Title: Effects of sexual dimorphism and estrous cycle on *C. difficile* infections in rodent models

Running title: *Sex effects on rodent C. difficile infections*

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Abstract

Clostridioides difficile infection (CDI) is responsible for the majority of identifiable hospital-related antibiotic-associated diarrhea. Susceptibility to CDI and severity of disease varies depending on a variety of factors such as aggressive use of broad-spectrum antibiotics, age, and immune status. Epidemiological studies have consistently shown that female patients are more at risk for CDI than their male counterparts. In this study, we show that female mice developed more severe CDI than their male counterparts when challenged with spores from three different *C. difficile* strains. CDI sexual dimorphism was still apparent when animals were placed under diet conditions that exacerbated CDI severity. Unlike male mice, females undergo the estrous cycle. Thus, female mice were challenged with *C. difficile* spores when they were at the estrus, metestrus, diestrus, late diestrus/early proestrus, proestrus, or late proestrus/early estrus stages. Animals were scored for CDI sign severity while continuously monitoring their estrous cycle stages. The resulting data showed a striking spike in CDI severity when animals were in proestrus the day before sign scoring. In contrast, animals who were in estrus the day before signs scoring were protected from CDI. Prophylactic treatment of CDI also showed sexual dimorphism with females responding better to treatment than males. Interestingly, infection sexual dimorphism was reversed in hamsters, with male hamsters developing more severe CDI signs than females. In conclusion, we have shown that mice recreate many of the conditions of sexual dimorphism of human CDI.

Background

Clostridium difficile infection (CDI) is responsible for approximately 25% of all antibiotic-associated diarrhea [1]. CDI has been designated as an urgent threat by the CDC [2]. In the U.S., over 223,900 CDI cases occur annually, with a mortality rate of up to 6.5% and costs estimated to be $6.3 billion [3]. The incidence of CDI is complicated by the appearance of highly resistant and hypervirulent strains [4, 5].

CDI treatments are limited. Recent guidelines discourage the use of cheap metronidazole due to the propensity for triggering relapses [6]. Oral vancomycin is now recommended for primary CDI [7]. Fidaxomicin is a newer antibiotic that causes fewer CDI relapses, but it is significantly more costly [8, 9]. Monoclonal antitoxins can reduce CDI morbidity and mortality, but do not reduce *C. difficile* colonization [10, 11]. Fecal transplantation has been used effectively as CDI treatment [12] but can transfer other deadly pathogens [13].

Epidemiological studies have shown that antibiotic use is the main risk factor associated with CDI [14, 15]. Immunocompetence is also strongly correlated with CDI symptom onset [16-18]. Furthermore, older populations are more susceptible to CDI than younger cohorts [14].

Bacterial infections have known sexual dimorphism [19] with human females tending to be more susceptible to genitourinary tract infections [20, 21], while human males are more prone to gastrointestinal [22] and respiratory infections [23].

When adjusted for age, immune status, and antibiotic exposure, sex is also an important risk factor in human CDI. Contrary to other GI bacterial infections [19], women are more vulnerable than men to both primary CDI and CDI relapse [24-31]. More recently, CDI has been flagged as an emerging threat to pregnant and peripartum women [32-35]. Similarly, older

women might be at higher risk of developing CDI due to permanent hormonal depletion after menopause. These last two conditions are associated with substantial changes in hormonal levels.

An obvious factor underlying human sexual dimorphisms is the fluctuating hormone concentrations during the interdependent ovarian cycle and uterine (menstrual) cycle [36]. Following menses, the follicular stage of the ovarian cycle is characterized by increased circulating estrogen that then triggers spikes of luteinizing hormone (LH), follicle stimulating hormone (FSH), and prolactin (PRL) just prior to the periovulatory period [36]. At the start of the luteal stage, ovulation occurs with concurrent small increases in PRL [37]. The mid-luteal stage is characterized by increases of both estradiol and progesterone, followed by a sharp decrease of both hormones in the premenstrual stage.

Similar to humans, female rodents undergo hormonal changes during the estrous cycle [38]. Estradiol levels increase during the proestrus stage, followed by spikes in LH and FSH [39]. The estrus stage is dominated by rapid increase of PRL, followed by a sharp decrease to trigger metestrus entry. The metestrus stage shows a sharp increase of progesterone, with a smaller simultaneous increase in estradiol. Finally, progesterone levels peak during the diestrus stage. Testosterone is produced uniformly throughout the estrous cycle, but its circulating concentration is 10-fold lower than in males [40]. Hence, similar to the human female menstrual cycle, each estrous cycle stage is characterized by a unique pattern of hormone levels.

C. difficile forms spores that act as infection vehicles for CDI [41]. Spores do not cause disease but recognize bile salts to germinate into toxin-producing cells [42-47]. The spore germination process is a required first step in establishing infection in both humans and animals. We have shown that steroidal sex hormones, but not prostaglandins, can directly interact with *C. difficile* spores and modulate germination [48, 49]. Similarly, since spore germination is a

necessary step for disease onset, we have found that sterane analogs can inhibit this process and prevent CDI onset in rodent models of disease [11, 50-59]. During our search for CDI prophylactics, we serendipitously observed that male mice tended to develop less severe CDI than their female counterparts.

In this study, we used rodent models of CDI to assess for sexual dimorphism during infection. We found that female mice developed more severe CDI signs than males when infected with three different *C. difficile* strains. Sexual dimorphism was still apparent under diet conditions known to trigger severe CDI. We also found that at any time point during disease progression, murine CDI sign severity correlated with the estrous stages that animals were on the prior day. As a result of this delayed effect, animals were protected from CDI onset if they were in estrus the day before. In contrast, animals developed deadly CDI signs if they were in proestrus the day before. CDI prophylaxis also showed clear sexual dimorphism. Even though male mice developed less severe CDI, they were also more refractory to treatment. On the other hand, anti-germinants protected female mice from developing CDI during most stages of their estrous cycle. Interestingly, infection sexual dimorphism was reversed in hamsters, with male hamsters developing more severe CDI signs than females.

Materials and Methods

Materials: *C. difficile* strains R20291 and 9001966 were donated by Professor Nigel Minton at the University of Nottingham, United Kingdom. *C. difficile* strain 630 was obtained from the American Type Culture Collection (ATCC). Synthetic bile salt analogs were prepared by Professor Steven M. Firestine at Wayne State University in Detroit, Michigan, or were previously synthesized in the Abel-Santos laboratory. Rodent diets were purchased from LabDiet (St. Louis, MO, USA). All growth of *C. difficile* strains was done in a Coy Laboratories vinyl anaerobic chamber (5% H_2, 5% CO_2, 95% N_2).

Animals: All procedures involving animals in this study were performed in accordance with the Guide for Care and Use of Laboratory Animals outlined by the National Institutes of Health. The protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Nevada, Las Vegas (Permit Number: R0914-297). Weaned C57BL/6 mice and weaned Golden Syrian hamsters were purchased from Charles River Laboratories (Wilmington, MA, USA). Mice were housed in groups of five per cage and hamsters were housed individually at the University of Nevada, Las Vegas animal care facility. Upon arrival at the facility, animals were allowed to acclimate for at least one week prior to the start of experimentation. All bedding, cages, food, and water were autoclaved prior to contact with animals. All post-challenge manipulations were performed within a biosafety level 2 laminar flow hood.

***C. difficile* spore harvest and purification:** *C. difficile* cells from frozen stocks were streak plated in an anaerobic chamber onto BHI agar supplemented with 2% yeast extract, 0.1% L-cysteine-HCl, and 0.05% sodium taurocholate (BHIS) to yield colonies (15). After 48 hours, a single colony was inoculated into BHI broth supplemented with 0.5% yeast extract and incubated

for 48 hours. The inoculated broth was then spread plated onto multiple BHI agar plates prepared as described above. Inoculated plates were incubated for 7 days at 37 °C.

Plates were then flooded with ice-cold deionized (DI) water. Cells and spores were harvested by scraping bacterial colonies from the plates. The cell and spore mixtures were pelleted by centrifugation at 8,000 ×g for 5 minutes, resuspended in DI water, and pelleted again. This washing step was repeated twice more. The cell/spore mixture was then centrifuged through a 20% to 50% HistoDenz gradient at 18,200 ×g for 30 minutes with no brake (22). Under these conditions, spores pelleted at the bottom of the centrifuge tube, while cell debris remained above the 20% HistoDenz layer. Pelleted spores were transferred to a clean centrifuge tube and washed five times before storing in DI water at 4 °C. To determine spore purity, selected samples were stained using the Schaeffer-Fulton endospore staining method (23) or were visualized by phase-contrast microscopy. Spore preparations used were greater than 95% pure.

