Evaluation of Helicobacter hepaticus bacterial shedding in fostered and strategically housed C57BL/6 mice

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EVALUATION OF *HELICOBACTER HEPATICUS* BACTERIAL SHEDDING IN FOSTERED AND STRATEGICALLY HOUSED C57BL/6 MICE

A Thesis

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Master of Science

in

The Interdepartmental Program in Veterinary Medical Sciences through the Department of Pathobiological Sciences

by

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May 2005
DEDICATION

For Chris
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ABSTRACT

The murine pathogen *Helicobacter hepaticus* has important confounding effects on research. Neonatal fostering has been studied in our laboratory for elimination of infection in mice. The purpose of our study was to examine fostering of pups from experimentally infected dams in male-absent parturition, and to determine the significance of gender and time on quantity of bacterial colonization in the cecum and feces of C57BL/6 mice. Approximately 20 C57BL/6 mice were fostered per day from one to four days of age. None of the C57BL/6 pups tested positive by PCR in fecal or cecal samples through four days of age. This data showed that removal of the male C57BL/6 mouse prior to parturition is crucial for extending the fostering period to obtain *Helicobacter*-free mice. In a second experiment, *H. hepaticus* infected mice were housed under varying arrangements to determine the effects of gender and housing on fecal and cecal colonization. Neither time or housing group affected bacterial fecal shedding. However, there was a significant overall effect of gender and a significant difference between male and female mice in both fecal and cecal bacterial copy number. Male fecal and cecal samples contained more copies of *H. hepaticus* than did female samples. Additionally, significant correlations between fecal and cecal *H. hepaticus* values were found both overall and by gender. Novel predictive algorithms were formulated to predict cecal bacterial colonization levels in fecal pellets. These findings should prove useful in *Helicobacter* elimination efforts, and in future work to further elucidate the role of *H. hepaticus* in transmission and disease.
INTRODUCTION

Significance

The role of Helicobacter as a cause of human gastritis came to the forefront of medicine in the early 1980's. One of the first Helicobacter organisms described was Helicobacter pylori in 1982 (Marshall et al. 1983). Helicobacter pylori is now recognized as the primary Helicobacter species responsible for gastroduodenal disease, ulcers, and gastric neoplasia in humans. It is a medical problem that crosses socioeconomic and geographical boundaries. Risk factors for humans are thought to include cultural characteristics such as sharing of food utensils, crowded living conditions, age, poverty, and fecal H. pylori-contaminated foodstuffs (Rodrigues et al. 2004). It has been estimated that approximately half of the world’s population may be infected (Fox et al. 2000). Prevalence in developing countries may be as great as 80% in middle-aged adults (Suerbaum et al. 2002).

Since the discovery of H. pylori, several other naturally occurring Helicobacter species have been isolated and characterized. One of these, H. hepaticus, is an enterohepatic Helicobacter first discovered in the 1990’s in association with chronic active hepatitis, hepatic adenomas, and carcinomas in A/JCr mice (Ward et al. 1994b). Helicobacter hepaticus affects mice both naturally and experimentally. Several of its disease characteristics closely resemble that of H. pylori in humans. Accordingly, it has become a commonly used animal model to examine the role of chronic inflammation and neoplasia. In both wild and laboratory rodents used for scientific research, the prevalence of H. hepaticus infection is thought to be widespread both in the United States (Shames et al. 1995), and in other parts of the world (Goto et al. 2004; Taylor et al. 2004). Infection presents a special problem in laboratory rodents since significant alterations in physiology can result, confounding the interpretation of experimental results. Modalities for the treatment, control, and eradication of Helicobacter remain a topic of current interest today due to the implications of the disease for both humans and rodents. For these reasons, study of the murine model of Helicobacter is intended to promote the understanding of the host-pathogen relationship, the role of the immune system in disease susceptibility, and the efficacy of treatment options. This study aims to further contribute to the body of scientific literature through exploration of H. hepaticus bacterial colonization and shedding, as well as the continued use of neonatal fostering as a strategy for eliminating the pathogen from mouse colonies.
Bacterial Characteristics

*Helicobacter hepaticus* is a pleomorphic, motile, gram-negative, spiral-shaped bacterium which colonizes the cecum, colon, and liver of mice. *Helicobacter hepaticus* grows best under microaerobic conditions at 37°C (Fox et al. 1994). It is notoriously difficult to isolate in culture because it forms a thin film-like sheen rather than distinct colonies. In addition, *H. hepaticus* may be out-competed by gastrointestinal bacteria in mixed culture samples due to slow growth that can take 48-72 hours or longer to evidence (Mahler et al. 1998). *Helicobacter hepaticus* is a urease, catalase, and oxidase positive bacterium with two bi-polar sheathed flagella (Fox et al. 1994). *Helicobacter hepaticus* lacks periplasmic filament bundles typical of other rodent *Helicobacter* species such as *H. muridarum* and *F. rappini* (Ward et al. 1994b). Under non-optimal growing conditions, *H. hepaticus* changes to a coccoid shape, which may be a non-culturable, metabolically active, and infectious form similar to that found in *H. pylori* (Osaki et al. 2002).

*Helicobacter hepaticus* is characterized by several virulence factors which are thought to contribute to its pathogenicity. These factors include urease, cytolethal distending toxin (CDT), and granulating cytotoxin (Young et al. 2000). A fourth putative virulence factor has also been recently described as the pathogenicity island HHGI1 (Suerbaum et al. 2003).

The first factor is urease. In *H. pylori*, urease alkalizes the cytosol and immediate environment around the bacterium by converting urea into ammonia and carbon dioxide (Sachs et al. 1997). This allows *H. pylori* to survive in the acidic milieu of the stomach. The dependence of *H. hepaticus* on urease for colonization as compared with *H. pylori* (Eaton et al. 1991) remains poorly understood since its principal environmental niche is not the acidic stomach (Suerbaum et al. 2003). Perhaps the urease allows *H. hepaticus* to survive transit through the stomach to make safe passage to more distal sites. Urease mutants of *H. hepaticus* have been developed that are unable to colonize mice (Whary et al. 2004a; Chin et al. 2004). Therefore, while urease plays a role in bacterial pathogenicity, further research is needed to clarify its importance for the bacterium’s in vivo function.

The second established virulence factor is the CDT holotoxin. It is encoded by three genes, *cdtA*, *cdtB*, *cdtC*, all of which are necessary for toxin activity (Chien et al. 2000). The *cdtB* gene is found in some, but not all *Helicobacter* species. It is thought to cause host cell distension and death through an
apoptosis-related mechanism (Kostia et al. 2003). It has been shown that CdtB alone has the DNase activity attributed to the enzymatic activity of the holoenzyme (Avenaud et al. 2004). Interestingly, CDT is not absolutely required for colonization (Young et al. 2004). While CDT knockout strains of *H. hepaticus* are still able to colonize C57BL/6 IL-10−/− mice, the resulting gastrointestinal lesions, with regard to degree of submucosal inflammation, are much less severe when compared to CDT-positive *H. hepaticus* strains (Young et al. 2004). Therefore CDT appears to play a role in pathogenesis of inflammatory bowel disease in C57BL/6 IL-10−/− mice (Young et al. 2004). The *cdtB* gene has also been utilized as a basis of quantitative PCR assays to quantitate *H. hepaticus* from mouse tissues (Ge et al. 2001; Whary et al. 2001a), and further work is underway to continue to examine its role in pathogenicity.

Granulating cytotoxin (GCT) is a third reported virulence factor for *H. hepaticus*. In the original study where it was first characterized, changes in the mouse liver cell line CCL9.1 were associated with dense granule accumulation and cytopathic effect as a result of GCT (Taylor et al. 1995). Granulating cytotoxin was originally postulated to be an independent virulence factor, but recent evidence shows that CDT may in fact mediate GCT activity (Young et al. 2004).

Suerbaum and coworkers (2003) described the putative pathogenicity island HHGI1, present in *H. hepaticus*, which appears linked to the induction of hepatic disease in mice, and contains several genetic components homologous to proteins involved with virulence in other bacteria. Hence, HHGI1 may represent a fourth virulence factor. HHGI1 proteins are structurally related to type IV secretion systems, which are a feature of the *cag* pathogenicity island (PAI) in *H. pylori* (Montecucco et al. 2001). The type IV secretion systems are thought to act in *H. pylori* by inserting *H. pylori* cellular elements into the cytoplasm of host cells to affect downstream signaling pathways that may ultimately play a role in carcinogenesis and inflammation (Nagai et al. 2003). It may be possible that *H. hepaticus* uses a secretion system from HHGI1 in a similar, yet distinct mechanism to that of the PAI in *H. pylori*, thereby contributing to its virulence.

**Potential Zoonosis**

While *H. hepaticus* is not considered zoonotic, experience with closely related *Helicobacter* species suggest the potential for human infections. Several enterohepatic helicobacters such as *H. felis*, *H. heilmanni*, *H. pullorum*, and *H. fennelliae* have been isolated from humans, and are therefore
potentially zoonotic (Fox 2002a). Hamsters can serve as a primary host for *H. cinaedi* which may also represent a zoonotic risk. *Helicobacter cinaedi* has been isolated from human blood and tissue, the liver of a rhesus macaque, and from dog and cat feces (Fox 2002b). Additional studies have suggested possible links between enterohepatic rodent *Helicobacter* sp. and humans. *Helicobacter bilis*, a bacterium closely related to *H. hepaticus*, has been associated with human cholecystitis, as well as with gall bladder and biliary duct cancer (Fox et al. 1998; Matsukura et al. 2002). Although no bacteria were observed grossly, a study of human patients with hepatobiliary cancer found PCR positive samples for *H. bilis* and *H. pylori* in bile and tissue samples (Fukuda et al. 2002). *Helicobacter bilis* (Murata et al. 2004) and *H. hepaticus* (Maurer et al. 2004) may also have a role in human cholelithiasis and biliary tract disease. Although further evaluation is needed, Nilsson and coworkers (2003) have demonstrated preliminary evidence of seropositivity to both *H. hepaticus* and *H. bilis* in a small percentage of persons with primary sclerosing cholangitis or hepatic autoimmune disease when either disease is present. Taken together, these studies provide strong supporting evidence that murine helicobacters, including *H. hepaticus*, may be zoonotic. This finding highlights the need for occupational health and safety programs that educate persons working with rodents.

