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Helicobacter Infection Significantly Alters Pregnancy Success in Laboratory Mice

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***Helicobacter* spp. are gram-negative, helically shaped bacteria that cause gastric and enterohepatic infections in mammalian species. Although *Helicobacter* infection frequently is implicated to interfere with reproductive success, few experimental data support these claims. We therefore retrospectively investigated the effect of *Helicobacter* infection on murine pregnancy outcome after the identification of endemic *Helicobacter* infection in an animal research facility. Multiplex conventional PCR analysis was used to characterize *Helicobacter* infection status in one inbred and 2 transgenic strains of mice in 2 self-contained rooms assigned to the same investigator. Outcomes of timed-mating experiments were compared among *Helicobacter* spp.-infected and uninfected mice of the same strain; *Helicobacter* infection was eradicated from the colony through fostering with uninfected dams. Although *Helicobacter* infection affected fecundity in only one strain of transgenic mouse, the total number of embryos per gravid uterus was significantly reduced in C57BL/6J mice that were infected with a single *Helicobacter* species, *H. typhlonius*. *Helicobacter* infection was also associated with a significant increase in the number of resorbing embryos per uterus and significant decreases in pregnancy-associated weight gain relative to uninfected mice in C57BL6/J mice and one transgenic strain. *Helicobacter* spp.-infected mice of all tested strains exhibited higher frequency of intrauterine hemorrhaging relative to uninfected mice. These results indicate that naturally-acquired *Helicobacter* infection not only reduces the productivity of a research animal breeding colony, but also negatively impacts embryo health. Despite these deleterious effects, these data suggest that colonies can be rederived to be *Helicobacter*-free by Cesarean section and fostering with uninfected dams. This paper provides the first evidence that *H. typhlonius* infection is sufficient to interfere with reproductive success and embryo health of C57BL/6J mice. Animal research facilities should therefore implement *Helicobacter* spp. surveillance and control practices to avoid confounding experimental results and to improve breeding colony efficiency.**

Abbreviation: GD, gestational day

Helicobacter spp. are gram-negative, helical bacteria that cause gastric and enterohepatic infections in mammalian species. Naturally acquired *Helicobacter* infections have been reported in all rodent species commonly used for biomedical research.[4,6,10,17,21,22,42,43,53]

Of the many *Helicobacter* spp., at least 9 are known to infect mice: *H. bilis*, *H. hepaticus*, *H. typhlonius*, *H. muridarum*, *H. rodentium*, *H. mastomyrinus*, *H. rappini*, *H. pullorum*, and *H. ganmani*.[1,10,21,22,32,57] Although *Helicobacter* infections are typically subclinical in immunocompetent mice, these bacteria can be pathogenic and immunogenic in multiple inbred, transgenic, or immunodeficient mouse strains. Notably, *Helicobacter* spp. induce liver,[48,56] colon,[30,36,38] and prostate[41] carcinomas; typhlitis and typhocolitis;[15,27,30,33,36,38,58] hepatitis;[16,36,37,51,56] proctitis;[15] and rectal prolapse[15,33] in various mouse strains used as animal models for biomedical research. Furthermore, *Helicobacter* infection induces a proinflammatory, Th1-biased immune response in infected mice that could confound the results of assays measuring an immune response to or assessing the phenotype resulting from an infectious or inflammatory disease.[1,3,11,29,34,35,44,45,54,58]

A 2007 study investigating the incidence and prevalence of *Helicobacter* infections in animal research facilities found that 84% of mice shipped from academic institutions worldwide tested positive for *Helicobacter* spp. by PCR analysis.[53] Furthermore, 64% of infected mice tested positive for *H. hepaticus*, which (with *H. typhlonius*) is among the *Helicobacter* spp. most commonly reported to be pathogenic in mice.[53]

Although *Helicobacter* infection is frequently alleged to interfere with reproductive success, few experimental data are available that support these claims. Intentional infection with *H. typhlonius*, *H. rodentium*, or both reduced the reproductive performance of mice deficient in IL10, a cytokine that normally limits proinflammatory responses, such as those induced by *Helicobacter* infection. This effect was mitigated by antibiotic treatment.[49] Another report showed that experimental infection of pregnant mice with *H. felis* results in maternal iron deficiency, although the number of pups born did not differ significantly between infected compared with uninfected mice.[18] *H. typhlonius* was found in the reproductive organs of naturally infected C57BL/6J, C3H/HeJ, and athymic *nu/nu* immunodeficient mice,[46] but the effect of this microbe on organ function was not assessed. Last, experimental infection with *H. pylori*, a species known to colonize the human upper gastrointestinal tract, reportedly increased the number of fetal resorptions and decreased average fetal weights compared with uninfected control mice.[45]

The 4 studies[18,45,46,49] just mentioned comprise the full extent of the available published literature that addresses how *Helicobacter* infection affects murine pregnancy. Importantly, 2 of these 4 studies were performed by using *H. pylori* and *H. felis*, which are not known to naturally infect mice and therefore may not be relevant from the perspective of colony maintenance. To fill this broad gap in knowledge, we retrospectively investigated the effect of naturally acquired *Helicobacter* infection, identified

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through routine sentinel testing, on pregnancy outcome in experimental mice derived from our breeding colony.