Estrous cycle tracking in mice: The estrous cycle tracking method performed in this study was adapted from *McLean et al., 2012* [39]. Mice estrous stages were tracked for at least one week prior to any manipulation to make sure animals were cycling normally. Animals were tracked daily for their estrous cycle stage by vaginal lavage at the same time each morning throughout the duration of the study. Briefly, excess urine is dabbed off using Kimwipes low-lint wipes. A micropipette was used to lavage 20 μL of DI water into the vaginal orifice of the animal. The vaginal orifice was flushed 3-4 times using the micropipette until the suspension appeared mildly cloudy. The 20 μL sample was then transferred onto a clean glass microscope slide and left to air dry for at least 1 hour. Animals were monitored to ensure there were no signs of vaginal infection.

Air-dried vaginal sample slides were dipped into a 2% crystal violet solution inside a Coplin jar for 1 minute. Slides were then moved to a Coplin jar containing fresh DI water to wash off excess stain. Slides were then dabbed gently with clean wipes, allowed to air dry, and examined using a compound light microscope at 100x and 400x magnification.

Using vaginal cytology and staining, estrous cycle stages can be identified based on the presence of either nucleated epithelial cells, cornified (keratinized) anucleated epithelial cells, leukocytes, or combinations of these cell types (Fig. S1). The proestrus stage is dominated by the presence of nucleated epithelial cells (blue arrows). In contrast, when animals are in the estrus stage, cornified anucleated epithelial cells are in abundance (yellow arrows). This stage can also show the presence of small specks of bacteria adhering to the epithelial cells (black arrow in the insert). In the metestrus stage, all three major cell types are present. When animals transition to the diestrus stage, samples contain mostly leukocytes (gray arrows) with scattered appearances of a few nucleated epithelial cells.

Cytologic characteristics can also show intermediate stages of the estrous cycle (Fig. S1) [60]. Small, irregular shaped cells (green arrows) can signify the formation of nucleated cells and are representative of late diestrus/early proestrus. Some lingering leukocytes may also be present at this stage. Late proestrus/early estrus is usually marked by a transition of nucleated cells to enucleation leaving a halo-like appearance (pink arrow) in the middle of the cell.

Murine CDI Model: The murine CDI model used in this study was adapted from Chen *et al.* [58]. Mice were given three consecutive days of antibiotic cocktail containing kanamycin (0.4 mg/ml), gentamicin (0.035 mg/ml), colistin (850 U/ml), metronidazole (0.215 mg/ml), and vancomycin (0.045 mg/ml) *ad libitum* (14). Mice were then given autoclaved DI water for the remainder of the experiment. On the day prior to *C. difficile* challenge (day -1), mice were given

an intraperitoneal (IP) injection of 10 mg/kg clindamycin. On the day of infection (day 0), experimental groups were challenged with 10^8 *C. difficile* spores by oral gavage. To test for CDI prophylaxis, mice were given 50 mg/kg daily gavage doses of either CamSA or CaPA dissolved in DMSO at 0-, 24-, and 48-hours post-challenge. As control, a group of animals were given neat DMSO excipient.

Mice were observed for signs of CDI twice daily and disease severity was scored according to a CDI sign rubric adapted from previously published work [53]. According to the rubric, pink anogenital area, mild wet tail, and weight loss of 8-15% were each given an individual score of 1. Red anogenital area, lethargy/distress, increased diarrhea/soiled bedding, hunched posture, and weight loss greater than 15% were each given an individual score of 2. After adding all scores, animals scoring less than 3 were indistinguishable from noninfected controls and were considered non-diseased. Animals scoring 3–4 were considered to have mild CDI. Animals scoring 5–6 were considered to have moderate CDI. Animals scoring greater than 6 were considered to have severe CDI and were immediately culled.

Hamster CDI Model: The hamster CDI model used in this study was adapted from procedures reported previously by *Howerton, et al.* [55]. Hamsters were orogastrically dosed with 30 mg/kg clindamycin 1 day prior to infection (day -1). On day 0, animals were challenged with 50 *C. difficile* strain 630 spores. Hamsters were also given a subclinical dose of 1 mg/kg vancomycin daily from day 0 to day 2 to prevent clindamycin-induced colitis. To test for CDI prophylaxis, hamsters were given a 300 mg/kg daily gavage dose of CaPA dissolved in DMSO for 11 days beginning on the day of infection. Animals were observed for CDI signs. Symptomatic animals were immediately culled.

Statistical Analyses: For the murine CDI model, severity of signs was analyzed by box-and-whisker plots with a minimum of three independent values ($n \geq 3$). Single factor ANOVA or one-tailed *t*-test were performed using the *R*-suite to assess differences between groups at every time point. ANOVA results with *p*-values <0.05 were analyzed *post hoc* using the Scheffe test for pairwise comparison between all groups. Statistical differences in Kaplan-Meier survival plots were performed by log rank test.

Results

Sexual dimorphism of CDI severity in mice: We infected male mice (n=5) and female mice (n=5) with three different *C. difficile* strains [50]. One cohort of male and female mice was infected with spores from *C. difficile* strain 9001966 (ribotype 106). This strain shows slow CDI onset (Fig. 1A). For both sexes, mice started to develop CDI symptoms on day 2 post-challenge. Male mice showed varied CDI sign severities during days 2-4, but all animals recovered by day 5. Female mice, on the other hand, showed more homogenous mild to moderate CDI symptomatology in days 2-3 and all animals recovered by day 4. Even though the CDI timeline was shorter in female mice, these animals developed statistically more severe signs than their male counterparts at the peak of infection.

A second cohort of male and female mice was infected with spores from *C. difficile* strain 630 (ribotype 012). In both male mice (n=10) and female mice (n=10) infected with *C. difficile* strain 630, CDI sign severity peaked on day 2 post-challenge. Male mice infected with *C. difficile* strain 630 spores showed less individual variability in CDI symptomatology and shorter infection progression compared to animals infected with *C. difficile* strain 9001966. Male mice infected with *C. difficile* strain 630 did not show any signs on day 1 after challenge but showed moderate CDI signs by day 2. CDI signs for male mice started to resolve by day 3 and completely disappeared by day 4 post-challenge. In contrast. female mice started to develop mild CDI signs one day after spore challenge (Fig. 1B). By day 2, female mice showed a notable increase in CDI sign severity. Similar to male mice, female mice returned to mild CDI signs by day 3 and were in complete remission by day 5 post-challenge. Notably, male mice showed mostly mild-to-moderate sign severity, while female mice showed statistically more severe symptomatology (Fig. 1B).

A third cohort of male mice (n=10) and female mice (n=10) was infected with spores from hypervirulent *C. difficile* strain R20291 (ribotype 027). In both male and female mice, CDI progression was faster than in mice challenged with either strain 9001966 or 630. Male mice challenged with *C. difficile* strain R20291 started to develop moderate CDI symptoms by day 1 post-spore challenge (Fig. 1C). Male mice remained moderately symptomatic until day 4, when CDI signs started to resolve. Similar to animals infected with *C. difficile* strain 9001966 and *C. difficile* strain 630, all male mice infected with *C. difficile* strain R20291 recovered and showed no CDI signs by day 5. In contrast, female mice challenged with *C. difficile* strain R20291 spores developed statistically more severe CDI signs than male mice the day after spore challenge (Fig. 1C and 2A). Indeed, while all males infected with *C. difficile* strain R20291 recovered from CDI, more than half of the female cohort succumbed to the infection (Fig. 2B, $p = 0.049$). Surviving female mice started to slowly recover from CDI and showed no symptoms by day 4.

Effect of diet on CDI severity in mice: We recently showed that an Atkins-type diet can exacerbate CDI severity in mice [53]. Male (n=5) and female (n=5) mice challenged with *C. difficile* strain R20291 while on this high protein/high fat (Atkins-type) diet showed delayed onset of CDI signs compared to animals fed a standard diet (Fig. 2A). Although female mice fed an Atkins-type diet did not show statistically more severe CDI signs (Fig. 2A) or survival (Fig. 2B) than female mice fed a standard diet, by day 3 post-challenge all female mice on the Atkins-type diet had developed severe CDI and had to be culled.