**Pathogenesis of Disease**

As with *H. pylori* in humans, *H. hepaticus* appears to colonize the host for an indefinite period despite the development of a strong immune response (Whary et al. 1998; Whary et al. 2004a). Infected susceptible mice develop chronic infections which may lead to typhlocolitis, hepatitis, and proctitis (Ward et al. 1994a; Hailey et al. 1998; Li et al. 1998). New evidence also defines the involvement of *H. hepaticus* in a causal role for murine cholelithogenesis in C57BL/6 lithogenic mice (Maurer et al. 2004). Disease in mice with *H. hepaticus* is highly dependent on environmental, genetic, and gender-related influences (Nilsson et al. 2004; Zenner 1999). In immunocompetent mice, infection is usually asymptomatic and does not measurably affect indicators of colony health, such as reproduction (Ward et al. 1994b). In contrast, immunocompromised mice may show clinical signs that reflect internal disease such as rectal prolapse, diarrhea, wasting, and death (Percy et al. 2001). For strains such as the immunodeficient C.B17/1cr-scid/NCr mouse, specific types of lesions, such as typhilitis, may be worsened (Ward et al. 1996a). Another strain effect relates to *H. hepaticus* bacterial burden. Whereas C57BL/6
mice are relatively resistant to development of *H. hepaticus*-induced hepatic or intestinal lesions, they have a higher cecal bacterial burden when compared to A/JCr mice (Whary et al. 2001). In contrast, A/JCr mice have a lower cecal bacterial burden, but have a greater tendency toward lesion development (Whary et al. 2001). The genetic effect appears directly related to the type of immune response generated by the particular strain of mice, and in turn, the type of lesions developed appears linked to the immune response.

Because of the noteworthy changes in immune function in susceptible infected mice, *H. hepaticus* has been evaluated as a model of human inflammatory bowel disease (IBD). Intestinal microflora in the host are thought to play a role in the immunologic response and pathology associated with IBD in *H. hepaticus* infection (Kullberg et al. 2001) and *H. hepaticus* may work in concert with other gastrointestinal bacteria to affect the response resulting in IBD (Burich et al. 2001). It has been theorized that lesions develop from two possible sources, either due to damage from self-reactive antigens elicited by *Helicobacter* infection such as heat shock proteins (Ward et al. 1996b), or instead from a direct host immune reaction to a toxin or virulence factor released from the *H. hepaticus* bacterium itself (Whary et al. 2001a). Work by Kullberg and coworkers (2003) suggests portions of the *H. hepaticus* flagellar hook protein (FlgE) may serve as one of three possible antigen recognition sites on the outer bacterial membrane protein responsible for inciting immunologic reaction by a specific clonal CD4+ T-cell response. Thus it is likely that a combination of host and bacterial factors play a role in the resulting pathology.

It is known that select mouse strains develop an immunological response to *H. hepaticus* that is predominately characterized by a Th1 T-cell response. Such mice include the C57BL/6 and A/JCr strains (Whary et al. 1998; Rogers et al. 2004). In general, the Th1 cell-mediated response is marked by increased levels of IL-12, IFN-γ, TNF-β, TNF-α, and IgG2a (Goldsby et al. 2003). Th1 T-cell responses in the intestine are characterized by transmural cellular inflammation, a feature shared with Crone’s disease (Strober et al. 2002). In contrast, other strains, including the BALB/c strain, are noted for their Th2 responses to *Helicobacter* species (Rogers et al. 2004; Watanabe et al. 2004). The Th2 response is marked by increased levels of IL-4, IL-5, IL-10, and IgG1 (Goldsby et al. 2003). The Th2 T-cell response results in superficial mucosal inflammation, similar to ulcerative colitis in humans (Strober et al. 2002; Whary et al. 2004c).
It has been shown that mice with concurrent infection have responses to *Helicobacter* that are up-regulated or down-regulated depending on the net total cumulative effect between the Th1 or Th2 T-cell responses (Fox et al. 2000). C57BL/6 mice co-infected with *H. felis* and the enteric nematode *Heligmosomoides polygyrus* shift immune reactivity away from production of Th1 cytokines and toward Th2 cytokines. This shift results in decreased gastritis and increased colonization at 16 weeks post-infection (Fox et al. 2000). Whary and coworkers have also demonstrated reduced lesion severity with co-infection of *H. hepaticus* and *H. polygyrus* in IL10−/− mice (Whary et al. 2004a). A similar shift towards a Th2 response has been described in humans with endoparasites, and has been postulated to account for the ‘African Enigma’ which theorizes that populations with certain endoparasite infections and concurrent *H. pylori* infection may have ameliorated disease progression and severity due to a shift away from the primary Th1 response incited by *H. pylori* in singular infection (Whary et al. 2004b). Helminths in particular are strong stimulators of the Th2 response (Palmas et al. 2003). Increases in specific IgG antibody against *Syphacia obvelata* in mice have been reported (Sato et al. 1995). A Th2 shift might also be expected to occur during infection with the common mouse pinworm, *Syphacia muris*, in *Helicobacter*-infected mice. This is important to note, because pinworms are relatively common in mouse colonies to this day, perhaps as a consequence of their prolonged infectivity period combined with sporadic shedding and other testing factors (Gaertner 2004). Therefore helminths compose a significant risk for co-infection with *H. hepaticus* and the described immunomodulatory effects. Likewise, concurrent infection of the IFN-γ mouse with mouse hepatitis virus (MHV) and *H. hepaticus*, causes an altered disease state as compared to MHV-G infection alone (Compton et al. 2003). With concurrent infection, Compton and coworkers (2003) found MHV-G related hepatitis and meningitis to be more severe in late stages, yet lessened in early stages of disease. This may reflect pathogen interaction as a function of co-localization in similar regions of the intestine, and interactions within the local immunological environment (Compton et al. 2003). These studies show that interactions with dual infections can affect the overall clinical disease outcome. Therefore general considerations of overall health status in research mice should also include screening and monitoring for *Helicobacter* infection.

In humans, gender appears to affect the pathogenicity of *Helicobacter* infection. It is established that there is a gender bias to certain forms of human disease, including inflammatory bowel diseases
(Livingston et al. 2004), so it is not surprising that this may also apply to Helicobacter infection as a result of gender-based differences in immunity. Studies on this topic conflict, but generally indicate that human males have a higher prevalence of infection (Repogle et al. 1995; Vu et al. 2000; Xu et al. 2000), and a greater percentage of gastric lesions compared to females (Lee et al. 2005). It is not known why these effects are observed, but they could be related to non-steroidal anti-inflammatory use, stress, or other unknown factors (Vu et al. 2000). Gender effects could potentially also be related to the influence of sex hormones, gender-influenced life-style behaviors, or subtle differences in physiology between males and females. Helicobacter infection is associated with increased human gastric and biliary cancer risk (Eslick et al. 1999; Bulajic et. al 2002). Since men show an increased susceptibility to both hepatic and gastric cancers in response to tumorigenic infectious agents, gender may affect the relative risk of human cancer development following Helicobacter infection (Rogers et al. 2004; Sugiyama 2004; Lee et al. 2005). One study by Kang and coworkers (2003) has found a significant increase in CpG methylation in a select group of genes in gastric tissue of men versus women, which might indicate a reason for increased cancer risk through gene inactivation via hypermethylation of CpG sites. However, this same report, when analyzed with respect to gastric cancer by gender, did not find significant differences in cancer risk based on gender. Perhaps more importantly, not all of the patient samples evaluated had confirmed H. pylori infection (Kang et al. 2003). This same study by Kang and coworkers (2003) also found that human colon and liver tissues, unlike gastric tissue, had a low frequency of CpG methylation, and they relate this finding in the stomach tissue to the comparatively overall high rate of gastric cancer in this tissue type. Therefore, it may mean it may be difficult to compare H. pylori gastric effects in humans to enterohepatic effects of H. hepaticus in mice due to tissue-type differences in cancer susceptibility. A study by Leung and coworkers (2004) found that male patients had a greater risk of progression from intestinal metaplasia towards cancer compared to females, and included alcohol consumption and water source as risk factors. This study also found that eradication of the bacterium through medical pharmacological treatment reduced the rate of progression to cancer, thus further highlighting the importance of eradication for humans and mice alike (Leung et al. 2004).

Gender effects on pathogenicity have been noted in the mouse model. Early on Ward and coworkers (1994a) found that certain strains of mice, including the C3H/HeNCr, SJL/NCr, BALB/cAnNCr,
and SCID/NCr, developed more severe hepatic lesions than were found in other strains. In addition, lesions were more severe in male versus female mice (Ward et al. 1994b). The finding that hepatic lesions are more severe in some strains of male mice has been supported by other reports (Fox et al. 1996a; Ward et al. 1994b; Hailey et al. 1998; Li et al. 1998; Livingston et al. 2004). These studies suggest that gender may affect disease outcome in mice as well as in humans. A recent study by Livingston and coworkers (2004) evaluating the cecum of chronically infected A/JCr mice at 3 months post-inoculation found that female versus male mice had a greater Th1 polarized immune response. This corresponded to increased lesion severity scores in the cecum, indicating a sex predilection for intestinal disease caused by *H. hepaticus*. This is in contrast to the findings of other researchers who have reported that lesions in the livers of male mice are more severe (Livingston et al. 2004). A recent article by Rogers and Fox (2004) indicated that with regard to hepatocellular carcinoma (HCC) ... “enterohepatic *Helicobacter* spp. are the only natural murine infectious pathogens known to induce HCC”, and that “a male-predominant tumor susceptibility [exists in mice], as is the case for HCC in humans”. A gender-influenced cancer effect has also been observed in male INS-GAS mice with *H. pylori* (Fox et al 2003).

In contrast to these reports, Whary and coworkers (2001) reported that gender did not affect lesion severity in *H. hepaticus*-infected A/JCr mice with hepatitis and typhlitis. Taken together, these reports suggest that gender affects *H. hepaticus* colonization, disease severity, and lesion development. Further study is needed elucidate the mechanisms by which some strains and sexes of mice develop lesions and neoplasia at different rates.

**Transmission and Detection**

*Helicobacter* sp. are transmitted primarily through the fecal-oral route, as a result of coprophagy (Figure 1). This has been clearly demonstrated through the use of acetylcholinesterase knockout mice, which lack normal prehensile strength and are essentially non-coprophagic (Duysen et al. 2002). Transmission of *H. hepaticus* among rodents has been documented to occur through exposure to contaminated bedding (Livingston et al. 1998). Based on the features of primary fecal-oral transmission as the predominant mode of transfer, it has been suggested that *H. hepaticus* infected mice can be safely housed in close proximity to negative mice with the implementation of strategies designed to minimize direct exposure to contaminated bedding (Whary et al. 2000). Aerosolization is another potential method
for pathogen transmission. A study involving transfer of *Pneumocystis carinii*, *Pasteurella pneumotropica* and *H. bilis* in SCID mice detected *Pneumocystis carinii*, but not *H. bilis* or *P. pneumotropica*, when methods to eliminate other possible mechanisms of spread were in place, suggesting that the latter two organisms are not easily transferable via aerosolization when mice are housed in negatively pressurized individually ventilated cages with filter tops (Myers et al. 2003). Although these two organisms represent different species, *H. bilis* is also thought to be spread by the same mechanism, via fecal-oral transmission. It is not known if this low risk of aerosolization also applies specifically to *H. hepaticus*. Therefore, attempts should be made to minimize aerosolization to reduce potential disease transmission (Whary et al. 2000). Other suggested routes of infection for *H. hepaticus* are via contaminated maternal mouse fur (Duysen et al. 2002), transplacentally (Li et al. 1998), via fomites, and through non-frozen human transplantable xenografts (Goto et al. 2001).

