	β	SE	χ^2	df	*P*	β	SE
Intercept				9.82	1.72				3.66	0.69
Dam weight (kg)	7.02	1	0.008	0.33	0.12	4.65	1	0.03	0.13	0.06
Age	28.21	1	<0.001	−0.01	0.01	30.55	1	<0.001	0.01	0.006
Inoculation [fZIKV]	0.02	1	0.9	0.11	0.75	0.1	1	0.75	0.1	0.33
Sex [male]	0.73	1	0.39	0.39	0.72	4.69	1	0.03	0.59	0.3
Age × inoculation [fZIKV]	2.32	1	0.13	0.06	0.02	0.07	1	0.79	0.02	0.01
Age × sex [male]	3.76	1	0.05	0.06	0.02	10.1	1	0.006	0.007	0.01
Inoculation [fZIKV] × sex [male]	3.21	1	0.07	−0.52	0.89	1.76	1	0.18	0.08	0.41
Age × inoculation [fZIKV] × sex [male]	6.53	1	0.01	−0.07	0.03	17.13	1	<0.001	−0.04	0.01
	R^2m = 0.49 R^2c = 0.81					R^2m = 0.36 R^2c = 0.82				

Given that, we evaluated the social behavior of fZIKV ($N = 10$) and CON ($N = 9$) infants by observations multiple times a week through 5-min focal observation from birth until they were 30 days old. During the first month of life, rhesus infants spent the vast majority of their time on their mothers, consistent with previous reports (*72*). Probabilities to be involved in any other social behavior (see table S4 for full ethogram) were too low (<1%) for a meaningful analysis, and we therefore limited the analysis to the time infants spent in contact with their mothers and the time spent being groomed by their mothers.

Overall, fZIKV infants were more likely to be observed on their mothers compared with CON infants, regardless of their age (probability: fZIKV = 0.98, SE_{fZIKV} = 0.01, CON = 0.90, SE_{CON} = 0.04; $\chi^2 = 4.51$, $P = 0.03$). Although the time spent on mother decreased over the first month of life for all infants, an interaction between ZIKV inoculation, age, and sex ($\chi^2 = 39.37$, $P < 0.001$) revealed that this decrease was smallest for fZIKV females compared with all other subjects (Fig. 5).

The magnitude of viral infection affected infant social behavior in a sex-dependent manner. The greater the magnitude of ZIKV RNA in amniotic fluid, the more time female fZIKV infants spent on mother; this was not observed in male fZIKV infants. However, although model selection retained this interaction between ZIKV infection and sex, this effect did not reach a traditional significance value (β = −4.65, SE = 1.92, $\chi^2 = 3.41$, $P = 0.06$). We did not find an overall effect of fZIKV exposure ($P = 0.92$), but there was an interaction between fZIKV, sex, and infant age ($\chi^2 = 12.99$, $P < 0.001$), revealing that the increase in being groomed with infant age was smaller for fZIKV females compared with all other animals (β = −0.68, SE = 0.19).

The infant neonatal assessment revealed subtle ZIKV-related developmental differences

Newborn human infants are regularly evaluated immediately after birth on a number of sensory and motor processes to ensure health and normal physiological and neuronal functions. One such evaluative test is the Brazelton Neonatal Assessment Scale, which evaluates infant responses to the environment and is typically carried out shortly after birth (*73*). To assess infant sensorial and motor development, we ran a battery of tests derived from the Brazelton Neonatal Assessment Scale but specifically tailored to infant monkeys, adapted from (*34*, *35*, *74*). As part of this assessment, infants were placed in a warmed incubator for a 5-min period, and their behavior was video-recorded and scored offline using a standard ethogram that includes behavior when active (e.g., standing, hanging, and exploration), vocalizations, orientation (e.g., visual orientation and attention), and "state control" (e.g., soothability and irritability) (see table S5 for full ethogram). Tests occurred when fZIKV ($N = 10$) and CON ($N = 9$) infants were 7, 14, 21, and 28 days old.