In contrast, once the infection started, challenged male mice fed an Atkins-type diet developed statistically more severe CDI signs compared to challenged mice fed a standard diet (Fig. 2A). Indeed, while all male mice on a standard diet survived CDI, 75% of male mice fed an

Atkins-type diet developed signs severe enough to require euthanasia (Fig. 2B). In fact, survival of male mice on an Atkins-type diet was statistically worse than male mice fed a standard diet (p=0.025), statistically identical to female mice fed a standard diet, but still statistically better (p=0.018) than females on the same Atkins-type diet.

Effect of estrous stage on CDI severity: Using vaginal smear cytology, we were able to monitor the estrous cycle of female mice (Figs. 3 and 4). Animals (n=60) were binned into estrus stage (blue columns), metestrus stage (orange columns), diestrus stage (green columns), and proestrus stage (magenta columns). Some animals could not be unambiguously assigned to a specific estrous stage [60]. These animals showed borderline cytology readings and were thus binned into two new categories: late diestrus/early proestrus (pink columns) and late proestrus/early estrus (yellow columns).

To determine the effect of the estrous cycle on murine CDI severity, female mice in distinct stages of the estrous cycle were challenged with *C. difficile* strain R20291 spores. We continued to simultaneously monitor the estrous cycle and CDI sign progressions.

As the estrous cycle does not stop during the course of the infection, animals could move across different estrous stages while CDI signs are being monitored. As a first approach, we tried to correlate daily CDI signs for each animal with their concurrent estrous stage, but we found no statistical significance between groups (Fig. 3A).

Intriguingly, when daily CDI severity was plotted against the estrous stages of the prior day, we found distinct daily patterns (Fig. 3B). The strongest statistical correlation between CDI signs and prior day estrous phases was found on day 1 post-infection. Animals that were on estrus when challenged with *C. difficile* spores (day 0), showed no CDI signs the following day

(day 1). Compared to animals that were in estrus on day 0, animals that were in metestrus, diestrus, and late diestrus/early proestrus during spore challenge developed statistically higher CDI severity the following day. Finally, animals that were at proestrus at day 0 developed the most severe CDI symptoms the day after challenge with more than half reaching the clinical endpoint.

The effect of prior-day estrous phases on CDI sign severity started to dissipate by day 2 post-challenge. Indeed, on day 2 post-challenge, animals that were on estrus on day 1 showed no CDI symptoms. In contrast, animals that were on metestrus, diestrus, or late proestrus/early estrus on day 1 post-challenge showed statistically higher CDI sign severity. Unfortunately, there were not sufficient animals in the proestrus and the late diestrus/early proestrus groups on day 1 for a complete statistical analysis of CDI severity on day 2 post-challenge. Furthermore, because of the high CDI-mediated mortality on days 1 and 2, there were not sufficient animals left for statistical analysis on CDI score vs estrous stages on day 3 post-challenge.

To determine if the prior-day estrous effect is independent of the infection time course, we combined the data for each estrous stage regardless of the day they were determined. This allowed us to increase the number of animals in each estrous stage for better statistical power. As expected, plotting CDI severity against their concurrent estrous stages showed no statistically significant differences (Fig. 4A).

In contrast, when CDI severity for each animal was plotted against their prior-day estrous stage, a similar delayed pattern was observed as in Fig. 3B. Indeed, when animals were in estrus at any day during infection, they showed few CDI signs the next day (Fig. 4B). Animals that were in metestrus, diestrus, and diestrus/proestrus at any time showed more varied but similar intermediate symptomatology 24 hours later. In contrast, animals in proestrus at any time during

infection became extremely sick the next day and had to be culled. Any animals in proestrus/estrus showed next-day CDI severity intermediate between proestrus and estrus.

Sexual dimorphism of mouse response to anti-germinant prophylactics: To evaluate the effect of sexual dimorphism in CDI prevention, male mice (n=5 per group) challenged with *C. difficile* strain 9001966 spores were treated with either DMSO excipient (Fig. 5A, solid blue columns), CamSA (dashed blue columns), or CaPA (checkered blue columns). Even though untreated male mice developed less severe CDI than untreated female mice (Fig. 1A), males did not respond to the CDI prophylaxis activity of either CamSA or CaPA at any time during the infection process (Fig. 5A).

In contrast, CaPA-treated female mice (Fig. 5B, checkered pink columns) showed statistically less severe CDI symptoms on both days 2 and 3 post-challenged compared to untreated controls (solid pink columns). CamSA treatment of female mice was less effective and showed protection only on day 3 post-infection (dashed pink columns).

CDI prophylaxis by CaPA in female mice (n=10 per group) was also evaluated against the more virulent *C. difficile* strain R20291 (Fig. 5C). Daily pairwise comparison shows that CaPA-treated female mice (checkered pink columns) developed less severe CDI symptoms at days 1 and 2 post-infection compared to control DMSO-treated animals (solid pink columns).

To understand the effect of estrous cycle on CDI prophylaxis, female mice were infected with hypervirulent *C. difficile* strain R20291 (Fig. 5D) and treated with excipient (n=60, solid-colored columns) or CaPA (n=18, checkered-colored columns). Pairwise comparisons showed that animals that were at any time in estrus (blue columns) or metestrus (orange columns) failed to respond to CaPA-treatment. In contrast, CaPA-treatment resulted in statistically significant

reduction of next-day CDI sign severity for animals that were at any point in diestrus (green columns), diestrus/proestrus (pink columns), proestrus (magenta columns), and proestrus/estrus (yellow columns).

Sexual dimorphism in a hamster model: Sexual dimorphism of CDI severity was also evaluated in the hamster model of CDI (n=5 per group). In contrast to mice, male hamsters showed slightly higher mortality than females (Fig. 6, p=0.06). Indeed, all male hamsters were deceased by day 3 (solid blue circles), while 40% of female hamsters survived to the end of the experiment (solid pink circles). Moreover, when hamsters were treated with CaPA, male hamster mortality was delayed but all animals became moribund by day 6 (checkered blue squares), whereas female hamsters treated with CaPA (checkered pink circles) showed statistically higher survival rate than CaPA-treated males (p=0.03).

Discussion and Conclusions

Few attempts have been made to understand how sex affects CDI onset and severity. To assess the clinical importance of sex on human CDI, we did an extensive PUBMED search for primary articles relating to CDI risks. We found that most studies (n=87) did not stratify for sex. Of the studies that assessed sex as a variable (n=24), most linked CDI to other comorbidities. These studies tended to have small sample sizes, making it difficult to determine if sex correlated to CDI or to the underlying comorbidity. However, when CDI was the primary diagnosis, multiple large cohort studies showed that women are at higher risk than men of developing symptoms [24, 25, 27, 28, 61]. Indeed, a multicenter retrospective cohort study of over 1 million patients at 150 US hospitals showed that the odds of CDI in women was 1.2 times as likely as in men [61]. Although the correlation between sex and human CDI is clear, testing for susceptibility in women is cost prohibitive and impractical. Yet patient outcomes could be dramatically improved if clinicians could adapt antibiotic treatments to match specific hormone levels.

Even though there is no standardized scale for human CDI symptomatology, CDI is not diagnosed unless symptoms are severe enough to warrant clinical intervention. In that sense, CDI diagnosis serves as a surrogate for symptoms severity in humans. Similar to other infection models, murine CDI provides an idealized and controlled (albeit imperfect) model to study disease onset, severity, and progression.

The murine CDI model presents symptoms progression similar to human CDI and responds to the same treatments [62, 63]. We have found that murine CDI follows a well-ordered progression of disease signs. Based on these observations, we have implemented a rubric for CDI severity development [50, 57].

In this study, the murine model recapitulates many of the differences in CDI outcome observed between men and women [24, 25, 27, 28, 61]. For instance, during our investigations into the factors affecting CDI progression and prophylaxis [50, 56, 57], we serendipitously found that female mice developed more severe CDI signs than males.

To further test for sexual dimorphism in the murine model of CDI, we infected mice with three different *C. difficile* strains. These strains represent different ribotypes and were selected because they show distinct timelines for CDI onset. *C. difficile* strain 9001966 (ribotype 106) is a clinical strain from the United Kingdom and shows delay onset of murine CDI. *C. difficile* strain 630 (ribotype 012) is a type strain [64, 65] that we have extensively tested for its capacity to induce CDI symptoms in mice [56, 57]. Finally, *C. difficile* strain R20291 (ribotype 027) is a hypervirulent strain that causes earlier infection onset and more severe murine CDI signs than other strains [50].