As noted above, it is difficult to grow and isolate *H. hepaticus* from mixed culture specimens. Therefore, PCR using fecal samples has become the standard method of screening mice for infection. These assays utilize portions of the 16S rRNA genome. PCR methods with reliable sensitivity and specificity for use with fecal and tissue samples have been developed (Beckwith et al. 1997; Shames et al. 1995; Compton et al. 2001; Nilsson et al. 2004; Battles et al. 1995). Although it is less commonly used, restriction enzyme analysis following PCR can also be used to differentiate between *Helicobacter* species (Riley et al. 1996). *Helicobacter hepaticus*-positive mice develop increased serum specific antibodies. These can be detected using serum ELISA, but this technology is seldom used, given the superiority of the PCR assay (Mahler et al. 1998). Urease breath tests, rapid screening blood assays, and stool antigen tests frequently reported in humans are not widely commercially available for cost-effective routine screening in mice. Additionally, because of the coprophagic nature of mice, some of the human assays, such as the $^{13}$C urea breath test, may not be accurate in mice for *Helicobacter* detection (Hammond et al. 1999).

**Treatments and Control**

*Helicobacter hepaticus* is undesirable in the laboratory mouse due to the immunomodulatory and pathologic effects previously discussed. *Helicobacter hepaticus* infection can act as a significant confounder for research involving the study of the immune system, liver, and gastrointestinal tract.
Furthermore, *H. hepaticus* has been shown to alter the genetic expression of multiple genes in the cecal tissue of A/JCr mice, including but not limited to those involved in immunological responses. The latter includes genes for MIP-1α, MIG, IP-10, serum amyloid A1, and IFN-γ-inducible protein (Myles et al. 2003; Livingston et al. 2004). *Helicobacter hepaticus* and *H. rodentium* co-infection in mice has also been shown to alter the genetic expression of genes involved in cholesterol homeostasis, and bile salt synthesis (Maurer et al. 2004). Two genes that are up-regulated in human cholangiocarcinoma (*Hspg2* and *Hmga1*) were found by Maurer and coworkers (2004) to be upregulated in murine *Helicobacter* spp. co-infections where *H. hepaticus* was a component. The repercussions of the complex downstream effects of this genetic alteration remain to be elucidated, however these reports clearly illustrate that the effects of *H. hepaticus* infection are far-reaching and beyond what was previously detectable by histology alone. Thus, the implications of *H. hepaticus* infection on research are serious, and it is highly desirable to derive an effective strategy to rapidly eliminate *Helicobacter* from rodent populations in order to maintain the highest standards of research.

Early methods used to eliminate *Helicobacter* sp. centered on the use of antimicrobial therapies. Typically, *Helicobacter* therapy combines antibiotics with proton-pump inhibitors to reduce gastric hydrogen levels. Studies involving antibiotic therapies in mice have usually relied on one or more pharmaceuticals, including amoxicillin, metronidazole, bismuth, or tetracycline (Foltz et al. 1995; Russel et al. 1995). A recent study involved administration of medicated Bio-Serv® tablets to *Helicobacter* infected...
rats. Tablets contained omeprazole, metronidazole, amoxicillin, and clarithromycin. Treatment was successful with and without the addition of cross-fostering of neonates (Jury et al. 2003). This combination of medical therapy and fostering may also have a place in the treatment of mice with *H. hepaticus*. Unfortunately, most of the treatments reported to successfully eliminate *H. hepaticus* from mice, under experimental conditions, have not been uniformly successful when applied “in the field”. Rederivation failures may occur due to difficulty in meeting the strict environmental control measures utilized under experimental conditions. Alternatively, failures may represent development of some degree of antibiotic resistance by the bacterium. In humans, a proportion of patients do not respond to treatment of *H. pylori* infection, and bacterial resistance and relapses are known to occur (Suerbaum et al. 2002). It is not known why some treatments are not completely effective. However, a recent study utilizing the *H. pylori* Sydney bacterial strain (SS1) mouse model demonstrated a “sanctuary site” at the antrum-body transitional zone. In this site, *H. pylori* appeared to be relatively refractory to therapy (Veldhuyzen van Zanten et al. 2003). It is not known if analogous privileged sites exist for *H. hepaticus* in murine extragastric tissues. Future medical treatment options may include strategies aimed at reducing the available hydrogen energy substrate for *Helicobacter*. This may be accomplished by inhibition of H$_2$-utilizing hydrogenases within the bacterium, and through host dietary carbohydrate modification (Maier 2003). Utilization of a vaccine designed to shift the T-cell response of susceptible mice from Th1 to Th2 represents another potential control strategy (Garhart et al. 2003). Such a shift in immune reactivity might be used to manipulate the host response in order to decrease lesion severity and perhaps alter bacterial colonization (Whary et al. 1998). This approach has been examined to some extent in *H. pylori*–infected mice, but the mechanism of protection observed does not appear to depend solely on IL-4 or IL-5 (Garhart et al. 2003). Effects of parasitic co-infection on *Helicobacter* lesion development have been studied (Whary et al. 2004b), but more research is needed to further explore this Th2-mediated mechanism of protection as a murine *Helicobacter* treatment strategy since co-infection models present additional confounding research issues and therefore appear impractical for use for eliminating infections with *Helicobacter* spp.

Non-medical techniques successfully used to eliminate *H. hepaticus* from mouse colonies include rederivation utilizing cross-fostering, embryo-transfer, or cesarean section (Duysen et al. 2002; Singletary
et al. 2003; Truett et al. 2000b). Improved sanitation and biosecurity is achieved through the use of microisolator caging, transfer forceps, and personal protective equipment. These improvements can limit the mechanical spread of *Helicobacter* within a room of infected mice and thereby facilitate rederivation efforts (Whary et al. 2000). Earlier research in our laboratory showed that successful cross-fostering of C57BL/6 pups from *H. hepaticus*-infected parents must be performed within 24 hours of birth, when both parents remain in the birthing cage following parturition (Singletary et al. 2003). In this same study, Singletary and coworkers (2003) demonstrated a gender effect. Male C57BL/6 *H. hepaticus*-infected mice had higher fecal shedding rates, which peaked prior to those of females (Singletary 2003). Due to previous reports of gender differences in the severity of *H. hepaticus*-induced lesions, and based on the gender differences in fecal shedding found in our laboratory, we sought to examine the possible effects of gender as a factor in the control and eradication of *Helicobacter* from a rodent colony. The purpose of our experiment was to: 1) evaluate the effects of gender on bacterial colonization and shedding in *H. hepaticus*-infected mice, 2) examine potential bacterial colonization relationships between sample types, and 3) determine whether removal of the *H. hepaticus*-infected sire prior to parturition would extend the time allowable for cross-fostering beyond 24 hours post-partum.
EXPERIMENTAL HYPOTHESES

Experiment 1

• $H_1 = \text{Removal of } Helicobacter\text{-infected C57BL/6 sires prior to whelping will reduce cage contamination with } H.\ hepaticus, \text{ thereby allowing newborn mice to remain uninfected beyond day 1 of age through fostering onto Helicobacter-negative BALB/c dams.}$

• $H_a = \text{Removal of male mice prior to parturition will increase infection rates in newborn C57BL/6 pups.}$

• $H_0 = \text{Removal of male mice prior to parturition will have no effect on newborn } H.\ hepaticus \text{ infection rates.}$

Experiment 2

• $H_1 = \text{Male C57BL/6 mice will have higher fecal and cecal bacterial } H.\ hepaticus \text{ load early in infection compared to females. This hypothesis is based on a previous demonstration by our laboratory of a cyclic shedding pattern where male mice have peak PCR positivity that precedes that for females (Singletary 2003).}$

• $H_{a(1)} = \text{Female C57BL/6 mice will have higher fecal and cecal colonization loads of } H.\ hepaticus \text{ compared to males.}$

• $H_{0(1)} = \text{There will be no difference in male versus female fecal and cecal colonization loads of } H.\ hepaticus.$

• $H_2 = H.\ hepaticus \text{ fecal colonization load will be greater in cages containing a larger number of male relative to female mice so that housing assignment of treatment will predict the following trend in descending order of bacterial quantity for C57BL/6 mice: paired males > male/female pair > paired females > single male > single female.}$

• $H_{a(2)} = H.\ hepaticus \text{ fecal colonization load will be greater in cages containing a larger number of female relative to male mice}$

• $H_{0(2)} = \text{There will be no difference in male versus female fecal and cecal } H.\ hepaticus \text{ quantity}$

• $H_3 = \text{Fecal pellet colonization load with } H.\ hepaticus \text{ will be related to cecal colonization levels.}$

• $H_{0(3)} = \text{Fecal pellet colonization load with } H.\ hepaticus \text{ will not be related to cecal levels.}$

• $H_4 = \text{Fecal and cecal } H.\ hepaticus \text{ load will decrease as a function of time.}$
• $H_{a(4)} = \text{Fecal and cecal } H. \text{ hepaticus load will increase as a function time.}$

• $H_{0(4)} = \text{Fecal and cecal } H. \text{ hepaticus load will not change as a function of time.}$
MATERIALS AND METHODS

Animals

Seven to eight week-old C57BL/6NHsd male and female mice originating from Harlan (Indianapolis, Ind.) were used in both experiments 1 and 2. Seven to eight week-old BALB/c male and female mice obtained from Charles River Laboratories (Portage, Mich.) were used in experiment 1. Vendor health surveillance reports indicated that upon arrival, all animals were free of common viral, parasitic, mycoplasmal, and bacterial pathogens, including Helicobacter species. The experiments described herein were approved the Louisiana State University Institutional Animal Care and Use Committee and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals. The university’s animal program and facilities are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC).

Housing and Husbandry

Upon arrival, all mice were allowed a two-week quarantine and acclimation period before being manipulated. Mice were group-housed during quarantine/acclimation, before separation into breeder pairs or assignment of housing strategy in accordance with experimental design. Mice were identified by stainless steel ear tags. Two weeks after arrival, fecal pellets were collected individually from each BALB/c and C57BL/6 mouse to be used in experiment 1. Likewise, one month after arrival, fecal pellets were collected from all C57BL/6 mice to be used in experiment 2. Mice were determined to be negative for Helicobacter species by fecal PCR using genus-specific primers as described herein.

Mice were housed in static polycarbonate Micro-Isolator™ caging with filter tops (Lab Products, Inc., Seaford, Del.) on standard stainless steel racks. Corn cob bedding cage litter was used (Bed-O’ Cob, Anderson’s Inc., Maumee, Ohio) for all animals in both experiments. C57BL/6 mice were fed Purina Lab Diet 5001 ad libitum, and BALB/c mice were fed Purina Lab Diet 5010 ad libitum (PMI Nutrition International, Inc., Brentwood, Mo.). All mice were given unrestricted access to tap water provided through standard water bottles and sipper tubes. Caging materials, food, and water used for BALB/c mice were autoclaved prior to use. Housing systems to be used for C57BL/6 mice were cleaned in a standard cagewash and were not autoclaved prior to use. A 12/12 hour light to dark period was used and environmental room temperature ranged 22-23°C. Humidity was periodically measured in two week
increments with HOBO® data logger units (Onset Computer Corp., Bourne, Mass.) throughout the duration of the study. Room humidity varied between 40-60%. C57BL/6 mice in both experiments 1 and 2 were given Nylabones® for environmental enrichment (Bio-Serv®, Frenchtown, N.J.). Nestlets (Ancare, Bellmore, N.Y.) were provided to all mice.