fZIKV and CON infants did not show significant differences in their behavior during the 5-min isolation test. Groups did not differ in activity ($mean_{fZIKV}$ = 191.41, SE_{fZIKV} = 30.31, $mean_{CON}$ = 166.39, SE_{CON} = 23.59 s, $\chi^2 = 2.06$, $P = 0.15$), the frequency of exploration ($mean_{fZIKV}$ = 0.80, SE_{fZIKV} = 0.49, $mean_{CON}$ = 3.13, SE_{CON} = 1.80, $\chi^2 = 0.38$, $P = 0.54$), or the frequency of vocalizations expressed ($mean_{fZIKV}$ = 146.40, SE_{fZIKV} = 18.04, $mean_{CON}$ = 122.60, SE_{CON} = 14.97, $\chi^2 = 0.05$, $P = 0.83$). Whether considered as a main effect or in interaction terms, ZIKV infection was never retained in the selected models for these factors. Factors related to orientation increased over the first month of life for both treatment conditions (table S6). fZIKV infants scored lower on visual orientation ($mean_{fZIKV}$ = 0.13, SE_{fZIKV} = 0.05, $mean_{CON}$ = 0.20, SE_{CON} = 0.04, $\chi^2 = 3.99$, $P = 0.05$) and attention ($mean_{fZIKV}$ = 0.36, SE_{fZIKV} = 0.11, $mean_{CON}$ = 0.53, SE_{CON} = 0.10, $\chi^2 = 3.90$, $P = 0.05$). The other factors associated with orientation did not differ between fZIKV and CON infants, and none of the factors related to state control were affected by treatment (table S5). We were not able to explore the sex-specific effects of prenatal infection on infant neonatal assessment results because of lack of statistical power (β range: 0.4 to 0.6).

There was no evidence of infant imitation in either group

In humans, it is widely believed that infants imitate behavioral actions generated by adults and that this early form of social processing has important implications for the development of social cognition [for reviews, see (*75*, *76*)]. Evidence in humans for

Table 4. Linear mixed model for femur, hand, and foot length according to prenatal exposure to ZIKV, infant sex, and age. fZIKV: $N = 10$, CON: $N = 9$. When predictors were not retained by model selection, dashes replace the values.

	Femur (mm)					Hand (mm)					Foot (mm)				
	χ^2	df	*P*	β	SE	χ^2	df	*P*	β	SE	χ^2	df	*P*	β	SE
Intercept				54.05	5.06				40.85	5.97				64.54	7.86
Dam weight (kg)	4.12	1	0.04	0.87	0.43	3.47	1	0.06	0.97	0.52	3.08	1	0.08	1.09	0.62
Age	183.85	1	<0.001	0.18	0.04	73.71	1	<0.001	0.13	0.03	81.49	1	<0.001	0.06	0.04
Inoculation [fZIKV]	0.01	1	0.97	−1.98	2.42	0.5	1	0.48	−0.006	2.14	0.03	1	0.87	−0.64	3.58
Sex [male]	0.33	1	0.57	−0.92	2.19	–	–	–	–	–	1.19	1	0.27	1.04	3.41
Age × inoculation [fZIKV]	0.2	1	0.65	0.12	0.05	5.47	1	0.02	0.1	0.04	0.8	1	0.37	0.17	0.06
Age × sex [male]	0.006	1	0.94	0.11	0.05	–	–	–	–	–	1.41	1	0.24	0.18	0.06
Inoculation [fZIKV] × sex [male]	0.02	1	0.88	3.18	2.95	–	–	–	–	–	0.21	1	0.65	1.55	4.18
Age × inoculation [fZIKV] × sex [male]	7.69	1	0.006	−0.2	0.07	–	–	–	–	–	11.03	1	0.001	−0.25	0.08
	R^2m = 0.47 R^2c = 0.85					R^2m = 0.37 R^2c = 0.86					R^2m = 0.34 R^2c = 0.90				

infant imitation is mixed (*77*), but imitation is consistently evaluated in neonatal studies in NHPs [e.g., (*34*, *78*–*80*)] as a developmental feature. For the sake of completeness of our neonatal battery [despite mixed evidence, see (*81*) re: monkeys], we investigated the influence of fZIKV on neonatal social skills development by assessing imitation of facial behaviors in fZIKV ($N = 10$) and CON ($N = 9$) infants at 3 and 7 days old.