When animals were infected with any of these strains, female mice developed statistically more severe CDI than their male counterparts. The timing of these differences varied, but in every case, the most significant differences in CDI severity coincided with the peak of disease severity in their respective infection timeline.

In contrast to hamsters [55, 66], murine CDI seldom tends to be lethal [50, 56, 57]. However, we recently showed that an Atkins-type (high-protein/high-fat) diet can greatly increase murine CDI sign severity and mortality [53]. When animals were fed this diet, mortality of male mice increased significantly. Even then, male mice were still better off than females fed the same diet. This shows that the sexual dimorphism of murine CDI is still present under conditions where CDI is severe.

The most obvious difference between male and female mice is the female estrous cycle. However, intraday correlation between estrous cycle and CDI severity showed no statistical differences at any time during the infection. In contrast, estrous stages seem to have a 24-hour delayed effect on CDI severity. This delay might be due to the time needed for hormones to affect host physiological responses during infection.

The interplay between estrous phase succession and CDI progression is quite complex. In female mice, CDI caused by *C. difficile* strain R20291 starts and reaches a maximum of severity on the day following spore challenge. Surviving animals completely recover by day 4 post-challenge. In contrast, the estrous cycle can take between 3-6 days to be completed. Furthermore, the duration of each estrous stage is variable, with proestrus and metestrus lasting as little as 8 hours and diestrus lasting as long as 72 hours [67]. Due to these differential timelines, most of the animals were infected during metestrus and very few were infected during diestrus. This was further complicated by the observation that estrous stages showed a 24-hour delayed effect on CDI severity. Thus, binning animals into different estrous stages for each day reduced our statistical power for some of the estrous stages.

Nevertheless, our data clearly shows that estrus decreases CDI severity and proestrus exacerbates CDI signs on the day following spore challenge. We have previously shown that *C. difficile* spores germinate between 6- and 9-hours post-challenge in the intestines of mice. The strong association between day 0 estrous phases and day 1 CDI severity could be due to estrous cycle influencing the initial stages of CDI onset.

On the other hand, the 24-hour delay of estrous cycle effects seems to continue throughout the infection timeline. This suggests that estrous stages can increase or decrease female mice susceptibility to CDI also at the later stages of the infection course. It would be

interesting to determine if this prior-day estrous effect is still present when animals are challenged with *C. difficile* strains (9001966 and 630) that cause slower CDI onset.

Anti-germination approaches represent a new therapeutic venue to prevent CDI onset. When evaluated against the slow onset *C. difficile* strain 9001966, we found sexual dimorphism in CDI prophylaxis. Indeed, even though males developed less severe CDI than females, anti-germination compounds did not affect the course of male infection. In contrast, female mice were protected from the most severe CDI signs by both CaPA and CamSA, although CaPA seems to be more effective over time.

CaPA was also active in preventing CDI in female mice infected with hypervirulent strain R20291. Intriguingly, the effect of CaPA on CDI sign severity shows the same 24-hour delay following each estrous stage. The only exception to CaPA prophylaxis activity were animals that came out of estrus and metestrus. The estrous stage independence from CaPA action is easily explainable since following estrus, animals naturally do not develop CDI, making CaPA treatment redundant. The refractivity to CaPA of animals coming from metestrus is baffling, since in all other estrous stages, CaPA was able to completely prevent CDI onset. The metestrus abnormality in the CDI prevention/estrous cycle axis needs to be investigated further.

Rodent models of CDI have been invaluable to study infection progression as well as to test for new treatments [62, 68]. Mice are quite resistant to CDI and require pre-conditioning with aggressive antibiotic treatment [58]. Nevertheless, once CDI is established, the murine model shows symptom progression that mimics human CDI [68].

Even though the hamster is the most used CDI model, disease onset is fulminant [69]. These conditions are rarely seen in human primary CDI. On the other hand, vancomycin treatment of infected hamsters results in delayed sign onset similar to human CDI relapse [69,

70]. Hence, the two CDI rodent models complement each other and cover the spectrum of human CDI symptomatology [55, 68].

Because CDI onset and symptomatology diverge in mouse and hamsters, it is not uncommon to find distinct responses to infection and treatments. Indeed, we recently found that carbohydrate-rich diets are protective in murine CDI [53], but result in delayed CDI onset in hamsters [66]. Here we found a similar mirror effect between the two models: while female mice developed more severe CDI signs, female hamsters were slightly more resistant to CDI-mediated mortality and responded better to CDI prophylaxis. The causes for these interspecies differences are currently under investigation.

The contrast between proestrus-mediated and estrus-mediated murine CDI severity could not be starker. While CDI is most deadly following proestrus, prior-day estrus offers strong CDI protection to animals. Furthermore, prior-day metestrus and diestrus correlate with more heterogeneous CDI symptomatology.

An obvious factor underlying CDI sexual dimorphism is the fluctuating hormone levels during female ovulation [36]. Indeed, CDI-exacerbating proestrus is characterized by large increases in estradiol, progesterone, LH, and FSH levels and low concentrations of PRL [39]. These spikes in hormone levels then allow entry into the CDI-protective estrus. At this point, the high concentrations of circulating estradiol, progesterone, LH, and FSH characteristic of proestrus are severely diminished followed by an increase of serum PRL. The metestrus and diestrus stages tend to have hormonal levels that fall in between those of proestrus and estrus [39].

Hormones have strong effects on both human and murine metabolisms [71] and can affect cell-mediated and humoral immune responses [72-74], as well as susceptibility to bacterial

infections [19]. Changes in sex hormones levels can also affect the gut microbiota [75-77], the main protective barrier against *C. difficile* colonization [78].

Previous work in the Abel-Santos laboratory has shown that steroidal sex hormones, but not prostaglandins, can directly interact with *C. difficile* spores and modulate the germination process [48]. Interestingly, sex steroidal hormones are present at low levels in the intestinal tract [49]. Due to the heterogeneous environment of the intestinal lumen, there could be localized areas of higher hormone concentrations where they could interact with *C. difficile* spores.

Together, these data suggest that the estrous cycle and its concurrent hormonal changes could affect CDI symptomatology directly by modulating spore germination in the intestine and/or indirectly by globally changing the host's gut microbiome and/or immune status. The effect of individual hormones on the biovariables that affect CDI severity needs to be established.

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Figure Legends

Figure 1. *Effect of sex on murine CDI severity*. Box-and-whisker plots of CDI sign severity in male mice (blue bars) and female mice (pink bars) challenged with *C. difficile* spores from (A) strain 9001966, (B) strain 630, and (C) strain R20291. Signs of severity were determined daily based on the CDI scoring rubric reported previously [53]. Animals with a score of <3 were indistinguishable from noninfected controls and were considered non-diseased. Animals with a score of 3 to 4 were considered to have mild CDI. Animals with a score of 5 to 6 were considered to have moderate CDI. Animals with a score of >6 were considered to have severe CDI and were immediately culled. Dashed line represents the clinical end point. Horizontal bold lines represent median values, asterisks represent mean values, bars represent interquartile ranges, whiskers represent maximum and minimum values, and dots represent inner point values. Daily statistical differences between males and females were determined by one-tailed *t*-test.

Figure 2. *Effect of Atkins-style diet on murine CDI severity and survival*. (A) Box-and-whisker plots of CDI sign severity and (B) Kaplan-Meier survival plots for female mice fed a standard diet (dark pink), male mice fed a standard diet (dark blue), female mice fed an Atkins-type diet (light pink), and male mice fed an Atkins-type diet (light blue) challenged with *C. difficile* spores from strain R20291 [53]. For panel A, single-factor ANOVA was performed at every time point to assess differences among the sign severity means of the different groups. ANOVA results with P values of <0.05 were analyzed *post hoc* using the Scheffe test for pairwise comparison between daily groups. Columns that are labeled with different letters are statistically different within the same day. For panel B, statistical survival comparisons of different groups were performed by log rank test.

Figure 3. *Time-dependent effect of estrous cycle on daily murine CDI severity*. Box-and-whisker plots of daily CDI sign severity plotted against (A) their concurrent estrous stages and (B) their prior-day estrous stages. Animals in estrus (blue bars), metestrus (orange bars), diestrus (green bars), late diestrus/early proestrus (pink bars), proestrus (magenta bars), and late proestrus/early estrus (yellow bars) were challenged with *C. difficile* spores from strain R20291 on day 0. CDI sign severity and estrous cycle stages were determined daily. Signs of severity were determined based on the CDI scoring rubric reported previously [53]. Single-factor ANOVA was performed at every time point to assess daily differences among the sign severity means of the different estrous stages. ANOVA results with P values of <0.05 were analyzed *post hoc* using the Scheffe test for pairwise comparison between daily groups. Columns that are labeled with different letters are statistically different within the same day.