Materials and Methods

Mice. C57BL/6J mice were originally purchased from The Jackson Laboratory and used to generate breeding stock and experimental animals in the University of Georgia Coverdell Vivarium. At the time of this study, the C57BL/6J breeders had been housed in our facility for 6 mo and were second-generation. Transgenic mice reported here include mice with floxed Tissue Factor (TF, *F3*), the initiator of the extrinsic pathway of coagulation, expressing Cre-recombinase under the control of the *Tek* (Tie2) or *Lyz2* (LysM) promoter.

Founder $F3^{flox/flox}$LysMCre breeder pairs were generously donated by Dr Nigel Mackman (University of North Carolina, Chapel Hill, NC) and were maintained by mating hemizygous $F3^{flox/flox}$LysMCre^{+} (LysMCre^{+}) sires with $F3^{flox/flox}$LysMCre^{-} (LysMCre^{-}) dams. $F3^{flox/flox}$Tie2Cre mice were initially generated by mating unfloxed hemizygous Tie2Cre^{+} sires from the Jackson Laboratory with $F3^{flox/flox}$, LysMCre^{-} dams bred inhouse from the line established by using the founders just described. Once a $F3^{flox/flox}$Tie2Cre line was established, it was maintained by breeding hemizygous $F3^{flox/flox}$Tie2Cre^{+} (Tie2Cre^{+}) studs with $F3^{flox/flox}$Tie2Cre^{-} (Tie2Cre^{-}) dams.

Tie2Cre^{+} mice have tissue-specific deletions of TF in vascular endothelial cells and likely Tie2Cre^{+} fetal-derived trophoblasts, given the reported expression of Tek kinase in trophoblasts.[13] Phenotypically normal Tie2Cre^{-} littermates were used as controls in experiments that included Tie2Cre^{+} mice without *Helicobacter* infection. *Helicobacter*-infected Tie2Cre^{-} mice were not tested because endemic *Helicobacter* infection was identified in the colony, and all experiments were discontinued prior to the initiation of experiments that included these mice. LysMCre^{+} mice have a specific deletion of TF in myeloid cells; phenotypically normal LysMCre^{-} littermates were used as controls in these experiments.

In the absence of *Helicobacter* infection, transgenic strains tested in these experiments were viable, fertile, normal in size and did not display any gross physical or behavioral abnormalities compared with C57BL/6J mice.

All experimental mice were virgin females (age, 8 to 10 wk). Stud males derived from the same breeders as the experimental virgin females were used until approximately 6 mo of age, when they were replaced with younger male mice of the same lineage and breeding stock. Because mice were initially intended for experiments that studied the effect of malaria infection on midgestational pregnancy outcome and that required an endpoint at gestational day (GD) 12, we assessed fecundity (calculated as no. of pregnancies successfully initiated / no. of observed plugs × 100%) and embryo outcomes on this day. These studies of gestational malaria were discontinued temporarily when *Helicobacter* was identified.

Mouse experiments were performed in accordance with the guidelines and with the approval of the University of Georgia IACUC (Animal Use Protocol no. A2015 03-005-Y1-A1).

Husbandry. Mice were fed an irradiated diet and kept under SPF conditions. Routine facility-wide sentinel testing was performed monthly to monitor the presence of common mouse pathogens. Fecal flotation was performed, as well as PCR analysis of fecal pellet DNA to detect mouse parvovirus, minute virus of mice, mouse hepatitis virus, *Clostridium piliforme*, mouse encephalomyelitis virus (strain GDVII), epizootic diarrhea of infant mice virus, and Sendai virus. Mice reported here were maintained in 2 rooms used solely by a single research team in a larger facility that served 20 investigators in 15 total rooms. At the time of this study, sentinels from 7 other rooms in the vivarium tested positive for *Helicobacter* infection by PCR analysis. Strict protocols to restrict traffic between rooms within the facility and maintain clean cages and bedding were implemented to prevent further spread of infection within the facility.

***Helicobacter* detection and species identification.** Initial observation of *Helicobacter* occurred during standard sentinel testing, which identified *Helicobacter* infection at the genus level. Species-specific *Helicobacter* identification was performed in our laboratory; fresh fecal pellets were collected from each breeder cage in the colony and pooled by strain. DNA was extracted from pooled samples (DNeasy Kit, Qiagen, Germantown, MD) according to the manufacturer's instructions. DNA was screened by multiplex conventional PCR analysis using published primer sequences[14] for the presence of DNA from 5 species of *Helicobacter* — *H. bilis*, *H. typhlonius*, *H. hepaticus*, *H. muridarum*, and *H. rodentium*. These species were selected because they reportedly are commonly found in mouse colonies and have been associated with pathogenesis in other mouse models.