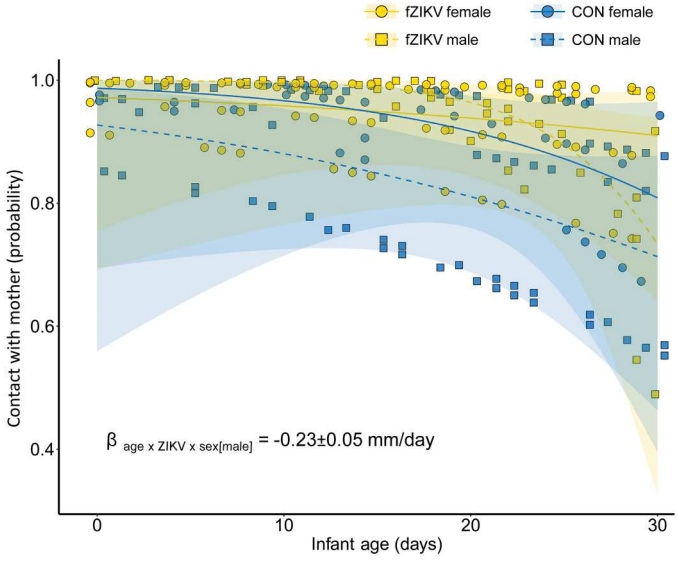

Fig. 5. Infants prenatally exposed to ZIKV spent more time in contact with their mother over the first month of life. The probability of being observed on mother during the first month of life is shown. ZIKV infants ($N = 10$) are plotted in yellow; CON infants ($N = 9$) are plotted in blue. Females are plotted with circles, and males are plotted with squares. Fitted curves are plotted in yellow for ZIKV animals and in blue for CON animals, with solid lines for females and dashed lines for males. Predicted probabilities are extracted from logistic binomial mixed model. Shaded areas represent 95% confidence intervals. Data can be found in data file S4.

The infants did not lipsmack at all during the experiment, so we focused the analyses solely on mouth open and tongue protrusion. Overall, there was no evidence for imitation for either the fZIKV or CON infants. Infants opened their mouth in 17.1% of baseline trials and 15.8% of dynamic stimulus trials. The odds of observing the infant open their mouth were consequently not influenced by demonstration of the behavior by the experimenter ($\chi^2 = 0.02$, $P = 0.89$), and this effect was not modulated by ZIKV exposure ($\chi^2 = 1.06$, $P = 0.30$). Infants pulled their tongue in 59.9% of baseline trials and 63.2% of the trials when this behavior was demonstrated by the experimenter. The odds of observing tongue protrusion were not influenced by demonstration ($\chi^2 = 0.20$, $P = 0.65$), and this effect was not modulated by prenatal exposure to ZIKV ($\chi^2 = 0.51$, $P = 0.47$).

DISCUSSION

Results from our study support the picture of ZIKV infection during pregnancy established by other reports in macaques (*14*, *21*, *40*, *43*, *82*, *83*) and then extend those findings by following infants though the first month of life (see table S7 for a summary of developmental effects). First, we recapitulated previous findings that like nonpregnant animals, pregnant animals infected with ZIKV have an early peak viremia (3 to 5 days postinfection); unlike nonpregnant animals, however, pregnant animals have a prolonged period of detectable ZIKV RNA in plasma and amniotic fluid, often with fluctuating magnitude and considerable individual variability, similar to the variability of viral trajectories seen in humans (*14*, *21*, *23*, *40*, *43*, *44*, *83*–*86*). Available data indicate that the source of the prolonged viremia is most likely spillover from virus replication in the placental-fetal compartment (*14*, *39*, *44*, *85*) given that when the placenta and fetus are removed, maternal viremia resolves essentially immediately (*87*). The variable patterns in ZIKV RNA magnitude observed in the current study may likely have long-term neural and psychobehavioral consequences for the fetuses despite them having been exposed to ZIKV at the same developmental stage (about GD64), because adverse outcomes are more common in neonates

that are born to mothers with prolonged viremia, even when adjusted for trimester of infection (*46*, *88*).

ZIKV infection had minimal clinical impact on features of maternal health, here indexed by maternal weight and hematological outcomes. Although all animals did gain weight across pregnancy, ZIKV mothers gained less weight and gained weight more slowly than CON mothers. Other evidence of infection included variation in hematological outcomes—although the changes were fairly modest and typically within normal ranges; thus, although they were statistically significant, it is not clear whether they were biological and clinically meaningful. ZIKV and CON females started with comparable magnitudes of white and red blood cells, hemoglobin, platelets, and hematocrit, and values decreased across gestation for ZIKV but not CON females. Together, the viral data and our health metrics demonstrate that we successfully infected mothers and that the mothers had minimal clinical symptomatology from infection.

By tracking fetuses across time using ultrasound, we were able to document the impact of ZIKV on their growth. Early in gestation, fetuses in the fZIKV and CON groups developed comparably, as measured by fBPD evaluated using ultrasound. However, the growth of fZIKV infants slowed across gestation such that their heads were smaller at the end of gestation and at postnatal day 3, and infant weight was lower at postnatal day 3. fZIKV infants continued to exhibit slowed head growth (measured by both BPD and occipital frontal diameter) as compared with CON infants through the first month of life. This slowed head growth was predicted by the magnitude of ZIKV RNA in maternal plasma and amniotic fluid; however, its impact was more pronounced in fZIKV males than in fZIKV females. This pattern revealed itself in other growth metrics as well such that the negative impact of infection was also stronger in males than in females in chest and arm circumference and femur growth. Potentially interesting sex differences emerged in infant weight and chest and biceps circumference metrics across the first month, because fZIKV males gained weight more slowly, and their chests and biceps grew more slowly than did CON males, whereas fZIKV females gained weight faster, and their chests and biceps grew faster than did CON females.