C57BL/6 and BALB/c mice in experiment 1 were housed in rooms maintained under positive pressure ventilation. C57BL/6 mice in experiment 2 were originally housed in a room under negative pressure ventilation from the time of their arrival to one week prior to inoculation, for total period of three weeks. After this time, room air flow was reversed, from negative to positive pressure, due to unrelated disease prevention issues within the animal facility. Cages housing C57BL/6 mice were changed twice weekly, on a portable stainless steel cart that remained in the room at all times. Cage changing surfaces were wiped down between every cage change with a 1:128 dilution of quaternary ammonia-based disinfectant (Super HDQ Neutral, Spartan Chemical Company, Inc., Maumee, Ohio). A fresh pair of non-sterile gloves were used to grasp the tail of each mouse during transfer from dirty to clean cages. BALB/c mice were housed in a room separate from C57BL/6 mice in order to reduce the risk of cross-contamination by Helicobacter. Breeder BALB/c mice (Figure 2) were housed on a three tier stainless steel cage rack in front of a positive flow laminar air bank (NuAire, Plymouth, Minn.). BALB/c foster dams with fostered pups were housed in the same room, in front of a laminar bank with negative air flow. Unexposed breeder BALB/c mice were changed out first, followed by BALB/c foster dams with litters. Disinfectant solution was used to clean all surfaces of the cage change-out area between cages. Gloves were changed between every cage. Disinfectant solution was also used in a footbath at the doorway to each mouse room. For all mouse rooms, personnel were required to wear a clean long-sleeved lab coat over clean employer-provided scrub uniforms. Once personnel entered the Helicobacter-positive C57BL/6 rooms, they were not allowed to enter the negative BALB/c room unless they showered and put on clean clothes and shoes. The doors to the animal rooms were kept locked at all times and only authorized personnel were allowed entry. All employees who worked in the room were trained concerning the project goals and husbandry requirements.
Figure 2. BALB/c Experiment 1 breeder mice. Static polycarbonate Micro-Isolator™ cages placed in front of a positive pressure laminar flow bank.

Bacterial Culture

*Helicobacter hepaticus* (American Type Culture Collection: ATCC No. 51449; 16S rRNA GenBank accession NO. U07573) was obtained and reconstituted in our laboratory, as previously described (Singletary et al. 2003). Frozen stock aliquots of pure *H. hepaticus* were prepared by suspension of bacterial colonies in *Brucella* broth with 5% fetal bovine serum (FBS) and 15% glycerol. Stock aliquots were frozen at -70°C and maintained at -20°C until use for culture. The frozen aliquots were incubated in a 37°C water bath for 5 minutes prior to direct plating onto trypticase soy agar with 5% sheep blood (Remel, Lenexa, Kan.). Inoculated blood agar plates were placed into vented incubation canisters (BBL™ GasPack 100™ and 150™ Anaerobic Systems; Becton Dickinson and Company, Sparks, Md.). Environmental gasses were evacuated to a negative pressure of 760 mmHg. An anaerobic gas mixture consisting of 80% nitrogen, 10% hydrogen, and 10% carbon dioxide, was added to create a microaerobic atmosphere. Gas canisters containing the cultures were placed in a Shel-lab Model 25 incubator (Sheldon Manufacturing, Inc. Portland, Ore.) at 37°C and grown for 72 hours before animal inoculation. Each gas canister underwent a microaerobic air exchange once every 24-36 hours during culture. All cultures were examined visually by darkfield microscopy for viability, motility, and contaminants before subculture or animal use.

Experimental Infection and Caging Assignments

*Helicobacter hepaticus* organisms grown on agar were suspended in *Brucella* broth with 5% FBS by application of a pre-moistened sterile cotton swab applicator tip to the colony surface. Total
concentration of bacterial inoculation dose was determined by the use of a spectrophotometric optical
density analysis at 660 nm. Optical density readings for the bacterial suspensions were taken in
duplicate and compared to an uninoculated control. Measurements were recorded in Ångströms. Each
C57BL/6 mouse in experiments 1 and 2 was inoculated with a 0.2 mL suspension of \textit{H. hepaticus},
administered by gavage using a 22-gage 1 ½ inch straight stainless steel needle with a 1 ¼ mm diameter
ball tip (Popper and Sons, Inc., Lincon, R.I.). Mice were gavaged every other day for a total of three
doses. All doses were administered at the same time of day on the first (day 0) and subsequent days of
infection (day 2 and day 4).

\textbf{Experiment 1.} The purpose of experiment 1 was to foster pups from \textit{Helicobacter}-positive
C57BL/6 parents onto BALB/c \textit{Helicobacter}-negative dams to obtain approximately 20 pups per day
representing days 1-4 of age. To achieve \textit{H. hepaticus} infection, C57BL/6 mice in experiment 1 were
gavaged with \textit{H. hepaticus} suspensions of optical densities 1.187Å, 1.191Å, and 1.089Å on infection days
0, 2, and 4 respectively. Mice were tested by traditional fecal PCR two weeks from the date of the last
inoculation in order to verify \textit{Helicobacter} shedding. Male and female breeder pairs were arranged
thereafter by placing one \textit{Helicobacter}-positive C57BL/6 male into a cage containing a single
\textit{Helicobacter}-positive C57BL/6 female mouse. The pair remained together 14 days. After 14 days, each
pregnant female was removed to a clean cage for single-housing until the next timed breeding event. A
minimum of two cage changes occurred prior to parturition. After parturition, female mice remained
single-housed for approximately 2-3 weeks before the next paired mating cycle. Mice were paired
multiple times until the required number of offspring was reached for each fostering age.

\textbf{Experiment 2.} In experiment 2, groups of mice were in arranged groups containing one to two
mice per cage after experimental infection with \textit{H. hepaticus}, in order to evaluate gender and housing
effects related to bacterial colonization and shedding over time. Two days prior to inoculation, individual
C57BL/6 mice were randomly assigned to five different housing groups based on gender. Housing
category assignments were permanent throughout the study. The experiment was designed so that one
identical set of mice from each housing group (a-e below) with a similar group size would be euthanized
for cecal and fecal collection at post-inoculation weeks 4, 8, and 12. The initial housing assignments at
each euthanasia interval were as follows: group (a) paired male mice (two per cage), (b) single male mice
(one per cage), group (c) paired female mice (two per cage), group (d) single female mice (one per cage), and group (e) male and female paired mice (one vasectomized male mouse with one female mouse per cage) (Table B). Several extra mice were similarly paired and simultaneously infected. These were intended to serve as replacements for any mice that needed to be removed from the study due to unforeseen problems. All mice were infected with *H. hepaticus* every other day for a total of three days as described in experiment 1. Spectrophotometric optical density analyses for the three doses were 0.401Å, 0.697Å, and 0.559Å for days 0, 2, and 4 of infection, respectively. Mice that became ill as a result of the gavage procedure were removed from the study. Mice of identical housing category assignment were introduced into the study groups as replacements. All permanent pairing relationships were established by two weeks following the final inoculation. Group sizes were not identical at all euthanasia time points when compared to the initial group sizes, even with utilization of replacement mice.

**Surgical Vasectomy Procedure**

Male C57BL/6 mice in group (e) of experiment 2 were vasectomized 11 days prior to pairing with intact female mice. Buprenorphine (0.075 mg/kg sq) was administered as an analgesic 30 minutes prior to surgery, and as needed post-operatively for pain relief. Anesthesia was induced by placing mice into a chamber with 5% isoflurane in O₂. Mice were then fitted with a mask and maintained on 2% isoflurane at an oxygen flow rate of 0.8 mL/minute for the remainder of the surgery. The surgical site was prepared over the lower abdomen by first shaving and cleaning to remove all hair, followed by a minimum of three alternating rounds of antibacterial cleansing using 2% chlorhexadine surgical solution (Nolvasan®) with 70% isopropyl alcohol applied on cotton-tip applicators. Body temperature was maintained with the use of a warm water circulating blanket and a forced air warming unit (Bair Hugger®: Arizant Healthcare Inc., Eden Prairie, Minn.). All surgical procedures were performed using aseptic technique as described elsewhere (Hogan 1986). A 1.0 cm longitudinal mid-line incision was made in the skin using a #15 blade. The linea alba was tented, and a longitudinal incision was made using iris scissors, ending approximately 0.5 to 1.0 cm proximal to the bladder. The vas deferens were identified bilaterally and isolated individually using stay sutures. Two circumferential ligatures were placed approximately 4 mm apart around each vas deferens using 6-0 absorbable polydioxanone suture (PDS II: Ethicon). The vas
deferens were transected mid-way between the two sutures and then returned to the abdomen. The abdominal musculature and skin layers were closed separately with five to six simple interrupted sutures of 6-0 PDS II in each layer. A thin strip of cyanoacrylate was applied topically over the skin sutures (Loctite® QuickTite® Super Glue Easy Squeeze Liquid, Marco Inc., Avon, Ohio ). Skin sutures were removed 10 days later.

**Fostering**

For experiment 1, C57BL/6 pups from *Helicobacter* infected parents were fostered from between 1 to 4 days of age onto lactating *Helicobacter*-free BALB/c dams. C57BL/6 dams were PCR tested a minimum of once every two weeks to detect *Helicobacter* fecal shedding (Table 1). Only the pups of C57BL/6 dams that had shed *Helicobacter* within 7 days of parturition were used for fostering onto BALB/c dams.

To achieve timed breeding, male and female BALB/c mice were paired four days in advance of pairing of C57BL/6 *Helicobacter*-positive mice. The C57BL/6 *Helicobacter*-positive female mice were evaluated daily at the same time of day so that the age of their pups could be determined. C57BL/6 pups that were between 0-24 hours of age were classified as day 1 pups. A total of 23 C57BL/6 pups were fostered on day 1, 20 pups each on days 2 and 3, and 19 pups on day 4. The technique used for fostering in this experiment has been described previously (Singletary 2003) and was the same procedure followed in experiment 1. C57BL/6 pups were fostered onto *Helicobacter*-negative BALB/c foster dams using a small amount of scented talcum powder (Kuddles, Winn Dixie Stores, Inc., Jacksonville, Fla.) to mask the scent of the C57BL/6 pups. The person delivering the C57BL/6 pups did not enter the clean room containing the BALB/c foster mice, and no bedding material was transferred with the fostering procedure. The recipient of the C57BL/6 pups did not touch the transport microisolator cage containing the C57BL/6 pups, or any other substrate aside from the bottle of talcum powder, prior to handling the newly arrived C57BL/6 pups. The outside of the plastic bottle of talcum powder was cleaned with disinfectant solution between every transfer of pups. If multiple cages of C57BL/6 pups from different C57BL/6 dams were fostered on the same day, the youngest pups were fostered first. After addition of the C57BL/6 pups to the BALB/c cage, the cage was placed on the rack in front of the negative flow air bank. A single remaining BALB/c natural litter pup was allowed to remain with the
fostered C57BL/6 litter until weaning at 21 days of age in order to encourage maternal acceptance of the fostered litter.