Initiation of pregnancy and clinical assessment. Timed pregnancy experiments and monitoring of experimental mice was performed according to a previously established protocol.[40] Briefly, the day on which time-mated 8- to 10-wk-old female mice had a vaginal plug was defined as gestational day 0 (GD0). Mice were left undisturbed peri-implantation and then were monitored daily from GD6 to GD12. Each mouse was weighed, and Hct was used as a measure of anemia. Blood collected from the tail vein into a heparinized capillary tube was centrifuged in a microhematocrit centrifuge and Hct (%) was calculated as:

$$(\text{Volume of packed RBC}) / (\text{total blood volume}) \times 100\%.$$

On GD12, mice of all strains were anesthetized by using 2.5% 2,2,2-tribromoethanol administered intraperitoneally and euthanized by exsanguination through the caudal vena cava. Embryo viability was assessed at necropsy as previously described.[2] Briefly, embryos exhibiting extensive intrauterine or intraembryonic hemorrhaging by gross pathology or lacking fetal heartbeat were scored as nonviable. Nongravid uteri were assessed for gross observation of resorption scars to indicate pregnancy loss during early gestation.

Fostering. *Helicobacter*-free Swiss Webster outbred mice (Charles River Laboratories) were used as foster mothers. A timed-mating scheme was implemented to ensure the presence of lactating foster female mice on the optimal day for caesarean section. Swiss Webster foster dams were paired with stud males 2 to 3 d before pairing the colony mice to undergo caesarean section. Successful mating of mice was indicated by the presence of a vaginal plug (GD0). At GD19, colony mice were anesthetized by using 2.5% 2,2,2-tribromoethanol. Under a laminar flow hood, the abdomen was opened, and 70% ethanol was liberally applied to the peritoneum. The peritoneum was then opened, and the gravid uterus was excised with sterile scissors and forceps. The uterus was placed in resealable zipper storage bags, which were sprayed twice with chlorhexidine and immediately moved to a clean facility. The uterus was opened longitudinally and pups were removed. Sterile cotton-tipped applicators were used to remove amniotic membrane and to clear mucus from the neonates' noses and mouths. Each neonate was gently massaged until it was moving independently and breathing. The umbilicus was removed, and neonates were placed on sterile paper towels laid over a hot-water bottle containing 37 °C water. Pups were covered to avoid loss of body heat and were

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massaged gently until sustained breathing without stimulation was achieved (approximately 10 to 15 min). Pups were rubbed with fresh fecal pellets from the foster mother's cage to give them a scent that the foster mother would accept.

The foster mother was then transferred to a clean holding cage and her pups removed. Pups to be fostered were transferred to the foster mother's original cage, and intimate contact between pups and bedding was established to further coat the pups with the appropriate scent. In our experience, keeping the numbers of pups in the endogenous and foster litters identical is essential; otherwise, the foster female rejects the new pups. When necessary, endogenous pups were added back to the foster litter to achieve the correct litter size. The foster mother was then returned to her original cage and monitored for approximately 15 min to ensure that she accepted the foster pups.

Statistical analysis. All statistical analyses were performed by using Prism (version 6.0, GraphPad Software, San Diego, CA). Correlation analysis according to the Spearman test was performed, and 2×2 contingency tables with corrections made for multiple comparisons were used for testing differences between proportions. Differences in pregnancy-associated weight gain and Hct were tested by AUC analysis.[26] Differences with *P* value of 0.05 or less were considered significant.

Case Report

Detection and characterization of *Helicobacter* infection. During an ongoing study of the effect of malaria infection on midgestational pregnancy outcome, *Helicobacter* infection was detected during monthly sentinel testing of our colony. This discovery prompted a cage by cage assessment for these bacteria using an inhouse multiplex conventional PCR analysis (Table 1). One inbred and 13 transgenic breeder lines housed in 2 separate rooms assigned to our research team tested positive for *Helicobacter* infection, although pregnancy outcome was assessed in the progeny from only 3 of those breeder lines (Table 1). DNA extracted from fecal pellets revealed that all 5 mouse strains derived from those 3 breeder lines assessed for pregnancy outcomes (Table 1) were infected with *H. typhlonius.* Except for C57BL/6J, all tested strains were infected with *H. rodentium*, whereas only mTF$^{flox/flox}$LysMCre^{+} (LysMCre^{+}), mTF$^{flox/flox}$LysMCre— (LysMCre—), and mTF$^{flox/flox}$Tie2Cre^{+} (Tie2Cre^{+}) mice were infected with *H. hepaticus* (Table 1). None of the strains tested were infected with either *H. muridarum* or *H. bilis*.

Effect of *Helicobacter* infection on fecundity in transgenic mice. To obtain a *Helicobacter*-free colony, pups from infected mothers were fostered with lactating, uninfected dams and kept in a separate facility until no infected mice remained. *Helicobacter* surveillance of breeder pairs was performed inhouse on a monthly basis to ensure our colony remained *Helicobacter*-free.