Figure 4. *Time-independent effect of estrous cycle on murine CDI severity*. Cumulative box-and-whisker plots of CDI sign severity caused by *C. difficile* strain R20291 plotted against (A) their concurrent estrous stages, and (B) their prior-day estrous stages. Animals that were at estrus (blue bars), metestrus (orange bars), diestrus (green bars), late diestrus/early proestrus (pink bars), proestrus (magenta bars), and late proestrus/early estrus (yellow bars) at any day were respectively pooled together. CDI sign severity and estrous cycle stages were determined daily. Signs of severity were determined based on the CDI scoring rubric reported previously [53]. Single-factor ANOVA was performed to assess differences among the sign severity means of the different pooled estrous stages. ANOVA results with P values of <0.05 were analyzed *post hoc* using the Scheffe test for pairwise comparison between all groups. Columns that are labeled

with different letters are statistically different.

Figure 5. *Effect of sex and estrous cycle on murine CDI prophylaxis.* (A) Box-and-whisker plots of CDI sign severity in male mice challenged with *C. difficile* spores from strain 9001966 and treated with DMSO (blue solid bars), CamSA (blue striped bars), or CaPA (blue checkered bars). (B) Box-and-whisker plots of CDI sign severity in female mice challenged with *C. difficile* spores from strain 9001966 and treated with DMSO (pink solid bars), CamSA (pink striped bars), or CaPA (pink checkered bars). (C) Box-and-whisker plots of CDI sign severity in female mice challenged with *C. difficile* spores from strain R20291 and treated with DMSO (pink solid bars), or CaPA (pink checkered bars). (D) Cumulative box-and-whisker plots of CDI sign severity caused by *C. difficile* strain R20291 plotted against their prior-day estrous stages and treated with DMSO (solid bars) or with CaPA (checkered bars). Animals that were at estrus (blue bars), metestrus (orange bars), diestrus (green bars), late diestrus/early proestrus (pink bars), proestrus (magenta bars), and late proestrus/early estrus (yellow bars) at any day were respectively pooled together. CDI sign severity and estrous cycle stages were determined daily. Signs of severity were determined based on the CDI scoring rubric reported previously [53]. For panels A and B, single-factor ANOVA was performed at every time point to assess daily differences among the sign severity means of the DMSO-treated, CaPA-treated, and CamSA-treated groups. ANOVA results with P values of <0.05 were analyzed *post hoc* using the Scheffe test for pairwise comparison between daily groups. Columns that are labeled with different letters are statistically different. For panels C and D, daily statistical differences between DMSO-treated and CamSA-treated animals were determined by one-tailed *t*-test.

Figure 6. *Effect of sex on hamster CDI survival*. Kaplan-Meier survival plot for DMSO-treated female hamsters (solid pink circles), CaPA-treated female hamsters (checkered pink circles), DMSO-treated male hamsters (solid blue circles), and CaPA-treated male hamsters (checkered blue circles) challenged with *C. difficile* spores from strain R20291. Statistical survival comparisons between groups were performed by log rank test.

Supplementary Information

Figure S1. *Vaginal cytology of murine estrous cycle stages:* Vaginal gavage samples were stained with crystal violet and examined using a compound light microscope. Estrous cycle stages can be identified based on the presence of nucleated epithelial cells (blue arrows), cornified (keratinized) anucleated epithelial cells (yellow arrows), associated bacteria (black arrow), leukocytes (gray arrows), Small, irregular shaped cells (green arrows), halo-like cells (pink arrows), or combinations of these cell types. Images for proestrus estrus, late diestrus/early proestrus, metestrus, and diestrus were acquired at 100X magnification. The image for late proestrus/early estrus and the insert in the estrus image were acquired at 400X magnification.

