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Contents

Comparison of Immune Function in Two Strains of Mice Infected with *Heligmosomoides bakeri* after Calorie Restriction

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Abstract

Direct calorie restriction (CR) or CR mimetics can potentially become therapies for many autoimmune and degenerative diseases, and studies are currently underway to test the use of CR by people. However, relatively little is known about the relationship between CR and response to pathogen infection. In this study I subjected laboratory mice (*Mus musculus*) to long term CR prior to infecting them with the intestinal nematode (roundworm) *Heligmosomoides bakeri* for 2, 4 or 6 weeks. Immune responses were measured in slow-responding (C57BL/6) and fast responding (SJL) mouse strains. Overall, my study showed that both C57 and SJL mice used cellular and immunoglobulin responses against *H. bakeri*, but the robustness, timing, and the effect of CR on those responses varied between the strains. The cellular responses fluctuated in C57 mice, with peaks at 2 (neutrophil numbers) and 4 weeks (eosinophil numbers, levels of mucosal mast cell protease-1 [mMCP-1]), but were similar for all infection durations in SJL mice. As expected, CR had no effect on eosinophils, but it did invigorate production of neutrophils. Interestingly, in C57 mice the invigorating effect of CR on neutrophils was observed only in the presence of infection, while the invigorating effect of CR on mMCP-1 was observed only in the absence of infection. CR invigorated white blood cell

responses regardless of infection in SJL mice, but it attenuated their mMCP-1 response. SJL mice may rely more on reactive oxygen species (ROS) which could have caused SJL to compensate for a CR-mediated decrease in other ROS by producing more ROS-producing neutrophils even in the absence of infection. SJL mice had a less robust mMCP-1 response than C57 mice, and this response was further attenuated by CR. Regarding immunoglobulin responses, my study showed that C57 had more robust non-specific responses (IgA and total IgG1), while the parasite – specific response (*H. bakeri*-specific IgG1) was similar in both strains. While CR invigorated IgA in C57 mice, it either had no effect, or an attenuating effect in SJL. CR had an attenuating effect on total IgG1 in both C57 and SJL mice, but CR had no effect on the *H. bakeri*-specific IgG1 response in either strain. Taken together, my study shows that calorie restriction can have both invigorating, attenuating, and no effect on various components of the immune system depending on mouse strain, with potentially important implications on the future CR and CR mimetics therapies for humans.

Introduction

Calorie restriction (CR) is documented to produce a variety of health benefits, including reduced occurrence of cancer (Weindruch and Walford, 1982), autoimmune diseases (Kubo *et al*., 1992, Mitzutani *et al*., 1994) and oxidative damage (Opalach *et al*., 2010, Qiu, *et al*., 2010). Effects of CR on immune function remain equivocal. Short to moderate duration calorie restriction (CR) ranging from 2-16 weeks can have either positive effects such as diminished age-associated decline in immune function (Kubo *et al*., 1992) or neutral effects related to cell mediated immunity (Peck *et al*., 1992). Short-term CR effects on the immune function can depend on degree of calorie restriction (Esquifino *et al*., 2007). Life-long CR has been shown to

preserve the number of naïve T-cells and maintain the proliferative capacity of antigen-specific T-cells as organisms age (reviewed by Nikolich-Zugich and Messaoudi, 2005). Although these studies provided valuable information on how CR affects the mouse immune system, they largely used *in-vitro* and antigen-based approaches.

Calorie restriction is controversial because in spite of studies that show a positive or neutral effect of CR on immunity when tested with antigens and mitogens *in vitro*, there are other studies that expose detrimental effects of CR when live pathogens are used. Calorie restriction has never been incorporated into a comparison of susceptibility among mouse strains to any intact pathogen. The overall aim of my research was to compare the effect of *H. bakeri* infection on the cellular and immunoglobulin immune responses of slow responding C57BL/6 and fast responding SJL strains of laboratory mice after they were given long term calorie restriction.