Strain fecundity rates were assessed for each strain of mouse shown in Table 1 in the presence and absence of *Helicobacter* infection (Table 2). Fecundity did not differ significantly between *Helicobacter*-infected C57BL/6, LysMCre^{+}, or LysMCre^{-} mice and their uninfected counterparts. However, the fecundity of *Helicobacter*-infected Tie2Cre^{+} mice was significantly ($P = 0.002$) affected and was 90% lower than that observed for uninfected mice of the same strain (Table 2). In addition, the fecundity of *Helicobacter*-infected Tie2Cre^{+} mice was lower ($P = 0.0003$) than that of *Helicobacter*-infected C57BL/6J mice; no *Helicobacter*-infected mice of this strain yielded gravid uteri despite the observation of a vaginal plug, indicating that mating had occurred. No significant difference was seen in the fecundity rates of the other strains compared with C57BL/6J. Pregnancy outcome data for TF-intact, *Helicobacter*-infected mTF$^{flox/flox}$Tie2Cre^{-} (Tie2Cre^{-}) littermates of Tie2Cre^{+} mice are not shown, because experiments were halted before this strain was included. However, this strain's fecundity rate in the absence of *Helicobacter* spp. is presented in Table 2 for comparison.

Effect of *Helicobacter* infection on weight gain in pregnant mice. C57BL/6J mice infected with *H. typhlonius* exhibited significantly ($P < 0.05$) reduced weight gain during GD6 through GD12, resulting in a final average weight gain that was 23.4% lower in *H. typhlonius*-infected C57BL/6J mice compared with uninfected mice of the same strain (Figure 1). Likewise, according to AUC analysis, LysMCre^{+} mice were affected by coinfection with *H. typhlonius*, *H. hepaticus*, and *H. rodentium*, exhibiting significantly reduced pregnancy-associated weight gain compared with that of *Helicobacter*-free mice of the same strain. Weights of pregnant LysMCre^{-} mice were unaffected by *Helicobacter* infection. Maternal Hct remained unchanged by infection status.

Effect of *Helicobacter* infection on midgestational embryo health. Postmortem gross pathologic assessment of uteri from *Helicobacter*-infected mice revealed increased intrauterine hemorrhage and embryo resorption relative to that in uninfected mice (Figure 2). In addition, uteri from *Helicobacter*-infected LysMCre^{+} mice were discolored compared with healthy uteri of the same strain, exhibiting a noteworthy gray-brown color on the embryo side of each horn.

At GD12, numbers of viable and resorbing embryos per uterus were counted and the presence or absence of intrauterine bleeding was assessed in *Helicobacter*-infected and -uninfected mice. *Helicobacter*-infected C57BL/6J mice had significantly smaller litter sizes (1.3 fold, $P = 0.0002$) than did uninfected mice of the same strain (Table 3). However, *Helicobacter* infection did not significantly influence the litter size of LysMCre^{+} mice. C57BL/6J ($P = 0.0108$) and LysMCre^{+} ($P = 0.0006$) mice infected with *Helicobacter* spp. had significantly more resorptions per uterus than did their uninfected counterparts (Table 3). *Helicobacter* infection had no significant effect on embryo viability or litter size in LysMCre^{-} mice. *Helicobacter* infection induced a significant increase in the incidence of intrauterine bleeding in all 3 strains (Table 3).

Pregnancy outcome data (Table 3) are shown for *Helicobacter* spp.-uninfected Tie2Cre^{+} mice. However, because no embryos were observed in the uteri of confirmed-mated, infected mice of this strain (Table 2), gross pathologic effects of *Helicobacter* infection on pregnancy outcome could not be assessed.

Discussion

Here we show that *Helicobacter* infection has negative effects on pregnancy, not only in immunodeficient and transgenic mice[18,44,45,48] but also in C57BL/6J mice. C57BL/6 mice are often referred to as being *Helicobacter*-resistant,[1,46] given the lack of obvious clinical signs of disease in *Helicobacter*-infected, nonpregnant C57BL/6 mice. However, the results we presented show that, although *Helicobacter*-infected C57BL/6J mice are able to carry pregnancies to term, endemic *H. typhlonius* infection in a mouse colony reduces reproductive success.