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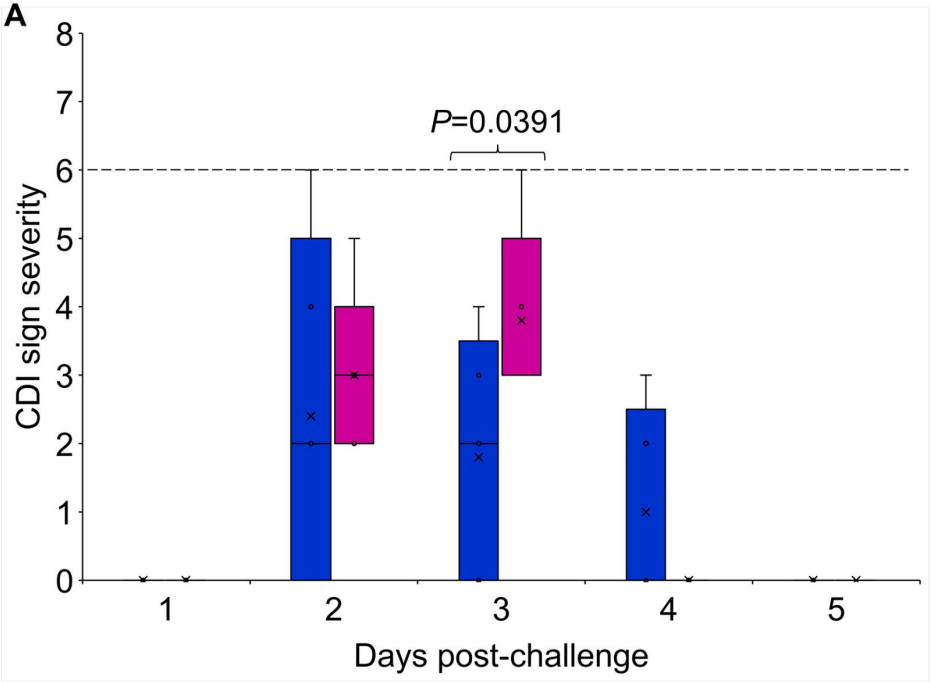
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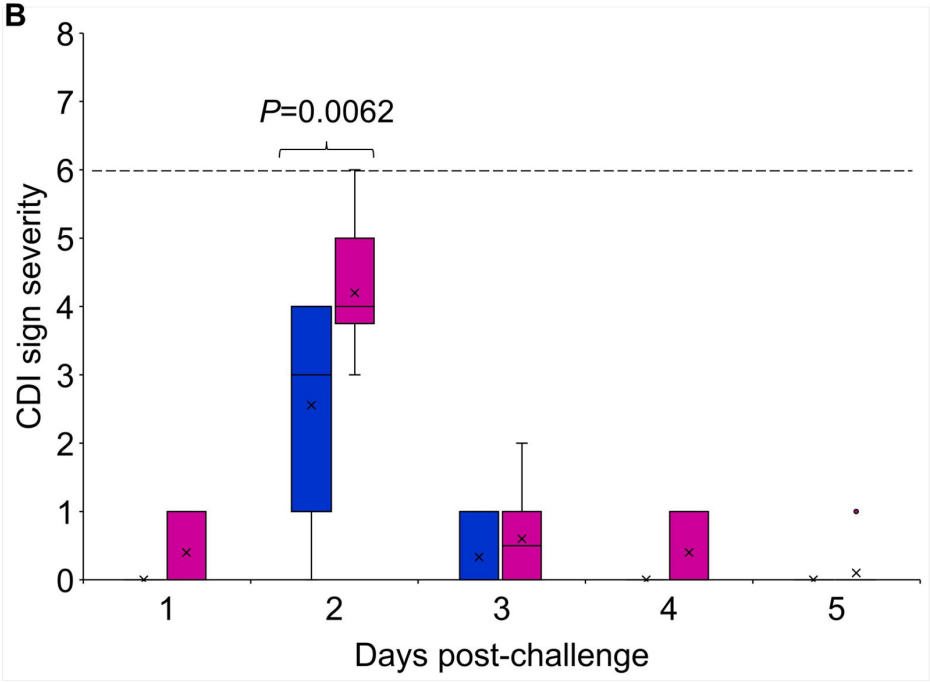
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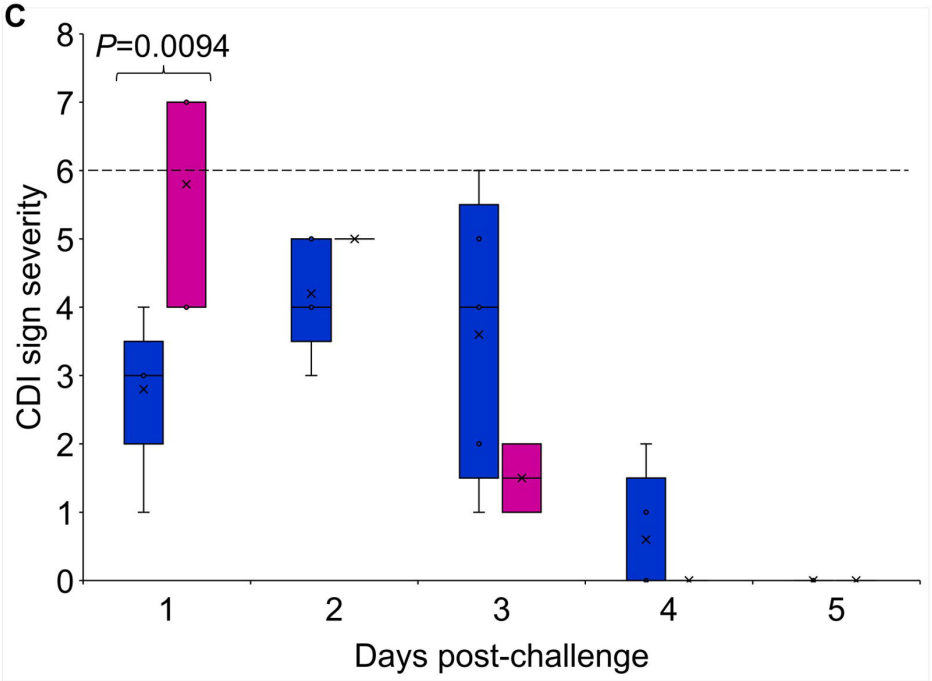
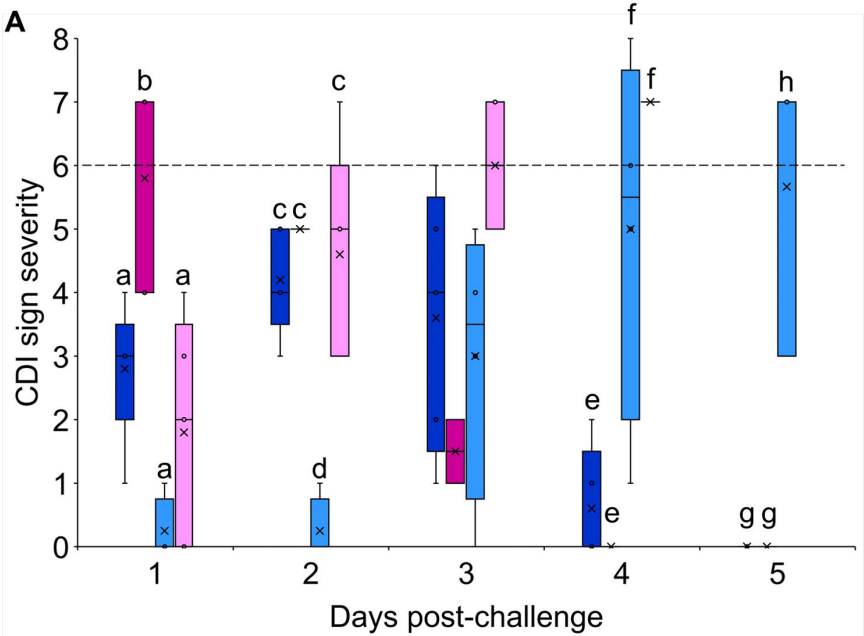
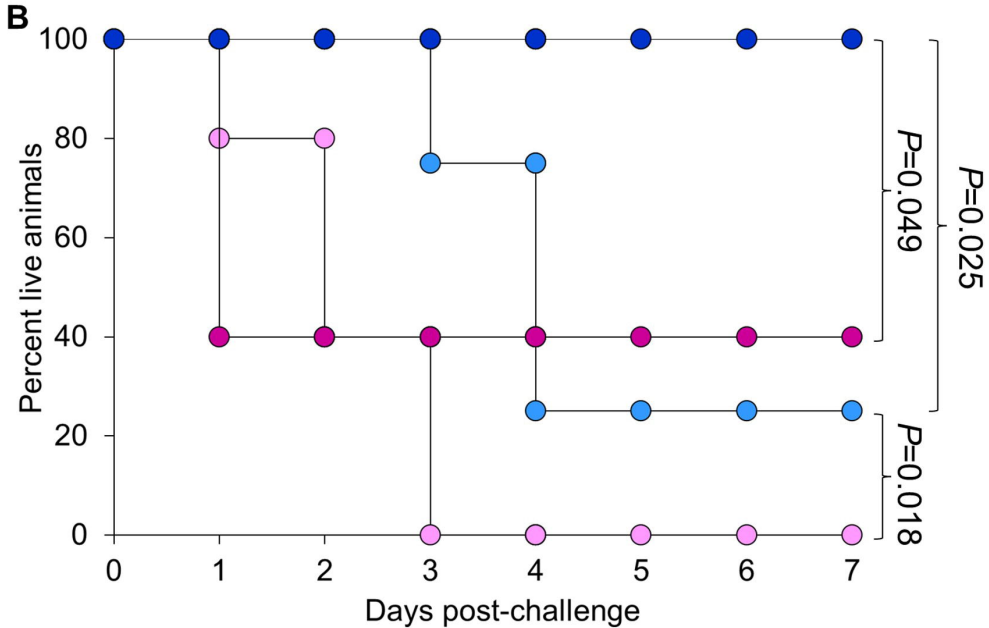
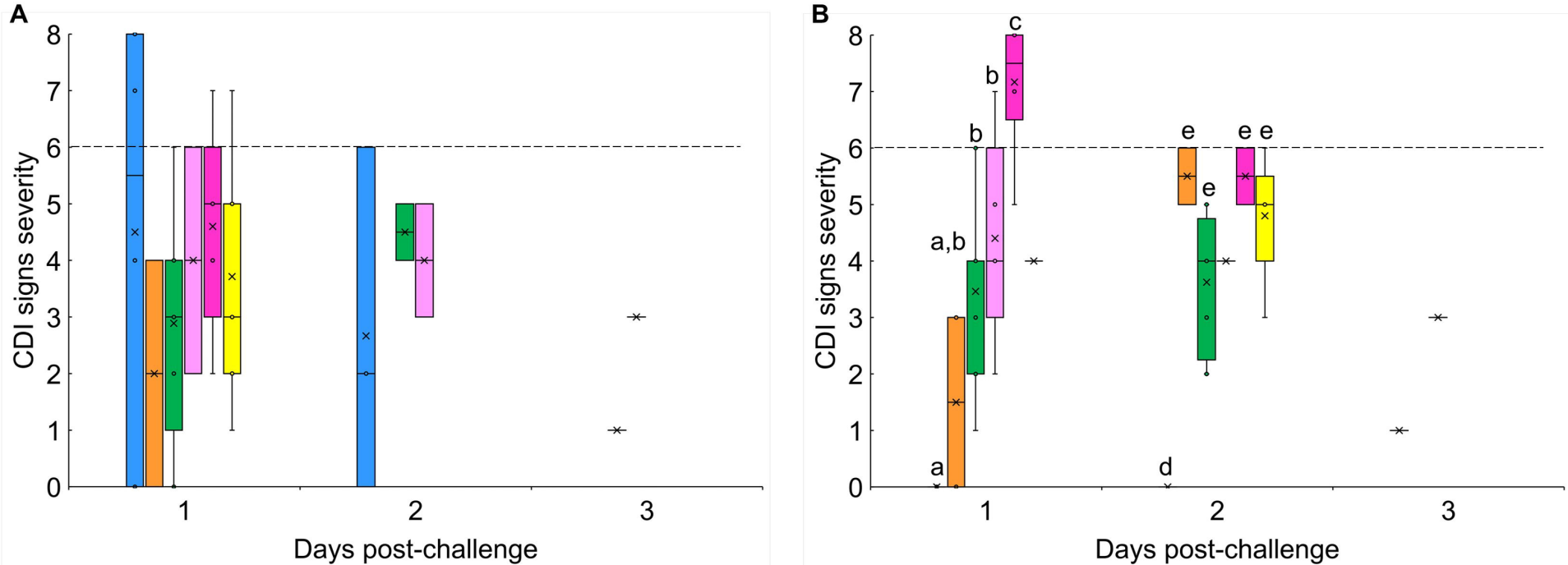
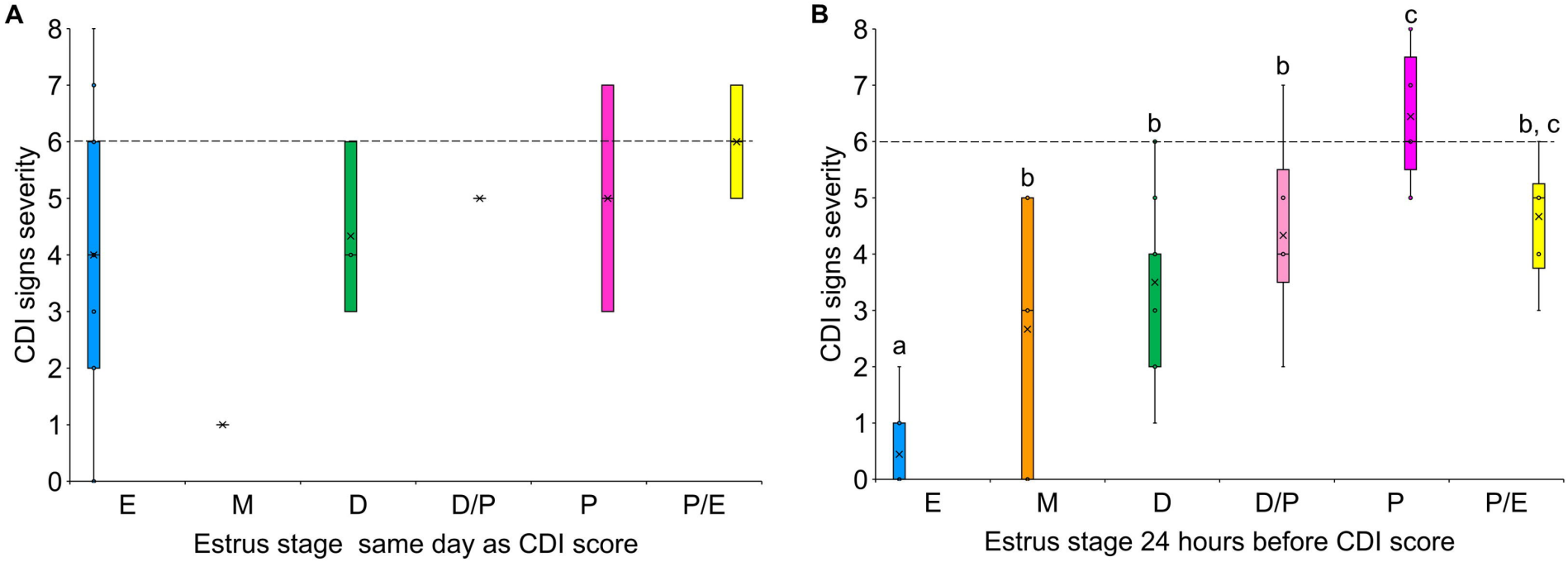
4
5