At 21 days of age, the fostered C57BL/6 pups were weaned and housed by sex in groups of 1 to 5 pups. The single natural BALB/c pup remaining was sacrificed at weaning and fecal samples were obtained for PCR testing. Fecal samples were also collected from the BALB/c foster dam and the weaned C57BL/6 pups at this time for this same purpose. After weaning of the foster litter, the BALB/c dam was either single or group-housed, depending on available space, for 21 more days. At 42 days of age, the C57BL/6 fostered pups, as well as their BALB/c foster dam were euthanized, and samples were collected in order to obtain a terminal cecal and fecal sample. Each BALB/c foster dam was used as a foster parent only one time. In addition, all C57BL/6 pups fostered onto one BALB/c foster dam were from the same Helicobacter-positive C57BL/6 mother. Large litters of C57BL/6 pups were occasionally split onto two different BALB/c foster dams as needed in order to fill out the required number of fostered C57BL/6 pups for a particular day of age. To verify the continued Helicobacter-negative status of the BALB/c dams, all breeder BALB/c males and females were tested every two weeks for the duration of the experiment until the female was utilized as a foster dam.

Sample Collection

For experiment 1, fecal pellets were collected by placing the lip of a sterile Falcon tube adjacent to the anus of the mouse without touching the fur so that the fecal pellet fell into the collection container without physical manipulation. Fecal pellets were collected from fostered C57BL/6 mice at the time of weaning on post-partum day 21. A fecal sample was simultaneously collected from each BALB/c foster dam and remaining natural litter pup. Fostered C57BL/6 pups were euthanized at 42 days of age. Fecal samples were again collected from these mice, using the method previously described, or were collected directly from the terminal rectum at necropsy using sterile technique. At the time of collection, samples were immediately refrigerated, and processed by Hot Shot DNA extraction within 24 hours (Truett et al. 2000a). All tissue samples obtained at euthanasia were collected in a Class II Type A1B3 biosafety cabinet (Model NU-425-200, NuAire, Plymouth, Minn.) under sterile conditions. Separate sterile instrument sets were used to open the skin, the abdominal muscle layer, for cecal sample collection, and for fecal collection. Instruments used were either autoclaved, or sterilized in a bead sterilizer (Germinator...
500, CellPoint Scientific, Inc., Rockville, Md.) for 2 minutes prior to use. A longitudinal half of the cecum was collected, and the fecal material was gently removed from the mucosal layer. Cecal tissue was placed in sterile Nunc™ CryoTubes™ (Nalge Nunc International, Rochester, N.Y.), frozen in liquid nitrogen, and held at -70°C until Qiagen DNA tissue extraction (Qiagen Inc., Valencia, Calif.) could be performed. Fecal pellets collected from the terminal colon or from the rectum prior to euthanasia, were immediately refrigerated and processed by Hot Shot DNA extraction within 24 hours, as described for day 21 fecal samples.

For experiment 2, C57BL/6 mice were divided into 3 sub-groups, representing mice euthanized at post-infection week 4, 8, or 12. Each subgroup contained a roughly equivalent number of mice (Table B). In this experiment, fecal samples were collected from all mice every two weeks post-infection, and at euthanasia. Cecal tissue samples were collected using sterile technique, as described for experiment 1. Fecal samples were collected directly from the anus of live mice, as described above, or by sterile collection at necropsy. Both fecal and cecal samples were immediately placed in liquid nitrogen after harvest, and stored at -70°C until processing by Qiagen DNA fecal extraction (Qiagen Inc., Valencia, Calif.).

Euthanasia

Mice were rendered unconscious by CO₂ inhalation, and euthanized via cervical dislocation and cardiac puncture. All animals were necropsied and examined for gross lesions. Euthanasia of neonatal BALB/c mice consisted of CO₂ asphyxiation followed by decapitation. Healthy mice that were not directly used for tissue samples in the project after euthanasia were donated for use as raptor food to support the LSU Raptor Rehabilitation Center.

DNA Extraction and PCR Analysis

PCR Reaction Conditions Experiment 1. Polymerase chain reaction testing was used to detect the presence of *Helicobacter* DNA in the fecal and cecal specimens obtained from BALB/c and C57BL/6 mice. DNA extraction of fecal samples was performed with the use of the HotSHOT method within 24 hours of sample collection (Truett et al. 2000a). A single fecal pellet from each mouse was tested at each collection time point. The HotSHOT protocol used in experiment 1 was performed as previously described (Singletary 2003). DNA extraction from mouse cecal tissue was performed using the
QIAamp® DNA Mini Kit, according to manufacturer’s instructions (Qiagen Inc., Valencia, Calif.). Following the September 2001 Qiagen protocol version of DNA extraction instructions, the RNase addition step was omitted, and the final elution step was made using a single addition of 200 µl Buffer AE following a 5 minute incubation period. Extracted DNA from the fecal and cecal samples were stored at -20°C until PCR assay was performed.

The genus-specific Helicobacter primers H276f and H676r were used for PCR (Beckwith et al. 1997). These primers amplify a highly conserved region of the 16S rRNA gene and produce a fragment measuring 375 base pairs in length. A 40-µl reaction volume contained 6-µl of DNA template, 4.0-µl PCR Buffer II (Applied Biosystems, Warrington, United Kingdom), 200 nM each of primers H276f and H676r (GeneLab, Baton Rouge, La.), 0.19 mM of dNTP (Applied Biosystems), and 1 unit of Taq Polymerase (Applied Biosystems). Each reaction volume was covered with 40-µl light paraffin mineral oil (Product 0121-1, Fisher Scientific, Fair Lawn, N.J.) prior to amplification. Amplification was carried out in a Robocycler (Stratagene, La Jolla, Calif.) under thermocycler conditions based on a modified protocol described by Truett and coworkers (Truett et al. 2000b). Briefly, an initial denaturation step of 95°C for 60s followed by 50 cycles of 95°C denaturation step at 60s, 57°C annealing step at 60s, and a 72°C elongation step for 30s, with a terminal elongation step of 72°C for 2 minutes were applied. Following amplification, 15-µl of PCR product from each reaction was mixed 5 µl of 1x Type I gel loading solution (Sigma, St. Louis, Mo.). Results were recorded after ethidium bromide staining on 1-2% Agarose 1™ gel electrophoresis at 94V (Amresco, Solon, Ohio). A 100 base pair DNA ladder (Bayou Biolabs, Harahan, La.) was used for comparison of standards. Positive controls consisted of DNA extracts of H. hepaticus spiked cecal tissue, or from pure bacterial culture. Results were recorded by direct optical visualization in conjunction with photography under ultraviolet illumination.

**PCR Reaction Conditions Experiment 2.** Cecal tissue samples were collected and DNA extracted using the QIAamp® DNA Mini Kit tissue protocol as described for experiment 1. For each mouse fecal sample, a single fecal pellet of variable mass was extracted using the QIAamp® DNA Stool Mini Kit following the August 2001 instructions for isolation of DNA from stool for pathogen detection (Qiagen Inc., Valencia, Calif.). Extracted fecal and cecal samples were shipped on dry ice overnight by courier from Baton Rouge, Louisiana, to Cambridge, Massachusetts. All samples were transported...
between destinations in less than 24 hours, and were unpacked immediately upon arrival. After DNA extraction and prior to PCR processing, both before and after shipment, all samples were kept in a non-defrosting -20°C freezer. All quantitative PCR assays were performed at the Massachusetts Institute of Technology in Cambridge, Massachusetts.

Extracted fecal and cecal samples were processed by fluorogenic quantitative PCR using the ABI Prism™ 7700 Sequence Detector (PE Biosystems, Foster City, Calif.) as previously described (Ge et al. 2001). The thermocycler was housed in a dedicated room within an isolated cubicle. For each sample, both the copy number of *H. hepaticus*, and amount of mouse 18S rRNA were quantified by measuring the change in fluorescence intensity (ΔR) using SDS software (PE Biosystems) as described by Ge and co-workers (2001). All samples were run in duplicate with a maximal allowable cycle threshold (Ct) difference of 1.0 for inclusion in the study. The thermocycler conditions used were as recommended by the manufacturer. These included the initial steps of 50°C for 2 minutes, then 95°C for 10 minutes, followed by 40 continuously replicative cycles of 95°C for 15s and 60°C for 60s. All chemical reagents used for both assays were purchased from Applied Biosystems (Foster City, Calif.) except for the DNAse/RNAse-free sterile water for PCR. All manipulations involved with PCR set-up and sample handling were performed within a class II type 2A SterilGARD® biosafety hood (Figure 3) (The Baker Co., Stanford, Maine) with a dedicated set of supplies in order to maintain optimal standards and reduce contamination for the sensitive assay.

The assay to detect copy number of *H. hepaticus* utilized 25 µl total reaction volume. This consisted of 10 nM each of forward and reverse cdtB primers, 5 nM cdtB probe, 12.5 µl TaqMan® 2X PCR Master Mix, and 5 µl of DNA template. The *H. hepaticus*-specific primers, 5’-CGCAAATTGCAGCAATACTT-3’ (forward), 5’-CACCTGTGCATTTTGGACGA-3’ (reverse) and the fluorogenic probe nucleotide sequence used (5’-FAM-AATATACGCGCACACCTCTCATCTCTGACCATTAMARA-3’) were based on the cdtB gene, and produce an 81 bp PCR DNA fragment based on the National Cancer Biomedical Institute (NCBI) nucleotide-nucleotide Blast sequencing from *H. hepaticus* ATCC No. 51449 (Ge et al. 2001). Aliquots of cecal DNA extracted from a day 1-fostered C57BL/6 *Helicobacter*-negative sibling pair were used as an age-matched control to create a 10-fold serial dilution of concentrations at 2 ng/µl, 200 pg/µl, 20 pg/µl, 2 pg/µl, 200 fg/µl, and 20 fg/µl for the generation of a
standard curve. The initial concentration of DNA from the mouse samples was determined using GeneQuant Pro (Amersham Pharmacia Biotech, Piscataway, N.J.) using optical density spectrophotometry readings at 260 Å. Prior to their use as standards, these mice were confirmed Helicobacter-negative based on PCR assay as described for fecal and cecal tissue in experiment 1, and additionally by the fluorogenic PCR assay described in experiment 2 for H. hepaticus. The upper limit of detection for the fluorogenic PCR assay was 5,000,000 copies of H. hepaticus, and the lower detection limit was 50 copies.