Despite their infection with fewer *Helicobacter* species, C57BL/6J mice exhibited more negative effects due to *Helicobacter* infection than did either TF-altered mouse strain, including significantly reduced pregnancy-associated weight gain, fewer embryos per gravid uterus, a higher proportion of resorbing embryos, and a higher incidence of intrauterine hemorrhage. No significant difference was seen in fecundity rate, litter size, gravid uterus weight, or incidence of uteroplacental hemorrhage

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Table 1. *Helicobacter* spp. in C57BL/6J, LysMCre, and Tie2Cre breeder lines

	H. typhlonius	*H. hepaticus*	*H. muridarum*	*H. bilis*	*H. rodentium*
C57BL6/J ($n = 28$)	+	–	–	–	–
mTF$^{flox/flox}$LysMCre ($n = 34$)	+	+	–	–	+
mTF$^{flox/flox}$Tie2Cre ($n = 23$)	+	+	–	–	+

Fecal pellets were collected from each strain and assessed for the presence of *Helicobacter* spp. DNA by species-specific multiplex PCR analysis.

between these strains in the absence of *Helicobacter* infection, indicating that the observed heterogeneity is not due to inherent differences in fecundity but rather to differential responses to *Helicobacter* infection among the strains considered. Insufficient data were collected during this retrospective study to definitively determine why C57BL/6J mice fared worse in these experiments than did their transgenic counterparts, despite their infection with only a single species, *H. typhlonius*, though 2 possible explanations exist that could explain these results.

The first possible explanation could lie in genetic differences existing among these mouse strains that are responsible for the production of different host responses to *Helicobacter* infection. Although all of these lines are on or derived from a C57BL/6 background, they originate from different substrains. C57BL/6J mice were procured from the Jackson Laboratory, founder LysMCre breeder pairs were obtained from an outside academic institution, and Tie2Cre mice were initially generated by mating unfloxed Tie2Cre+ breeders from the Jackson Laboratory with LysMCre⁻ breeders. Reports indicate C57BL/6 substrains acquired from different commercial sources and university animal facilities have genetic differences[25,31] and variations in gut microbiome composition[23,28,55] that can have a significant effect on these substrains' immune responses to infection.[8,24] All of these possibilities—genetic differences, immunologic differences, and microbiome-mediated modulation of disease severity—could explain our observations. It is noteworthy that both strains of mice in which at least one member of the original founder breeder pair was obtained from the Jackson Laboratory exhibited similar phenotypes, whereas the outlier strain was obtained from an entirely different source. However, no definitive conclusions can be drawn from the available data.

Alternatively, because mice with altered TF expression exhibit differential host responses to disease compared with TF-intact mice,[7,12,39,47,52] this relatively worse outcome could be a by-product of intact TF activity inducing a more robust inflammatory response in C57BL/6J mice than in the TF-altered mouse strains. As the initiator of the extrinsic pathway of coagulation, TF plays an important role in inducing thrombin-mediated proinflammatory responses; reduced production of proinflammatory cytokines due to ablation or impairment of TF activity has been seen in animal models of endotoxemia,[7,12,47,52] sickle cell disease[9] and arthritis.[59] While the effects of TF deletion on the immune response to infection in pregnant LysMCre+ and Tie2Cre+ mice have yet to be reported, procoagulant activity and the extent of TF deletion in the *F3*$^{flox/flox}$, LysMCre+ and *F3*$^{flox/flox}$, Tie2Cre+ strains used in these studies have been assessed in detail in a mouse model of endotoxemia. In this model, LPS-induced procoagulant activity of peritoneal macrophages isolated from LysMCre+ mice and plasma Thrombin-Anti-Thrombin (TAT) complex levels, a marker of activated coagulation, were significantly reduced compared with LysMCre- littermates.[39] Using this same model, it was found that LPS-induced TF expression and activity are significantly reduced in both endothelial and hematopoietic cells isolated from Tie2Cre+ mice.[39] Thus, alterations in TF expression would likely affect immune signaling following infection.

Table 2. Strain-specific fecundity rates in the presence and absence of *Helicobacter* infection

	No. gravid uteri/no. observed plugs		
	Helicobacter-positive	*Helicobacter*-negative	*P*
C57BL6/J	20/22 (90%)	5/6 (83%)	0.5
LysMCre⁻	9/12 (75%)	4/5 (80%)	1.00
LysMCre⁺	7/11 (64%)	5/6 (83%)	0.60
Tie2Cre⁻	not tested	7/8 (88%)	—
Tie2Cre⁺	0/5 (0%)[a]	9/10 (90%)	0.002

Mice were euthanized and evaluated on gestational day 12. Fecundity rates among *Helicobacter*-uninfected mice were comparable ($P > 0.05$).
[a]$P = 0.002$ compared with uninfected Tie2Cre+ mice; $P = 0.0003$ compared with *Helicobacter* spp.-infected C57BL/6J

Two notable effects of *Helicobacter* infection on pregnancy outcome observed in the TF-altered strains in the presence of *Helicobacter* infection were the significantly reduced fecundity rate and significantly greater proportion of resorbing embryos observed in *Helicobacter* spp.-infected Tie2Cre+ and LysMCre+ mice, respectively, compared with C57BL/6J mice. As the fecundity rate was drastically reduced in the Tie2Cre+ mice in the presence of infection, these data implicate endothelial or trophoblast-derived TF as playing a significant role in protecting against *Helicobacter* during the earliest stages of pregnancy. In addition, the fact that fecundity rate was unchanged but embryo health was significantly reduced in *Helicobacter* spp.-infected LysMCre+ mice indicates that the different outcomes observed among *Helicobacter*-infected strains are possibly due to different host responses to infection, and that myeloid cell-derived TF may play a role in preventing *Helicobacter*-induced pathology during midgestation. However, since more detailed analyses could not be performed on these mice, precise determination of the mechanisms underlying these processes cannot be made.