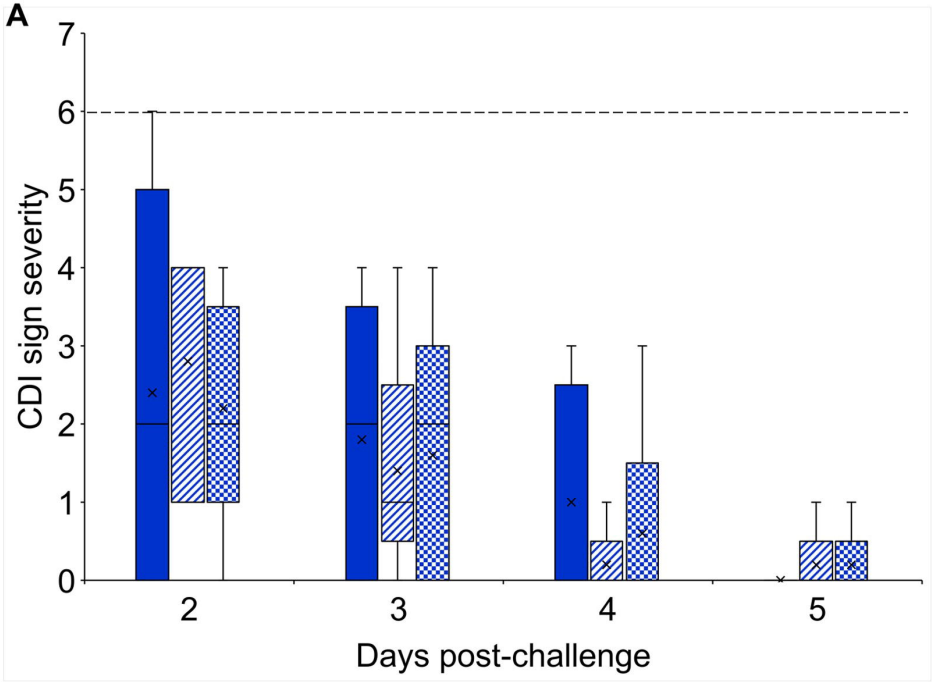

C
P=0.0094
CDI sign severity
Days post-challenge
0
1
2
3
4
5
6
7
8
1
2
3
4
5

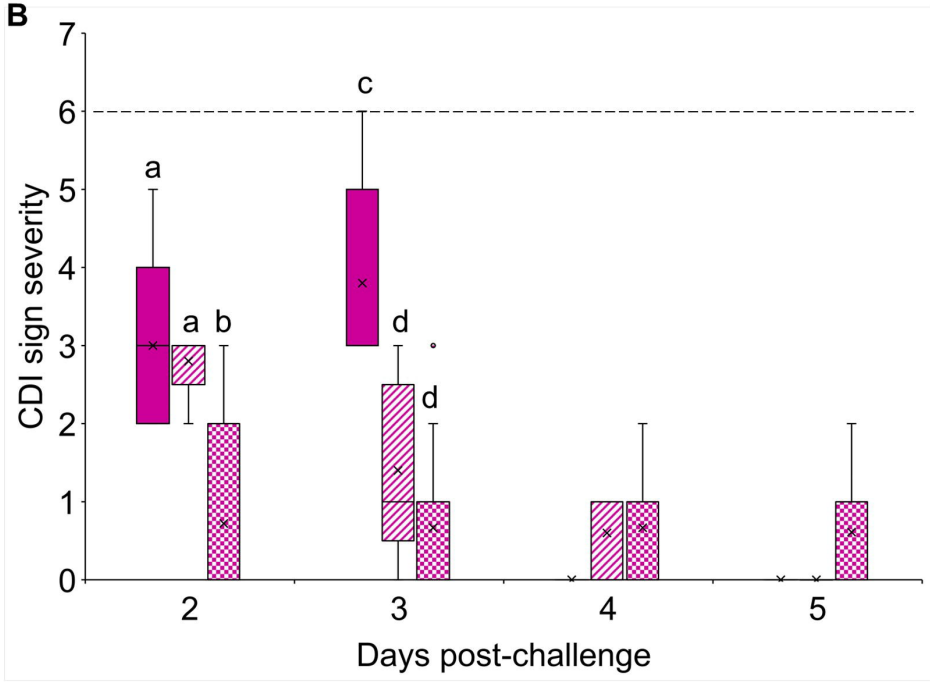

A
CDI sign severity
Days post-challenge
0
1
2
3
4
5
6
7
8
1
2
3
4
5
a
b
a
a
c c
c
d
e
e
f
f
g g
h