To quantify the mouse 18S rRNA gene in the fecal and cecal DNA-extracted material, a total reaction volume of 25 µl was used with the same thermocycler cycling conditions as in the cdtB assay. The reaction consisted of 1.25 µl 20x 18S rRNA Pre-Developed Taqman® Assay Reagents: Control Primers and Probe, 12.5 µl TaqMan® 2X PCR Master Mix, and 5 µl of DNA template. Cecal DNA extract from the same Helicobacter-negative noninfected C57BL/6 sibling pair were used as controls for the mouse DNA assay. A standard curve was generated using 20 ng/µl, 2 ng/µl, 200 pg/µl, 20 pg/µl, and 2 pg/ul so that the upper and lower detection limits of the assay were 100,000 and 10 copies, respectively.

**Figure 3.** Class II biosafety hood at Massachusetts Institute of Technology. Sample handling and set-up for PCR were performed within the hood using dedicated supplies.
STATISTICAL ANALYSIS

Data collected in experiment 2 was processed using SAS statistical software (version 8.2, SAS Institute Inc., Cary, N.C.). Replicate values were averaged and expressed as number of copies of *H. hepaticus* per microgram of mouse DNA. Raw data were transformed using a log_{10} transformation prior to analysis to stabilize variance terms. Data from experiment 1 did not require statistical analysis.

One-way analysis of variance (ANOVA) in a repeated measures design was used to evaluate fecal content of *H. hepaticus*. A univariate approach analyzed as a split plot arrangement of treatments was used. The ANOVA evaluated mean log_{10} fecal *H. hepaticus* quantitation data (average copy *H. hepaticus*/µg mouse DNA of the duplicate samples) values derived from mice within the same treatment category (housing group assignment). The ANOVA used mouse identification (ID), treatment group (TREATMENT), and time (post-inoculation WEEK) as the three classification effects. The ANOVA used TREATMENT, as well as ID within TREATMENT as the error term on the main plot to evaluate between subject factors. WEEK and TREATMENT by WEEK interaction terms were on the subplot or within subject portion of the experiment. When the overall ANOVA tests indicated results at or approaching significance (p<0.05), Tukey’s Studentized Range Test (Tukey’s HSD Test) was used as a secondary test for main effects comparisons. Tukey’s HSD Test was applied to conduct pairwise comparisons of both the time and treatment main effects.

Similarly, a second ANOVA using a repeated measures design was used to evaluate significance between gender and time with respect to fecal *H. hepaticus* copy number. For this test, GENDER as well as ID within GENDER, were used on the main plot. WEEK, and GENDER by WEEK terms were used on the sub-plot. Where significance was found, least squares means were evaluated to make pairwise comparisons for significance between both genders, and week of fecal collection.

A factorial ANOVA in a non-repeated measure design was additionally used to test for significance between fecal and cecal *H. hepaticus* copy number between each gender, regardless of treatment group, for the euthanasia time points of 4, 8, and 12 weeks. Because the cecal samples were collected at necropsy, samples from mice within the same euthanasia group were evaluated separately, and simultaneously compared between euthanasia groups. At each time point, the cecal and fecal samples were from the same group of euthanized mice. Between different time points, the fecal and
cecal samples were from different cohorts of euthanized mice. The 2x2x3 factorial arrangement evaluated sample type (fecal and cecal), gender (male and female), and time (4, 8, and 12 weeks). Where significance was found, least squares means were evaluated to make pairwise comparisons for significance.

Correlation and regression analyses were conducted to evaluate potential linear relationships between *H. hepaticus* bacterial copy number in fecal and cecal samples taken at the same time from within identical individual mice in post-inoculation weeks 4, 8, and 12. Pearson product moment correlation coefficients ("r") and regression models were analyzed for both statistical and practical significance in the overall data, as well as for each treatment, housing arrangement, and gender. SigmaStat® and SigmaPlot® 2000 statistical software (version 2.03, Systat Software Inc., Point Richmond, Calif.) were used to analyze data to create graphs using the standard error of mean.
RESULTS

Experiment 1

C57BL/6 mouse pups were successfully fostered onto BALB/c dams. No fostered C57BL/6 pups were rejected by their foster dams at any time. The BALB/c foster dams accepted the new pups immediately and began rearranging the pups and adjusting the nestlets within the first minutes of exposure.

None of the C57BL/6 pups fostered between 1 and 4 days of age tested positive by fecal or cecal PCR, for *H. hepaticus* at 21 or 42 days of age. Fecal and cecal samples had been collected on BALB/c dams at time points coinciding with similar collections from C57BL/6 fostered pups. Because C57BL/6 pups were fostered at times that varied between 1 to 4 days of age, fecal samples were taken once from BALB/c dams at a post-fostering point which varied between day 18 to day 21. Similarly, cecal samples were collected from fostered pups, at intervals ranging from post-fostering day 39 to 42. All fecal and cecal samples collected from BALB/c foster dams tested negative for *H. hepaticus* by PCR. A total of 15 BALB/c foster dams were used in experiment 1. A total of 83 C57BL/6 pups, including 46 females and 37 males were used in experiment 1. The largest litter of C57BL/6 pups fostered was comprised of 10 pups, and the smallest was comprised of two pups. None of the fecal pellets collected from the individual BALB/c pups left with their dams tested positive at weaning. One BALB/c pup died within the first few days of life and so was not available for testing at weaning. The tissues from the deceased BALB/c pup were not available for necropsy, consequently cause of death could not be determined. Of the remaining 14 BALB/c pups tested, 5 were males, 8 were females, and one pup’s gender was not recorded.

No *Helicobacter*-positive BALB/c foster parents were detected at any time during the bi-monthly fecal testing. Repeated testing of fecal pellets excreted by C57BL/6 sires and dams initially revealed shedding by all breeders. Over the course of the experiment however, shedding rates gradually declined for dams (Figure 4). Fecal shedding of *H. hepaticus* was lowest at week 34 for both male and female C57BL/6 breeder mice.

Experiment 2

In the first ANOVA using the repeated measures design, neither the effect of time, nor housing strategy significantly affected fecal shedding of *H. hepaticus* in C57BL/6 mice of experiment 2. However,
Figure 4. Fecal shedding of Helicobacter hepaticus in adult C57BL/6 mice after experimental inoculation. The blue line (diamonds) represents the percentage of males shedding the bacteria. The pink line (squares) represents the percentage of females shedding the bacteria. The data shown is from the Experiment 1.

A non-significant trend emerged towards weeks 10 and 12 where housing groups containing female mice were lower than the two groups containing male-only mice (Figure 5). In the second ANOVA using repeated measures design, the overall effect of gender was found to be significant p<0.001 when compared regardless of housing group (Figure 6). Time was not found to be significant as a main effect, although the TIME by GENDER interaction term was significant p=0.0056. Results were confirmed by Tukey’s studentized range test (HSD). Least squares means found significant interaction effects in this second ANOVA at weeks 6, 10, and 12. Male fecal samples contained a greater copy number of H. hepaticus compared to females (p =0.0397, p=0.0017, p<0.001 respectively) at these time points. No significance for differences between male and female fecal samples was found for weeks 2, 4, 8.

The factorial ANOVA using a non-repeated measures design found overall significance for the effects of both gender and sample type (p=0.0001, p=0.0003 respectively), but did not detect significance for time as a main effect (Figure 7). These results were confirmed by Tukey’s studentized range test (HSD). Least mean squares detected significance at week 12 where the combined H. hepaticus bacterial copy number of cecal and fecal samples were greater for males than for females (p<0.0001). With regard to overall sample type the least mean squares showed that male cecal bacterial load was significantly greater than male fecal bacterial load (p=0.0103) when evaluated across all time points, and this finding
was similar for female samples (p=0.0075). Additionally, male cecal samples had a significantly higher bacterial load than did female cecal samples when evaluated across all time points (p=0.0117), and again, this same male-biased finding was true with the fecal samples (p=0.0113).

When all mice were compared regardless of gender, housing strategy, or time, Pearson’s correlation coefficient detected a statistically significant (p<0.001) correlation (r=0.684) between fecal \textit{H. hepaticus} shedding and cecal bacterial colonization levels. Regression analysis likewise yielded significant parameter estimates for slope (m) and intercept (b) so that the equation model \( y=mx+b \) was generated where \( y=\log_{10} \text{cecal copy} \ H. \textit{hepaticus}/ \mu\text{g mouse DNA}, m=0.60545, x=\log_{10} \text{fecal copy} \ H. \textit{hepaticus}/ \mu\text{g mouse DNA} \) and \( b=2.98005 \). Both the slope and intercept parameters for this regression model were significant at \( p<0.0001 \).

When mice were examined by gender (Figures 6-7), regardless of treatment group or time, Pearson’s correlation coefficients were again statistically significant for both female (p\leq0.001, r=0.83078) and male mice (p=0.0149, r=0.33609). Regression models for these same animals were significant and determined \( m=0.93063 \) (p<0.001) and \( b=0.93671 \) (p=0.0753) for females, and \( m=0.12578 \) (p=0.0149), \( b=6.21823 \) (p<0.001) where \( y=mx+b \) when \( x \) and \( y \) are as defined as indicated above. Finally, correlation and regression models based on treatment group found significant Pearson’s correlation and regression models for the female/female and the male/female housing group. For the female/female group, \( r=0.75414 \) (p=0.0003); \( m=0.68686 \) (p=0.0003), \( b=2.38272 \) (p=0.0205). For the male/female housing group, \( r=0.90670 \) (p<0.0001); \( m=1.01087 \) (p<0.0001), \( b=0.44298 \) (p=0.3804).
Figure 5. Mean $\log_{10}$ fecal *H. hepaticus* copy per $\mu$g mouse DNA (± standard error of mean) in C57BL/6 mice on Experiment 2 over time. No significant overall effects were found for either time or housing strategy based on ANOVA statistical analysis. However, the effect of mouse within treatment group approached significance at $p=0.059$ when alpha levels were arbitrarily set at $p<0.05$. 
Figure 6. Mean log$_{10}$ fecal H. hepaticus copy per µg mouse DNA (± standard error of mean) in C57BL/6 mice on Experiment 2 over time with treatment groups merged. ANOVA found a significant difference between male and female mice at weeks 6, 10, and 12.
Figure 7. Bar graph of the mean log₁₀ fecal and cecal data [H. hepaticus copy per µg mouse DNA] compared by gender irrespective of treatment group for experiment 2. Error bars indicate the standard error of mean. Week 4, 8, and 12 data sets were each obtained from separate populations of mice taken from samples collected at euthanasia. Within each individual time point, the fecal and cecal values were from the same population of mice. Factorial ANOVA showed an overall significance of both gender and sample type p=0.0001, p=0.0003 respectively. Least mean squares found a significant difference between averaged fecal and cecal values for males as compared to females p<0.0001. Pearson’s correlation coefficients were statistically significant (p≤0.001) for male and female mice regardless of treatment group or time for the correlation of fecal to cecal data scores.
DISCUSSION

*Helicobacter hepaticus* is an important murine pathogen which can cause significant pathologic changes (Ward et al. 1994a; Hailey et al. 1998; Li et al. 1998) and genetic dysregulation in susceptible animals (Myles et al. 2003; Maurer et al. 2004; Livingston et al. 2004). Infection can result in confounding effects on research involving the immune and gastrohepatic systems of mice (Beckwith et al. 1997; Hailey et al. 1998). Because of these consequences of infection, many researchers, laboratory animal veterinarians, and commercial vendors desire rapid and complete methods to eradicate the bacterium from infected mice (Compton et al. 2001). Traditional medical therapies can be expensive, and time-consuming. Furthermore, studies show that treatment of *Helicobacter* spp. can be subject to recurrence, bacterial resistance, or treatment failure (Fox 2002b; Veldhuuyzen van Zanten et al. 2003; Suerbaum et al. 2002). In addition, pharmacological medical treatments generally require either frequent handling of mice for gavage, or cage disturbance for compound administration in water or feed (Russel et al. 1995; Foltz et al. 1995). Physical manipulation in and of itself can increase stress to the animal, alter reproductive performance, and introduce variability into research (Linder 2003; Meek et al. 2001; Hale et al. 2003; Brown et al. 2000; Peters et al. 2001). Previous work in our laboratory has shown that fostering is a reliable, reproducible, and cost-effective alternative to anti-*Helicobacter* medical regimens and other re-derivation approaches when performed within 24 hours of birth (Singletary et al. 2003). The study reported here sought to further define the fostering paradigm in the hope of making fostering of neonatal mouse pups more practical.