This study is a retrospective analysis of experiments that were discontinued once *Helicobacter* infection was detected. Although our data clearly establish *H. typhlonius* as a pathogen that affects reproduction and embryo survival in infected wildtype mice, additional studies are required to establish the specific effects of other *Helicobacter* species on reproduction and embryo health in the absence of *H. typhlonius*, given that we did not assess either the bacterial burden in these mice or the effect of single-species infection. To avoid further contamination of our facility, tissue samples generated from infected mice were discarded before they could be analyzed; as such, retroactive histologic assessment and culture of uteri lumen to positively identify the presence of *Helicobacter* spp. in the reproductive tract were not performed. Therefore, other common mouse pathogens that could have been eliminated through fostering might have been responsible for the phenotypes observed in these experiments; however, this scenario is unlikely. Records of sentinel testing from 6 mo before, during, and 6 mo after these dates indicate that the following parasites were absent from our colony during this time period: mouse parvovirus, minute virus of mice, mouse hepatitis virus, *Clostridium piliforme*, mouse encephalomyelitis

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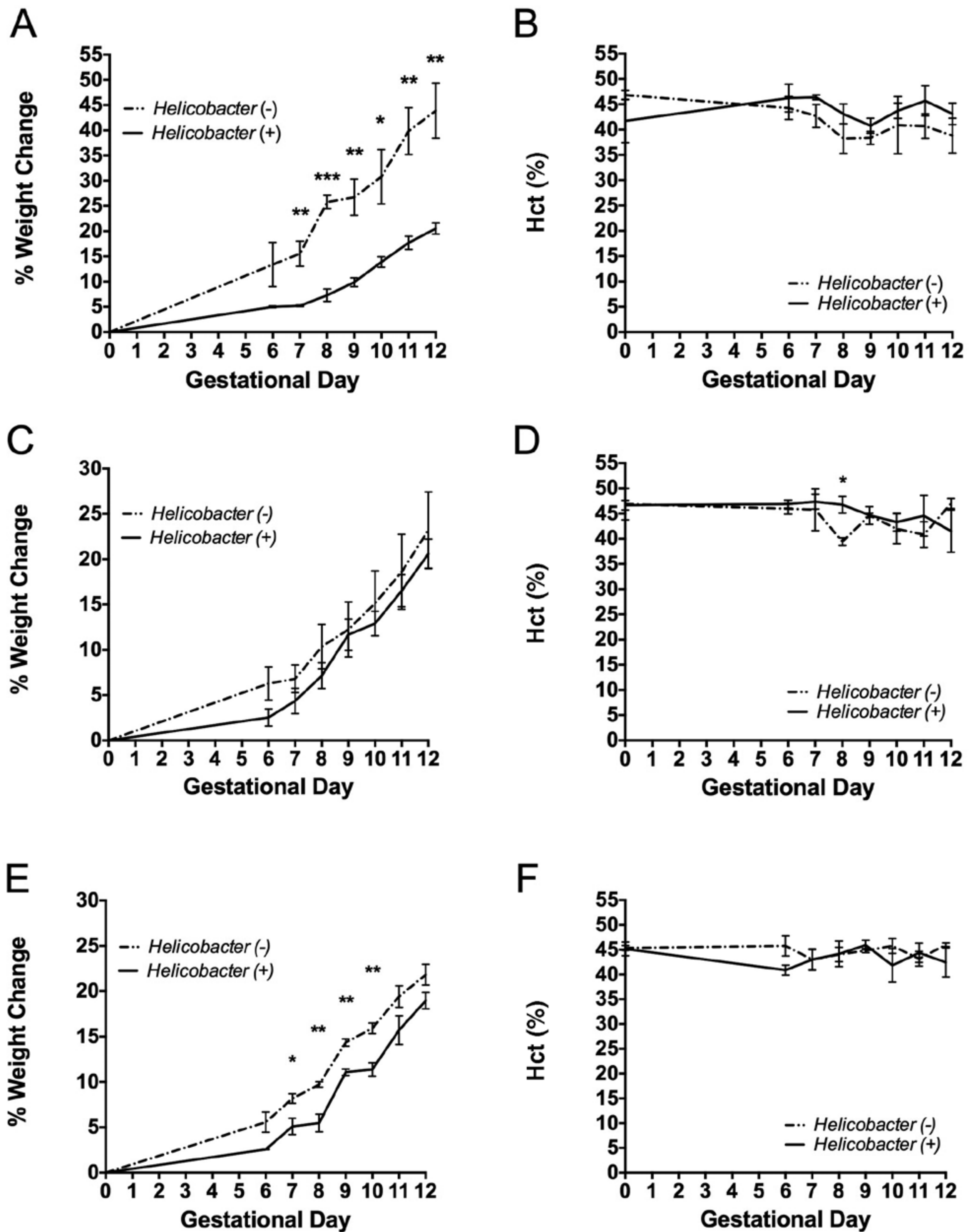