The overall negative impact of fZIKV on growth in our study is consistent with some evidence from humans (*8*–*10*) and monkeys (*14*), although many studies do not find growth differences after fZIKV infection at all in normocephalic children (*89*, *90*) or in monkeys (*46*, *47*). Little extant literature detailing sex differences in growth in fZIKV children exists; however, a review of the growth data in a cohort of children from birth up to 2.5 years revealed interesting sex differences in weight gain (*10*). In this cohort, both boys with microcephaly and normocephalic boys with neurological abnormalities gained less weight across the 2.5 years of study as compared with normocephalic boys who did not present with neurological abnormalities. In contrast, only girls with microcephaly evidenced less weight gain than both normocephalic girls with and without neurological abnormalities (*10*). This finding parallels what we observed in our study: fZIKV infection affected males′ weight gain more than females′ weight gain. Our data point to one possible reason for this discrepancy. Many of our growth metrics, including weight gain, were predicted by the magnitude of infection during gestation, and the magnitude of infection in amniotic fluid was higher for male as compared with female fetuses. It is possible that in previous documentations of sex differences in morphometrics (*10*), those differences arose from high viral loads in utero. These findings suggest that reducing fetal infection magnitude and duration might have positive developmental consequences.

Our primary interest for this long-term project is understanding how fZIKV infection influences psychosocial, behavioral, and neural development from infancy to childhood, and here, we evaluate the first month of these infants′ lives. Notably, newborn rhesus macaques spend essentially all of the first month of their life in physical contact (typically ventral-ventral contact) or within arm′s reach of their mothers (*72*, *91*), and mothers typically restrict other social contact of their infants to female kin (*91*). Because the frequency and duration of infants′ interactions with their mothers are highest during infants′ first month of life (*91*), our analyses focused on the time spent on their mothers. Although the time spent on mother decreased over the first month of life for all infants, fZIKV infants were more likely to be observed in contact with their mothers than CON animals. This effect was affected by sex such that the change in time that fZIKV females spent in contact with mother decreased the least across the first month of life. It is possible that some of the sex-specific differences in growth that were noted in our study (e.g., fZIKV females gained more weight across the first month of life than did CON females) were a result of time spent on mother. Staying on mother would result in more limited activity expenditure and possibly greater access to breast milk that might promote growth.

We also carried out a few additional standard evaluations of neonatal infant development that yielded minimal group differences. We isolated infants for a brief period (5 min) in a small incubator and used a standardized ethogram to measure motor coordination and affective behavior. On the basis of the human literature documenting motor developmental delays and disorders in CZS (*5*, *92*–*97*), we hypothesized that we might see variation in motor coordination or activity in our subjects. No such differences were observed. There were also no group differences in affective behavior, contrasting reports from the human literature that infants with CZS are irritable (e.g., exhibiting excessive crying and inconsolability) (*95*). We did carry out the standard subjective sensorimotor and behavior ratings widely used in macaque infant development laboratories as well (*35*, *46*) and found that fZIKV infants had impaired attention and visual orientation, similar to findings presented in (*46*). Unfortunately, our dataset was affected by year 1–to–year 2 cohort effects that were the result of experimental staff changes, and although our models account for this, we are cautious about the interpretation of these results.

Our behavioral evaluations of infant imitation also evidenced no group differences, perhaps because we found no evidence that any of the infants imitated. Although other groups do find evidence of imitation in neonates using this task (*34*, *98*), when data are re-analyzed in ways that ensure that infants are making meaningful responses that differ from chance (called cross-target analyses), these claims do not hold (*81*, *99*). As a result, it is unclear whether rhesus neonates actually imitate in general and whether performance on this task is meaningful. Despite these null findings on this evaluation of social interaction, interactions with mother remain the most important psychosocial feature of this developmental phase, and so the documented group differences are an important feature of the fZIKV syndrome we are documenting here.