Results from experiment 1 indicate that fostering of C57BL/6 pups from experimentally infected *H. hepaticus*-positive dams can be extended through day 4 of age if the sire is removed from the birthing cage approximately 1 week prior to parturition. This finding is important from an animal husbandry standpoint as it allows three additional days before C57BL/6 pups must be fostered in an *H. hepaticus* eradication program. This is advantageous because it allows pups to be fostered after weekends, holidays, and during unexpected personnel shortages. Looking at the trends in fecal shedding (Figure 4), it appears that males versus females continued to have a greater percentage of *H. hepaticus* shedding in their feces, as was in agreement with the study by Singletary and coworkers (2003). However in experiment 1, although a cyclic pattern of fecal shedding was found (Figure 4), the plateaus and peaks
appeared to correspond to similar time points for both genders, rather than the males preceding females as had been demonstrated by Singletary and coworkers (2003). This difference may be related to the smaller sample sizes of C57BL/6 female mice used at given bi-monthly collections (n=5-12) over the course of the breeding experiment as compared to that of Singletary and coworkers (n=10-20) (Singletary 2003).

Possible explanations for the differences between the findings of this study and those from Singletary and coworkers (2003) with regard to the prolonged fostering interval to generate *Helicobacter*-free pups, may be that the removal of the sire C57BL/6 mouse prior to parturition causes a reduction in overall *H. hepaticus* cage contamination within the cage due to decreased fecal exposure. In other words, having only one adult mouse in the cage versus two, may have reduced the amount of feces produced in the cage by at least half. Reduced numbers of available *Helicobacter*-infected fecal pellets in the cage constitutes lower potential exposure levels to pups. Therefore, lower exposure in the absence of the male C57BL/6 mouse may have facilitated fostering pups out to day 4 of age in experiment 1.

Differences in results observed between the two studies might also be explained by the altered family structure. When the sire remains in the cage, he may contribute to grooming and socialization of the pups, thereby increasing horizontal bacterial transfer to the pups. To the best of the authors’ knowledge, the amount and type of parental behavior of the male C57BL/6 mouse has not been quantified, however several studies indicate that male mice do participate in neonatal rearing in variable degrees depending both on strain, and on housing strategy (Wright et al. 2000; Schradin et al. 2003; Gubernick et al. 1987). Additionally, studies on behavior of CD-1 Swiss Webster albino mice show that female mice do not alter the amount of care given to the pups in male-absent conditions, and except for nursing, male mice often perform the same parenting activities as do females including grooming and licking (Wright et al. 2000). Differences in time spent on parental care, and types of care, may depend on the gender of the pup, with male pups receiving more licking and nursing behavior than females (Alleva et al. 1989). Transmission of *H. hepaticus* is primarily via fecal-oral route (Duysen et al. 2002), but because mice are coprophagic, grooming with the mouth of the male sire, could transfer the bacteria from his mouth to the areas being groomed on the pups if he had recently consumed infected fecal pellets. Gubernick and coworkers (1987) in fact documented that male *Peromyscus californicus* mice spent more
time licking their pups than did females. Therefore while it is not known if the C57BL/6 sire’s parenting behavior influenced the results, this does represent one possible difference in the care given to the pups between the current study, versus that of Singletary and coworkers, where both parents were present (Singletary et al. 2003).

Experimental procedures between the two projects, including types of reagents used in DNA extraction and PCR processing conditions, were the same in both studies, and only small differences between the experimental infection doses were present. In the current study (experiment 1) *H. hepaticus* inoculum ranged 1.087 to 1.191 Å, whereas in the previous study, spectrophotometric optical density of inoculum ranged between 0.75 Å and 1.172 Å (Singletary et al. 2003) using the same spectrophotometer and measuring techniques. However, due to the high sensitivity of PCR in bacterial detection (Compton et al. 2001), and since all mice used in both experiments initially tested positive for experimental *H. hepaticus* infection, these relatively small differences in gavage bacterial concentrations are not thought to be a contributing factor sufficient to explain the differing results of the two studies in terms of the prolonged fostering interval. Therefore it appears that the only important difference between the design of the study by Singletary and coworkers (2003), and experiment 1 described herein, was the absence of the C57BL/6 sire, and the differences in fecal output exposure and parenting effect that his presence or absence would have generated.

To accurately determine the day by which C57BL/6 pups must be fostered to prevent infection with *H. hepaticus* beyond day 4 of age, it would have been ideal if the study had been carried out long enough for neonatal infection to occur. Since no PCR *Helicobacter*-positive fostered C57BL/6 pups were detected in this study, it is unknown on precisely what date pups would have become infected after day 4 of age. Increases in age-dependent neonatal coprophagic behavior after day 4 of age may contribute to a steady increase in exposure, however to the best of the authors’ knowledge, it is not known at which day of age true neonatal coprophagic behavior begins in the C57BL/6 mouse, or reaches high enough levels to facilitate *Helicobacter* transfer. One study in male ICR mice has shown coprophagy beginning in 17 day old mice (Ebino et al. 1987) but equivalent data is not available for the C57BL/6 mouse. It is therefore possible then that fostering of C57BL/6 pups can be carried out past 4 days of age without
Helicobacter infection, when parturition is in the absence of the male. This question will be addressed by additional experimentation.

The frequency of bedding changes may also have an impact on *H. hepaticus* transmission by reducing the quantity of infected fecal pellets present within the cage. Additionally, type of caging and bedding used may be important due to altered intra-cage humidity and other environmental effects on the feces in individually ventilated cages versus standard open-top caging systems (Reeb-Whitaker et al. 2000). In this study, cages were cleaned twice weekly, and a minimum of two cage changes occurred prior to birth of the C57BL/6 pups. Although *Helicobacter* DNA remains stable in feces for up to 5 days (Beckwith et al. 1997), it is unknown how long *H. hepaticus* bacteria in fecal pellets remains infective, and if a reduction in the frequency of cage changes would have increased transmission to the C57BL/6 offspring. On the other hand, frequent cage changing is also stressful to mice, and has been associated with increased pup mortality (Reeb-Whitaker et al. 2000). Nevertheless, the fostering method employed in this study, based on the described experimental testing conditions, proved effective in elimination of *H. hepaticus* from C57BL/6 pups derived from infected parents through fostering on or before day 4 of age.

Neither mouse housing strategy or time affected fecal *H. hepaticus* population levels as measured in the first repeated measures ANOVA. The failure of meeting significance at p<0.05 in the ANOVA analysis may have been affected by the different sizes of treatment groups, since some treatment groups contained dissimilar animal numbers due to study design, and unexpected necessary euthanasia (Appendix B), of several non-replaceable mice early on either due to gavage-related issues, or failure to become infected (n=2). While a small number of replacement mice were included into the study design to allow for these potential problems, the number of extra mice added proved inadequate. Because only mice with identical housing strategy assignments could serve as replacements, the number of available replacement mice was reduced as a function of housing assignment. For example, a pair-housed male could not serve as a replacement for a single housed male. Aside from unequal sample size as a factor, size itself may have played a role in the result outcome. Incorporation of larger treatment groups may have provided a more robust outcome through reduction in variability. Additional studies utilizing larger sample sizes, and/or extending out measurement for longer periods are needed to answer this question. However both the repeated measures and non-repeated measure results of the ANOVAs
with regard to fecal *H. hepaticus* did show an effect related to gender. The finding that male cecal samples and fecal samples contained greater copies of *H. hepaticus* than in similar female samples may be related to lesion severity in the cecum. Although we did not examine histology of the cecum in this study, the findings by Livingston et al (2004) in A/JCr mice revealed that females had greater cecal lesion scores than males. It may have been that our C57BL/6 females had greater cecal lesions than the C57BL/6 males in our study. If this were the case, the findings of lower bacterial load in our females could possibly be explained by a study comparing A/JCr and C57BL/6 mice, where lesion scores were found to be inversely related to *H. hepaticus* bacterial quantity in the cecum (Whary et al. 2001a). In other words, the C57BL/6 female mice on our study having lower cecal *H. hepaticus* copy numbers would have been expected to have increased lesions relative to males with greater bacterial colonization.

The results of this study have important application for mouse husbandry, particularly for breeding programs where *H. hepaticus* is present in the colony. Some laboratory animal facilities make use of harem breeding schedules where one male is paired with more than one female mouse so that this relationship is maintained indefinitely through repetitive parturition events until a decline in reproductive efficiency emerges, requiring the replacement of one or more harem members. This breeding regime (1 male:2 females) is used because it could result in increased maternal sharing of rearing responsibilities, increased pup body weight (Reeb-Whitaker et al. 2000), and a reduction in the overall requirement for number of male mouse breeders. The findings of both experiment 1 and 2 suggest that harem breeding, where multiple mice are in the home cage at parturition, will likely increase *H. hepaticus* infection of offspring through increased exposure to infected fecal pellets. Hence the use of harem breeding schemes may be contraindicated in instances where elimination and control of *H. hepaticus* is desired.