Figure 1. *Helicobacter* infection significantly affects pregnancy-associated weight gain (%, relative to baseline) but does not induce maternal anemia. Pregnant mice were weighed on GD0 and GD6 through GD12. *Helicobacter* infection significantly reduced weight gain in (A) C57BL/6 and (C) LysMCre$^+$ but not (B) LysMCre$^-$ mice. Hct (%) was largely unaffected by the presence or absence of *Helicobacter* infection in these strains. Data are presented as mean ± SEM (n = 4 mice per group). *, P < 0.05; †, P < 0.01; ‡, P < 0.001.

virus (strain GDVII), epizootic diarrhea of infant mice, Sendai virus, and any parasites whose eggs or cysts are detectable by fecal flotation. These results rule out those specific common mouse pathogens as possible causes for the observed phenotypes. Routine testing for murine norovirus was not performed; we cannot rule out that this pathogen was responsible, although limited research suggests that orally acquired virus does not infect the uterus.[20]

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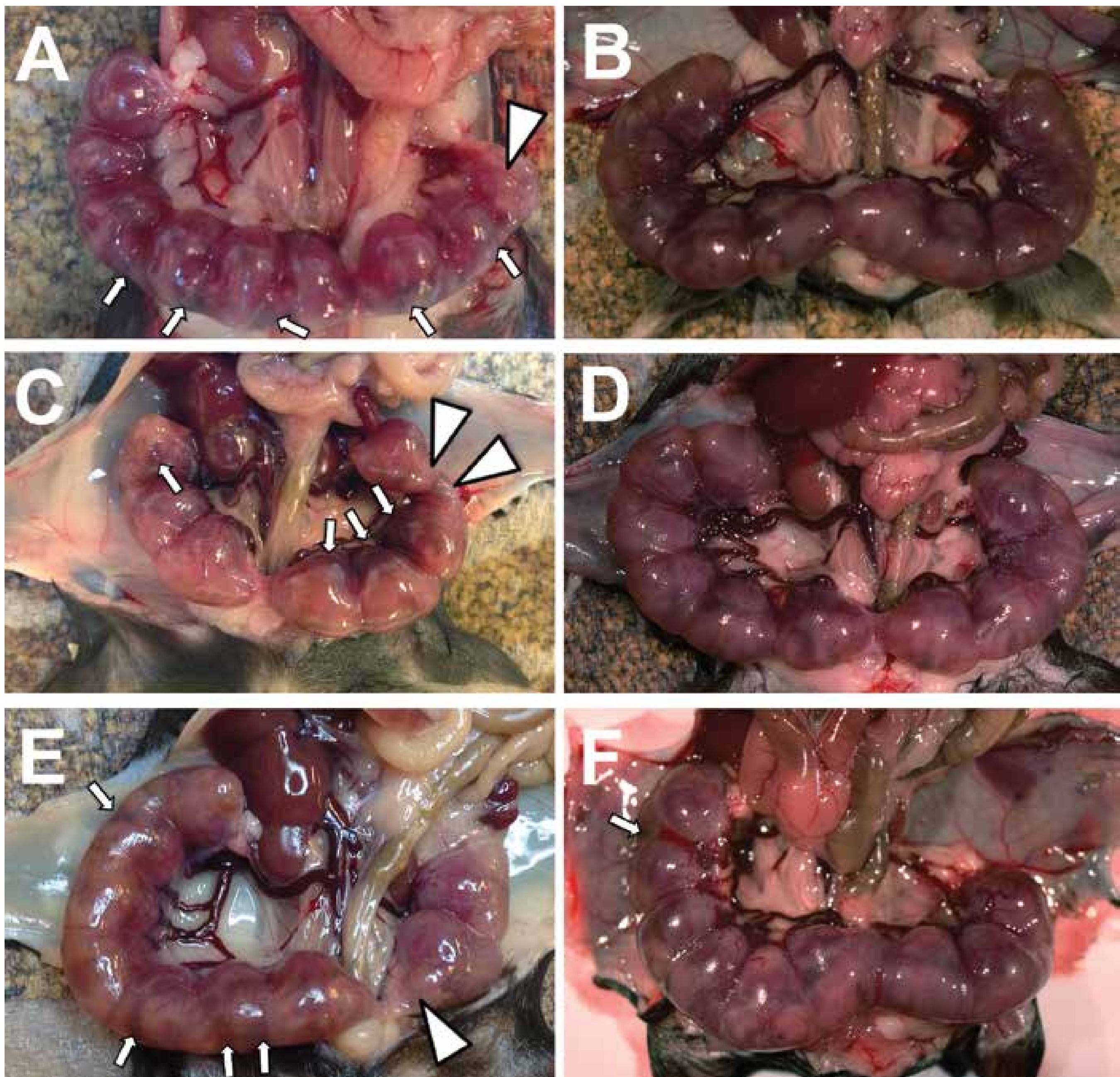