Effects of Calorie Restriction on Immune Response to Parasitic Infections

Immunological effects of CR during pathogen infection depend on the duration of CR. While short term CR of mice improved response to bacteria (Peck *et al*., 1992; Stapelton *et al*., 2001), long term CR was detrimental to infection with intact virus (Gardner, 2005) and bacteria (Sun *et al*., 2001). For example, in the Gardner study (2005) mice that underwent long term CR had greater virus titer and died earlier from influenza than their *ad libitum* (AL) fed counterparts despite the delay in senescence of lymphoproliferation that was measured *in vitro* after stimulation with the concanavalin-A mitogen. CR mice that were subjected to polymicrobial peritonitis also died earlier than their AL counterparts, likely due to increased inflammation (Sun *et al*., 2001). These findings were surprising when considering equal or

increased immune responses in CR mice that were measured *in vitro* in these same studies, such as decreased interleukin (IL) 6 and IL 12 expression and similar tumor necrosis factor (TNF) α expression in macrophages after lipopolysaccharide stimulation, although macrophages from CR mice had decreased phagocytic activity. In addition to viral and bacterial infections, CR was also shown to have detrimental effects on mice infected with a nematode worm. A single published study on the topic (Kristan, 2007) investigated the effects of chronic CR on C57BL/6 mice that were infected with intestinal nematode *Heligmosomoides bakeri*. Interestingly, worms in CR mice established in greater numbers and produced more eggs both *in vivo* and after removal from the host *in vitro* despite the finding that CR mice had enhanced or equivalent immune response as their AL counterparts. Both CR and AL mice mounted an equal eosinophilia response, and CR mice had 10% greater total IgG1 prior to infection. After infection with *H. bakeri* both CR and AL mice mounted an equal total IgG1 response. Findings from Gardner (2005), Sun *et al*. (2001) and Kristan (2007) were counterintuitive, since traditional measures of enhanced immune function did not translate into greater protection from intact pathogens.

The mechanism for how CR affects susceptibility to nematode worms is still unknown. One potential mechanism may be changes in reactive oxygen species (ROS) and antioxidants because CR has been shown to alter antioxidant production and reduce ROS damage. For example, Wu *et al*. (2003) showed that long term administration of both 20% and 35% CR increased antioxidants such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) that catalyze the decomposition of ROS. Long term 40% CR was also shown to decrease mitochondrial hydrogen peroxide (H_2O_2) production and oxidative damage to

mitochondrial DNA in rat liver (Lopez-Torrez *et al*., 2002). Since CR may increase antioxidant production (Wu *et al*., 2003) and reduce ROS damage (Lopez-Torrez *et al*., 2002.), possibly by decreasing ROS production, it is possible that CR mice did not have enough ROS available to attack the nematode, which is a common host response to infection (Ben-Smith *et al*., 2002). However, it should be noted that CR does not always increase antioxidant production and reduce oxidative damage (Mura *et al*., 1996, Gong *et al*., 1997). These conflicting findings by Wu *et al*. (2003), Lopez-Torrez *et al*. (2002), Mura *et al*. (1996) and Gong *et al*. (1997) show that CR does not always change antioxidant capacity and ROS damage, but when it does the direction of change can vary, possibly due to genetics of different mouse strains (Mura *et al*., 1996). The role of CR in antioxidant and ROS changes that may apply to susceptibility of different strains of mice to *H. bakeri* infection remains to be elucidated, and this study is currently underway in our lab.

Heligmosomoides bakeri – Parasite Life Cycle and Immune Responses in the Host

Heligmosomoides bakeri is an intestinal nematode that infects laboratory mice and wild house mice (*Mus musculus*). It was initially named *Nematospiroides dubius*, after which it was simultaneously referred to as *Heligmosomoides polygyrus* and *Heligmosomoides polygyrus bakeri* for approximately four decades (Behnke *et al*., 2009). In 2006 it was discovered that *Heligmosomoides polygyrus bakeri* was a different species from *Heligmosomoides polygyrus* that infects European wood mice (Cable *et al*., 2006), so the name was changed once again to *Heligmosomoides bakeri*. Despite the name change, Behnke and Harris (2010) have noted that some authors still refer to the parasite that infects *M. musculus* as *Heligmosomoides polygyrus*. Throughout my thesis, I will use *H. bakeri* to refer to studies of the parasite that infects North

American *Mus musculus* although some references may still refer to *H. polygyrus* or *N. dubius*, depending on their publication date.