Like all studies, ours has a number of limitations. Because of constraints at our center and beyond our control, we conducted the

study over two birth cohort years with odd numbers of groups in each cohort. As a result, we elected to balance the sample with two ZIKV and one CON groups in year 1 and one ZIKV and two CON groups in year 2. This complicated analyses where there was an experimenter effect across year 1 and year 2 (for the neonatal assessment battery) because there were different numbers of subjects per condition each year. Additionally, although a sample size of 19 is a fairly large NHP study, it is, compared with typical epidemiological studies, a small sample. Luckily, we were able to maintain relatively equal group sizes (10 fZIKV and nine CON) and fairly equal sex distributions per group (five fZIKV females, five fZIKV males, four CON females, and five CON males), and use of our timed breeding colony allowed for precise experimental control over infection time point. Although our original sample size was planned to have sufficient statistical power, we did not anticipate sex differences in developmental effects. Power associated with these analyses was limited, which means that, although we are confident in the effects that we reported, it is possible that we did not identify additional weaker effects.

Ultimately, the power of the monkey model is that we can track these infants across their infancy and childhood. We are able to carry out frequent repeated measures to maximize power and paint a robust picture of development after fZIKV infection that includes both behavioral and neurobiological assessments. Whether infection dynamics predict long-term outcomes, sex differences in development persist, and fZIKV affects psychosocial and behavioral development into childhood and the extent to which behavioral development relates to central nervous system insult remain open questions.

MATERIALS AND METHODS

Study design

The aim of this study was to investigate the early developmental consequences of prenatal ZIKV exposure in a rhesus macaque (*M. mulatta*) model. To do that, we inoculated pregnant females and their fetuses with ZIKV during the second trimester of fetal development. Pregnant adult females were randomly assigned to a condition as groups of monkeys were formed, and the sex distribution of their fetuses by condition was random. We evaluated ZIKV RNA concentrations in both maternal plasma and amniotic fluid until birth and compared hematological parameters between inoculated mothers and noninoculated control mothers. The effects of prenatal ZIKV exposure on offspring were explored by comparing ZIKV-exposed ($N = 10$) and control ($N = 9$) infants during the prenatal phase and during the first 30 days after birth. Outcomes included prenatal head growth, postnatal body growth, social behavior with their mothers, imitation, and sensory motor development. When possible (e.g., evaluation of prenatal ultrasounds and scoring of behavior from video), experimenters were blind to condition as described in Supplementary Materials and Methods. All methods were approved by the University of California, Davis Institutional Animal Care and Use Committee under protocol #22389.

Statistical analysis

Data were analyzed using R 4.0.3 (*100*). We compared the weights and the hematological parameters between ZIKV and CON dams collected at a single time point immediately before inoculation using *t* tests. *T* tests were also used to compare the weights of fZIKV and CON infants at birth. The influence of fZIKV infection was explored using mixed models with the lme4 package (*101*). The identity of the individual monkey was used as the random factor, DPI or infant age (for variables measured prenatally or postnatally, respectively) was used as a continuous predictor, and infant sex was used as a categorical predictor. Quadratic terms (DPI^2 or age^2) were also included to check for nonlinear relationship between the dependent variable and time. Final models were selected on the basis of Akaike information criterion (*102*) after model simplification from the full factorial model. In some cases, the final models retained predictors that did not reach conventional significance values ($P < 0.05$). However, because those variables critically contribute to the model fitting, we decided to note when they were retained if their *P* values were <0.1. We clarify in the text which predictors reach conventional significance values and which do not. This approach is consistent with modern analysis approaches that have begun to de-emphasize the importance of *P* values (*103*). For ZIKV-infected animals, the magnitude of ZIKV RNA in plasma and amniotic fluid [indexed as area under the curve of the vRNA (AUC)] was used as an additional predictor for maternal and infant outcomes in similar factorial models. For analytic purposes, viremia values that did not reach the LOD were set to 0, and these values were not plotted in our figures (replaced by LOD value).

All models were built controlling for the year (1 or 2) when the animals were born and for the identity of the experimenter. Models for infant morphometrics were built controlling for the influence of maternal weight. The package emmeans (*104*) was used to calculate and compare estimated marginal means, and *P* values were corrected for multiple comparison using the Tukey method. We report Wald χ^2 from the models and estimates with their SEs. Means, estimated marginal means, and estimates are presented with their SEs. Where linear models were used, we checked for the normal distribution of fitted residuals using the qqmatch function of the lattice package (*105*). Details about model building and selection can be found in Supplementary Materials and Methods. Data files are also available at https://osf.io/dv3xe/.

Supplementary Materials

This PDF file includes:
Materials and Methods
Figs. S1 to S3
Tables S1 to S7
References (*106*, *107*)

Other Supplementary Material for this manuscript includes the following:
Data files S1 to S4
MDAR Reproducibility Checklist

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