Figure 2. *Helicobacter* spp. negatively affects midgestational embryo health. Gross pathologic view of (A and B) C57BL/6J, (C and D) LysMCre⁻, and (E and F) LysMCre⁺ uteri of (A, C, and E) *Helicobacter*-infected and (B, D, and F) uninfected mice. Macroscopic evaluation of uteri revealed a significant increase in intrauterine hemorrhage (small white arrows) in *Helicobacter* spp.-infected C57BL/6J, LysMCre⁻, and LysMCre⁺ mice relative to uninfected mice of the same strain (quantified in Table 3). *Helicobacter*-infected C57BL/6J and LysMCre⁺ mice exhibited significantly greater numbers of resorbing embryos (white arrowheads) than did uninfected mice of these strains (Table 3).

The C57BL/6J mice we evaluated here originally were imported *Helicobacter*-free from the Jackson Laboratories and then were housed in our facility for more than 2 generations (6 mo) before use. In addition, mice from other sources, including investigators at other institutions, were maintained in our colony (that is, in the other 18 rooms of the facility) during this time frame. Due to the retrospective nature of the present observations, it is not possible to discern the source or time course of the observed *Helicobacter* infections.

A decrease in breeding colony efficiency due to the *Helicobacter*-induced reductions in pregnancy success demonstrated in the current study could lead to costly increases in the time and labor required to generate sufficient numbers of experimental mice. However, the effects of these results also might extend beyond a simple increase in lost time and funds. Evidence in the literature indicates that *Helicobacter* infection has the potential to confound the results of various types of experiments, particularly those investigating inflammatory diseases and the gastrointestinal system. However, when *Helicobacter* infection slows, stunts, or otherwise interferes with embryo development, the resulting reduction in embryo health might have lasting consequences for the embryos as they grow to mature mice used in an experimental setting. In both mice and humans, evidence suggests that many major chronic diseases, including coronary heart disease, hypertension, and type II diabetes, originate in impaired intrauterine growth and development, a phenomenon

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Table 3. Effect of *Helicobacter* infection on implantation frequency and embryo viability in pregnant mice

	Mean no. of embryos / uterus (range)			Total no. of resorptions / total no. of embryos			No. of uteri with intrauterine hemorrhage / no. examined		
	Helicobacter-positive	*Helicobacter*-negative	*P*	*Helicobacter*-positive	*Helicobacter*-negative	*P*	*Helicobacter*-positive	*Helicobacter*-negative	*P*
C57BL6/J	9 (6–10)	11 (10–12)	0.0002	14/102 (14%)	0/44 (0%)	0.01	11/12 (92%)	0/4 (0%)	0.003
LysMCre⁻	8 (6–10)	9 (9–10)	0.2	22/96 (23%)	2/28 (7%)	0.1	12/12 (100%)	0/3 (0%)	0.002
LysMCre⁺	8 (7–11)	9 (8–11)	0.4	34/93[a] (37%)	1/45 (4%)	0.0001	11/11 (100%)	1/5 (20%)	0.003
Tie2Cre⁺	—	8 (6–11)	—	—	3/52 (6%)	—	—	2/6 (33%)	—

Data were collected on gestational day 12; no *Helicobacter* spp.-infected Tie2Cre+ mice successfully implanted.
C57BL6/J *Helicobacter* spp.-infected, $n = 12$; *Helicobacter* spp.-uninfected, $n = 4$.
LysMCre⁻ *Helicobacter* spp.-infected, $n = 12$; *Helicobacter* spp.-uninfected, $n = 3$.
LysMCre⁺ *Helicobacter* spp.-infected, $n = 11$; *Helicobacter* spp.-uninfected, $n = 5$.
Tie2Cre⁺ *Helicobacter* spp.-infected, N/A; *Helicobacter* spp.-uninfected, $n = 6$.
[a]$P < 0.0002$ compared with *Helicobacter* spp.-infected C57BL/6J.

commonly referred to as 'programming' or the 'fetal origins hypothesis'.[5,19,50] Thus, the negative effects of *Helicobacter* infection in utero may also have long-term repercussions that could affect future experiments using those mice.

Here we provide the first evidence that *H. typhlonius* infection is sufficient to interfere with the reproductive success and embryo health of C57BL/6J mice. Animal research facilities should therefore implement *Helicobacter* surveillance and control practices to avoid confounding experimental results and to improve breeding colony efficiency. Should *Helicobacter* infection be discovered, fostering and caesarean delivery of pups is a viable option to clear the infection.

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