The *H. bakeri* life cycle includes both free-living and parasitic stages (Bryant, 1973). After being eliminated through mice feces, *H. bakeri* eggs hatch 36-37 hours later, and enter the first free living larval stage (L_1). L_1 larvae molt two more times to give rise to larval stage 3 (L_3). L_3 is the infective larval stage that enters mice through the oral route, and moves into the muscle layer of the small intestine. The larvae then progress into larval stage 4 (L_4) within 90-96 hours. At seven to eight days post-infection adults enter the intestinal lumen where they feed on mucosal tissue and produce thousands of eggs on daily basis (Kerboeuf, 1982). L_4 and adult components of the *H. bakeri* life cycle can elicit host immune responses.

In order to protect themselves against *H. bakeri* infection, mice employ a number of immune responses, including immunoglobulin production, leukocytosis (i.e. increased ratio of white to red blood cells) and enhanced ROS production. The immune response to gastrointestinal nematodes is typically biased toward a T helper cell type 2 (Th2) response (Finkelman *et al*., 1997), although Th1 responses to *H. bakeri* also occur (Ben-Smith *et al*., 2003). Th2 cells produce IL4, IL5, IL9 and IL10, but not IL2, interferon (INF) gamma, and lymphotoxin. IL4 helps to expel *H. bakeri* adults by increasing gut contractility and might possibly play role in damaging parasites through lipid peroxidation, since IL4 has been linked to lipid peroxidation in humans (Belkner *et al*., 1993). IL4 also increases the amount of fluid in the intestinal lumen through increasing permeability and sensitivity of intestinal epithelial cells to prostaglandin E_2 (Anthony *et al*., 2007) which can interfere with adult worm adherence and feeding. IL4 also is essential for antigen-specific Th2 cell expansion and differentiation that is

triggered by the parasite's lipids, proteins, and lipoproteins. The cytokine response is local, as evident from increased expression of IL3, IL4, IL5, IL6, IL9 and IL10 in Peyer's patches of the small intestine and mesenteric lymph nodes, but not in splenic tissues (Urban *et al*., 1992). Cytokines also are released from T cells, as well as eosinophils, mast cells and basophils (Anthony *et al*., 2007) and enter the general circulation where they will contact B cells.

B cells are important for the immune response against *H. bakeri* because they support Th2 cell development, present antigen, activate mast cells, and prevent the attachment of adult worms to mucosal tissue through enhancing mucous secretion (Finkelman *et al*., 1997). B cell deficient mice are unable to mount an immunoglobulin response which may play a role in *H. bakeri* expulsion during secondary infections (McCoy *et al*., 2008); however, they are still able to suppress *H. bakeri* fecundity (Liu *et al*., 2010). B cells' most important role in *H. bakeri* infection might be immunoglobulin production.

Some of the most important immunoglobulins in response to *H. bakeri* are total IgG1 and *H. bakeri* specific IgG1. Total IgG1 (which includes parasite specific IgG1 plus non-specific IgG1) was found to be negatively correlated with *H. bakeri* worm burden in some (Ben-Smith *et al*., 1999), but not all (Behnke *et al*., 1987; Cypress *et al*., 1975; McCoy *et al*., 2008) studies. Administration of IgG1 *in vivo* caused a reduction of *H. bakeri* worm length and numbers (Pritchard *et al*., 1983). This immunoglobulin also may mediate a cellular response to *H. bakeri*, since addition of IgG1 to a mixture of worms and mouse peritoneal exudate cells caused the exudate cells to attach to the worm surface (Pritchard *et al*., 1983). *H. bakeri* specific IgG1 might be more important than non-*H. bakeri* specific IgG1 (McCoy *et al*., 2008) as evidenced by the observation that a passive immunization with serum that contains *H. bakeri* specific IgG1

resulted in protection while immunization with a serum that contains non-specific IgG1 did not. *H. bakeri* specific IgG1 is locally secreted in the small intestine at the site of worm attachment (Ben-Smith *et al*., 1999), but is also present in the peripheral blood, and it occurs at highest levels in later stages of infection when adults are present (McCoy *et al*., 2008, Molinary *et al*., 1978).