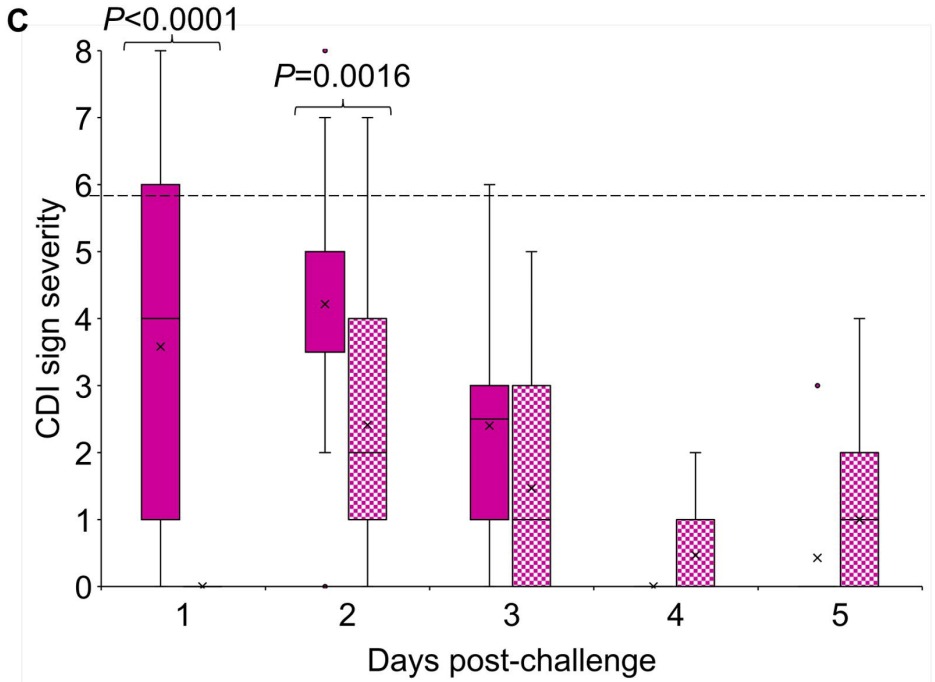

B
Percent live animals
Days post-challenge
0
20
40
60
80
100
0
1
2
3
4
5
6
7
P=0.049
P=0.025
P=0.018

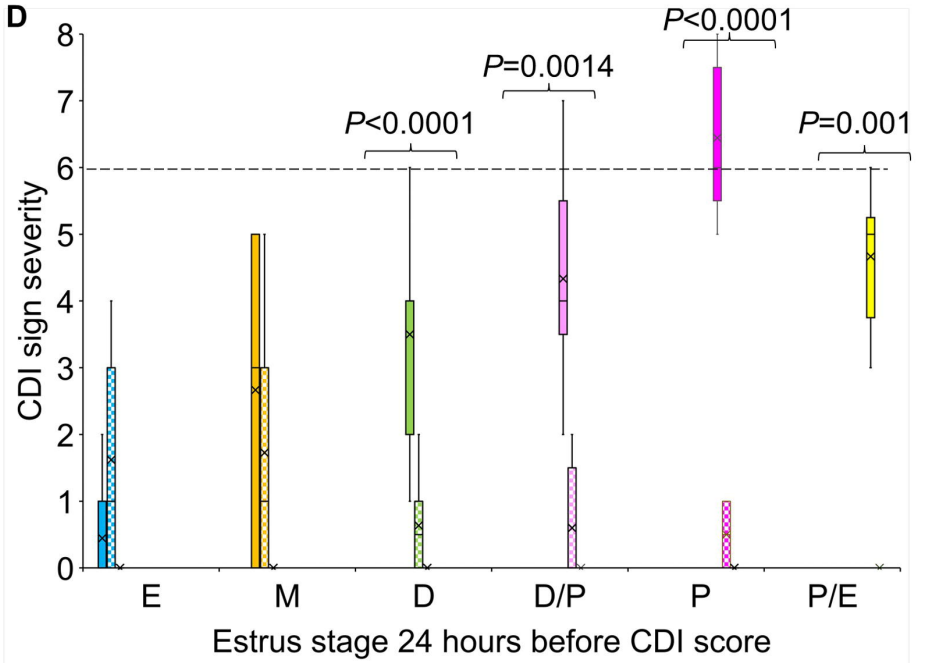

A
B
CDI signs severity
Days post-challenge
0
1
2
3
4
5
6
7
8
a
a,b
b
b
c
d
e
e
e
e

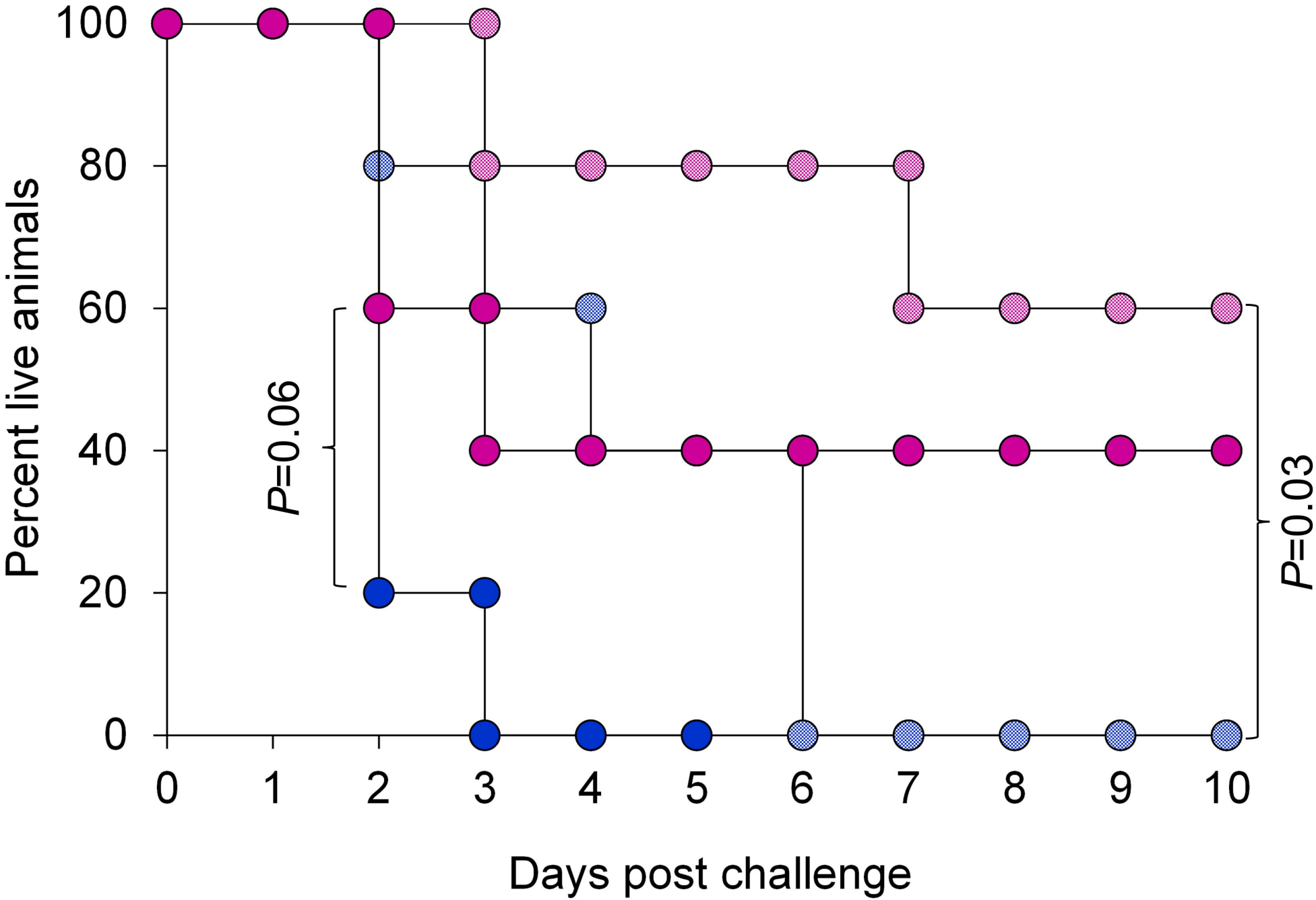

A
CDI signs severity
0
1
2
3
4
5
6
7
8
E
M
D
D/P
P
P/E
Estrus stage same day as CDI score
B
CDI signs severity
a
b
b
b
c
b, c
E
M
D
D/P
P
P/E
Estrus stage 24 hours before CDI score


A
CDI sign severity
Days post-challenge
0
1
2
3
4
5
6
7
2
3
4
5


B
CDI sign severity
Days post-challenge
a
a b
c
d
d
0
1
2
3
4
5
6
7
2
3
4
5


C
P<0.0001
P=0.0016
CDI sign severity
Days post-challenge
0
1
2
3
4
5
6
7
8
1
2
3
4
5


D
P<0.0001
P=0.0014
P<0.0001
P=0.001
CDI sign severity
Estrus stage 24 hours before CDI score
0
1
2
3
4
5
6
7
8
E
M
D
D/P
P
P/E


100
80
60
40
20
0
Percent live animals
0
1
2
3
4
5
6
7
8
9
10
Days post challenge
P=0.06
P=0.03