Correlation and regression analysis of fecal *H. hepaticus* levels in experiment 2 proved useful in creating mathematical algorithms that can predict cecal bacterial load from fecal bacterial load. This information may be helpful since fecal samples can be collected in the live animal, whereas cecal samples can generally only be obtained through invasive surgery or in euthanized animals. These linear equation models predict that cecal bacterial load will be greater than fecal load when fecal quantitation data is in the low range, whereas fecal quantitation data may equal or exceed quantitation of cecal load at the high end of the range. One possible weakness of these models is that they incorporate a y-intercept
term, that is when $x \ (\log_{10} \text{fecal copy } H. \ hepaticus/ \mu g \text{ mouse DNA}) = 0$, the predicted $y$ value ($y = \log_{10} \text{cecal copy } H. \ hepaticus/ \mu g \text{ mouse DNA}$) does not $\neq 0$. This model may be a good fit however since our findings showed that cecal $H. \ hepaticus$ copy values were greater than fecal values in our factorial ANOVA. Therefore it is plausible that the intercept model of regression is a better fit than a no intercept model, since it is certainly feasible that a mouse with a low bacterial cecal colonization of $H. \ hepaticus$ may not have detectable copies of $H. \ hepaticus$ in its feces. Furthermore, the intercept model is arguably a better choice for predicting cecal values in this situation, since the no intercept model can often be misleading since it forces the regression response to pass through the origin and in doing so uses an uncorrected sum of squares (Freund et al. 1991). In spite of these limitations, these models for estimation of cecal $H. \ hepaticus$ load may still be useful for future work involving therapeutic efficacy, pathogenesis of $H. \ hepaticus$ associated lesion and cancer development, as well as for epidemiological studies. To the best of the authors’ knowledge, no such predictor relationships based on fecal $H. \ hepaticus$ quantitation data have thusfar been reported in the literature.

It is notable to mention that the results for experiment 1 may not be directly transferable to other strains of mice, where shedding is greater than that for C57BL/6 mice. Although many strains of laboratory animal mice utilize the C57BL/6 background (Linder 2003), genetically altered mice, or mice on different background strains could potentially shed more fecal $H. \ hepaticus$ than C57BL/6 mice. Additional studies should be performed to address this question. Because the exact bacterial level required for $H. \ hepaticus$ fecal transfer to neonates is unknown, it is possible that even if the sire of a non-C57BL/6 strain is removed from the birthing cage prior to parturition, a high shedding female mouse may be able to transfer the bacterium to the neonatal offspring earlier than four days of age. We recently conducted a large field trial involving several strains of mice, using the first day of age as the time by which fostering was performed. In that study, mouse strain did not appear to affect the success of neonatal fostering for deriving Helicobacter-free mice (Singletary et al 2003).

In conclusion, results of this study did not support the hypothesis that Helicobacter-positive feces from male C57BL/6 mice can influence the quantity of $H. \ hepaticus$ in the feces of their cage-mate, and no statistical significance at $p<0.05$ was shown for the effects of time or housing strategy in experiment 2. However our results did support our hypotheses that gender affects fecal and cecal Helicobacter load,
with males having an overall higher cecal and fecal *H. hepaticus* copy number. Additionally, we have proposed mathematical formulas based on regression analysis to estimate cecal *H. hepaticus* bacterial counts when fecal quantities of *H. hepaticus* are known. This paradigm may be useful for predicting cecal bacterial load, and may thereby reduce the need for euthanasia of C57BL/6 mice for cecal tissue collection required for bacterial load quantification. Finally, our results show that the technique of fostering C57BL/6 mice through day four of age can be applied to eliminate *H. hepaticus* from experimentally infected mice based on the DNA extraction and PCR techniques described within this study. This finding will facilitate rederivation efforts by allowing for a greater number of days by which fostering can occur. Future studies should evaluate fostering of C57BL/6 mice past day four of age with paternal separation prior to parturition, as well as consideration of the minimum bacterial *H. hepaticus* burden necessary to produce infection in neonatal animals.
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Table 1. Percentage of adult breeder Experiment 1 C57BL/6 mice shedding *H. hepaticus*, based on fecal PCR analysis, at the indicated week following final inoculation. These mice were used as sires and dams to produce progeny used in the fostering experiment to attempt derivation of *Helicobacter*-negative pups from *Helicobacter*-positive parents. The numbers in parentheses indicate the percentage of mice shedding the bacteria for that given week.

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<td>9/11 (81.82%)</td>
<td>4/6 (66.67)</td>
<td>13/17 (76.47)</td>
</tr>
<tr>
<td>12/26/03</td>
<td>20</td>
<td>8/11 (72.73%)</td>
<td>5/6 (83.33)</td>
<td>13/17 (76.47)</td>
</tr>
<tr>
<td>1/10/03</td>
<td>22</td>
<td>7/11 (63.64%)</td>
<td>3/6 (50)</td>
<td>10/17 (58.82)</td>
</tr>
<tr>
<td>1/23/04</td>
<td>24</td>
<td>11/11 (100)</td>
<td>4/6 (66.67)</td>
<td>15/17 (88.24)</td>
</tr>
<tr>
<td>2/6/03</td>
<td>26</td>
<td>9/11 (81.82%)</td>
<td>2/6 (33.33)</td>
<td>11/17 (64.71)</td>
</tr>
<tr>
<td>2/20/03</td>
<td>28</td>
<td>5/11 (45.45%)</td>
<td>2/6 (33.33)</td>
<td>7/17 (41.18)</td>
</tr>
<tr>
<td>3/5/04</td>
<td>30</td>
<td>10/11 (90.91%)</td>
<td>3/6 (50)</td>
<td>13/17 (76.47)</td>
</tr>
<tr>
<td>3/19/04</td>
<td>32</td>
<td>11/11 (100)</td>
<td>3/6 (50)</td>
<td>14/17 (82.35)</td>
</tr>
<tr>
<td>4/4/04</td>
<td>34</td>
<td>3/11 (27.77%)</td>
<td>0/5 (0)</td>
<td>3/16 (18.75)</td>
</tr>
</tbody>
</table>
APPENDIX B: EXPERIMENT 2 HOUSING DESIGN

Table 2. Male and female C57BL/6 mice were arranged into housing assignments prior to experimental inoculation with *H. hepaticus* in experiment 2. Several cage-mates within pairs were replaced prior to two weeks post-inoculation, however housing assignment category remained the same throughout the project for all mice. Group sizes at euthanasia were not equal at all cecal collection points for each housing assignment category.

<table>
<thead>
<tr>
<th>HOUSING ASSIGNMENT CATEGORY (PER CAGE)</th>
<th>CECAL COLLECTION POST-INOCULATION DATE (EUTHANASIA)</th>
<th>GROUP SIZE (n) AT EUTHANASIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) MALE/MALE PAIR</td>
<td>4 WEEKS (WKS 2, 4 FECAL)</td>
<td>6</td>
</tr>
<tr>
<td>b) SINGLE MALE</td>
<td>4 WEEKS (WKS 2, 4 FECAL)</td>
<td>5</td>
</tr>
<tr>
<td>c) FEMALE/FEMALE PAIR</td>
<td>4 WEEKS (WKS 2, 4 FECAL)</td>
<td>6</td>
</tr>
<tr>
<td>d) SINGLE FEMALE</td>
<td>4 WEEKS (WKS 2, 4 FECAL)</td>
<td>5</td>
</tr>
<tr>
<td>e) MALE/FEMALE PAIR</td>
<td>4 WEEKS (WKS 2, 4 FECAL)</td>
<td>12</td>
</tr>
<tr>
<td>a) MALE/MALE PAIR</td>
<td>8 WEEKS (WKS 2, 4, 6, 8 FECAL)</td>
<td>6</td>
</tr>
<tr>
<td>b) SINGLE MALE</td>
<td>8 WEEKS (WKS 2, 4, 6, 8 FECAL)</td>
<td>5</td>
</tr>
<tr>
<td>c) FEMALE/FEMALE PAIR</td>
<td>8 WEEKS (WKS 2, 4, 6, 8 FECAL)</td>
<td>6</td>
</tr>
<tr>
<td>d) SINGLE FEMALE</td>
<td>8 WEEKS (WKS 2, 4, 6, 8 FECAL)</td>
<td>6</td>
</tr>
<tr>
<td>e) MALE/FEMALE PAIR</td>
<td>8 WEEKS (WKS 2, 4, 6, 8 FECAL)</td>
<td>12</td>
</tr>
<tr>
<td>a) MALE/MALE PAIR</td>
<td>12 WEEKS (WKS 2, 4, 6, 8, 10, 12 FECAL)</td>
<td>6</td>
</tr>
<tr>
<td>b) SINGLE MALE</td>
<td>12 WEEKS (WKS 2, 4, 6, 8, 10, 12 FECAL)</td>
<td>6</td>
</tr>
<tr>
<td>c) FEMALE/FEMALE PAIR</td>
<td>12 WEEKS (WKS 2, 4, 6, 8, 10, 12 FECAL)</td>
<td>6</td>
</tr>
<tr>
<td>d) SINGLE FEMALE</td>
<td>12 WEEKS (WKS 2, 4, 6, 8, 10, 12 FECAL)</td>
<td>7</td>
</tr>
<tr>
<td>e) MALE/FEMALE PAIR</td>
<td>12 WEEKS (WKS 2, 4, 6, 8, 10, 12 FECAL)</td>
<td>12</td>
</tr>
</tbody>
</table>
VITA

Robin Crisler Roberts grew up in Greenwood, Arkansas, as a young girl. It was there she was exposed to and first developed an interest in animal care through trips with her parents to county fairs, and visits to the local veterinary hospital with her pet Golden Retrievers. During her high school years at Houston High, Germantown, Tennessee, she was inspired by several of her teachers as well as her parents to actively pursue a career in science, and in particular, veterinary medicine. After graduation, she moved to Lafayette, Louisiana, where she attended the University of Louisiana at Lafayette (ULL) on partial academic scholarship. During these years, she adopted her grandfather, Dr. Robert M. Crisler, a former ULL geography department head and former Louisiana state representative, as a mentor. Her grandfather’s father, Otto S. Crisler, had been a veterinarian who graduated from a private veterinary school in Indiana around 1914. Many of her grandfather’s stories about his father’s life intrigued her, and furthered her interest in becoming a veterinarian. During her undergraduate studies, she worked part-time at Doiron-Plauche Veterinary Hospital where she solidified her desire to attend veterinary school through her interactions with staff veterinarians who served as positive role models. After graduating *cum laude* with a Bachelor of Science in animal science at ULL, she entered into the veterinary medicine program at Louisiana State University (LSU) which led to a doctorate in veterinary medicine. Because of her continued interest in science, an introductory veterinary school course on alternative careers in veterinary medicine at LSU, and based on the experiences of a personal friend and fellow veterinary student (Dr. Kem B. Singletary), she applied to a residency program in laboratory animal medicine at LSU. After completing the residency portion of the combined master of science degree program, she successfully found employment as a clinical veterinarian at a major university in Indiana where she currently resides. Despite the long road to achieve qualifications to become employed in this field, she has found the journey both fulfilling and worthwhile, and enjoys her career immensely. After completion of the Master of Science degree at LSU, she plans to work towards becoming a Diplomate of the American College of Laboratory Animal Medicine. By becoming specialized in this particular veterinary branch, she hopes to make a lasting contribution to both research and animal care through her active participation in this challenging and rewarding field. She is deeply grateful for the support over the years
that she has received from the many people who have encouraged her in this endeavor, and who have thereby given her this unique and lasting opportunity in life.