In addition to IgG1, IgA (Pritchard *et al*., 1983), IgE (Ben-Smith *et al*., 2003, Finkelman *et al*., 1997) and IgG2a (Ben-Smith *et al*., 2003) also increase in response to *H. bakeri* infection. IgA is produced by B cells and partially mediates immune response using a $CD4^+$ T cell-independent pathway (McCoy *et al*., 2008). IgA is associated with early and late stages of infection, peaking at 3 days after both primary and secondary infection, and peaking again 14 days after primary infection (Molinary *et al*., 1978). IgE was increased throughout infection, and it was expressed in greater quantities in strains of mice that responded faster to infection (Ben-Smith *et al*., 2003). IgG2a was expressed locally (Ben-Smith *et al*., 2003), but could not be detected in serum (Molinary *et al*., 1978). Other immunoglobulins, such as IgM did not change in response to *H. bakeri* (Ben-Smith *et al*., 1999).

Additional cellular immune responses include eosinophilia and neutrophilia (Cypress, 1972). Eosinophils might aid the immune response by releasing IL4 and IL13, presenting antigens to T cells, and aiding in the intestinal tissue healing process (Anthony *et al*., 2007). Eosinophils have been shown to permanently bind to the surface of *N. dubius* via an antibody-independent manner *in vitro* (Penttila *et al*., 1983). While *H. polygyrus* can use secretory-excretory products to evade the host immune system (Segura *et al*., 2007, Telfold *et al*., 1998), eosinophils might be able to prevent this evasion by binding to the head and tail region of the

intestinal parasite (Penttila *et al*., 1984b), which is the main location of secretory-excretory products. Although eosinophils did not kill *N. dubius in vitro* (Penttila *et al*., 1984a), they were able to damage L_3s and reduce their infectivity (Penttila *et al*., 1984b, Prowse *et al*., 1978). In helminth infections the binding of parasite specific immunoglobulin to the Fc receptor leads to an antibody-dependent cytotoxicity pathway that activates eosinophils and causes them to release toxic proteins implicated in tissue damage (Makepeace *et al*., 2012). Cypress (1972) noted eosinophilia after third and fourth, but not first and second infections with *H. bakeri*. However, they used a very low inoculation dose of 50 L_3s. It is possible that if authors used higher doses, eosinophilia would have been noted during primary and secondary infections as well, as seen by Kristan (2007). They also noted a slight neutrophilia after primary infection, but after a third and fourth infection blood smears revealed more neutrophils than lymphocytes. This could be considered an "extreme" neutrophilia, since lymphocytes are the most numerous leukocytes in uninfected mice (Wilkinson *et al*., 2001). Cypress (1972) suspected that neutrophilia was the sign of inflammation and expulsion of worms and also noted leukocytosis (increased numbers of total white blood cells).

Besides white blood cells, small intestine goblet cells and mast cells also become more numerous following *H. polygyrus* infection (Behnke *et al*., 2003). Mast cells come from the same cell progenitor as eosinophils, and can also produce IL4 and IL13 (Anthony *et al*., 2007). Mast cells also make and secrete mucosal mast cell protease 1 (mMCP-1) which increases intestinal permeability and facilitates worm expulsion (McDermott *et al*., 2003). Repeated *H. bakeri* infection increased goblet cells, mast cells and levels of mMCP-1 (Behnke *et al*., 2003). The mMCP-1 levels peaked on week 2 and 3 post infection (Behnke *et al*., 2003), and were

increased both in serum and in the small intestine lumen (Ben-Smith *et al*., 2003). Mast cell deficient mice were not able to suppress *H. bakeri* fecundity, but a bone marrow transplant was able to increase mMCP-1 and then suppress worm fecundity and decrease survival of worms at 3 weeks post infection (Hashimoto *et al*., 2010).

Mouse Strain and Susceptibility to *H. bakeri*

When *Mus musculus* are infected with *H. bakeri*, immune responses can vary depending on mouse strain (Zhong and Dobson, 1996). For example, the RH strain has a strong immune response (aka a "fast responder") against nematodes, allows fewer parasites to establish in the intestine, and decreases the fecundity of worms in comparison to other inbred and outbred strains. The SL strain, on the other hand, was selected for generations for its weak immune response (aka a "slow responder") towards *H. bakeri*. Other inbred lab strains that were tested by Zhong and Dobson (1996) such as SJL/J, BALB/C, CBA and SH3 showed a range of responses. These mice were bred for reasons other than their susceptibility to intestinal worms, but the SJL strain was found to be particularly fast responding, and C57BL/6 was found to be particularly slow responding (Behnke *et al*., 2006). These are the two strains used in my research.

Fast responding strains often rely heavily on the mast cell response. When Behnke *et al*. (2003) administered 8 repeated infections to fast responding SWR mice and to slow responding CBA mice, they found that SWR showed a strong Th2 dependent response with higher mast cell counts and associated mMCP-1 levels. SWR mice had smaller worm burdens and were able to develop resistance to *H. polygyrus bakeri*, while CBA mice had high worm burdens and remained susceptible throughout the experiment. A study by Ben-Smith *et al*. (1999) that

involved a single infection dose also showed that fast responding strains, including SJL, had higher serum mMCP-1 and smaller worm burdens than the slower responding strains. There is additional evidence that the resistance of mouse strains to intestinal nematodes is related to mMCP-1. When Brown *et al*. (2003) took stem cells from BALB/c (intermediate responder) and C57BL/10 (slow responder closely related to C57BL/6) and forced them to differentiate into mast cell homologues, they noticed that BALB/c cells expanded more rapidly and started producing mMCP-1 earlier than C57BL/10 cells. A study by Lawrence and Pritchard (1994) showed that fast responder SJL mice had a seven fold increase in mast cell counts following *H. polygyrus* infection, while the intermediate responder BALB/c showed only a two fold increase, and the slow responder CBA showed no increase in mast cell counts. Fast responding strains also have a more pronounced immunoglobulin response. Behnke *et al*. (2003) showed that fast responding SWR mice had higher anti-adult worm IgG1 levels when repeatedly infected with *H. polygyrus bakeri*. There also were higher IgA (Ben-Smith *et al*., 1999), IgE and IgG2a (Ben-Smith *et al*., 2003) levels in fast responding mouse strains.

In addition to host immune differences, there is evidence that worms from fast responding strains must repair more damage to tissues as evidenced by proteomic analyses that showed greater amounts of actin, myosin, and troponin in the worms (Morgan *et al*., 2006). Worms from faster responding hosts had slower development and greater levels of the antioxidant catalase (Ben-Smith *et al*., 2002) compared to worms from slower responding mouse strains. Overall, mouse strain affects the host immune response to infection and also the subsequent biology of *H. bakeri* worms. These differences in mouse strain responses have not been fully explored in the context of long-term CR.

To examine this relationship, I tested two mouse strains (SJL: fast responder; C57BL/6: slow responder) that were infected with *H. bakeri* for either two, four or six weeks after they had been subjected to six months of CR. I hypothesized that after infection, the SJL mice would produce more eosinophils and have more mucosal mast cell protease 1 (mMCP-1) than C57BL/6 mice in both calorie restricted and *ad libitum* groups because SJL are fast responders and C57BL/6 are slow responders. I also hypothesized that after infection, the SJL mice would produce more immunoglobulins than C57BL/6 mice in both calorie restricted and *ad libitum* groups because SJL are fast responders and C57BL/6 are slow responders. Last, I hypothesized that calorie restriction would have no effect on eosinophil counts because eosinophilia was not induced by CR in a previous study (Kristan 2007), but it would increase other white blood cells because CR increases the immune response in general. On the other hand, I expected CR to increase the immunoglobulin response because CR has been shown to increase the number of B and T cells (Mitzutani *et al*., 1994) in mouse spleen. I hypothesized that CR-induced increase in immunoglobulins would further translate into increased mMCP-1 since immunoglobulins can activate mast cells (Wang *et al*., 2008). Changes in immune responses for each strain were tested separately to determine effects of calorie restriction, infection with *H. bakeri*, and duration of infection. Between strain differences were qualitatively evaluated because there would be too many factors in the analysis making the interpretation of results problematic.

Methods

Experimental Design

My experiment used two mouse strains (C57BL/6, SJL). For each mouse strain there were three treatments (food treatment [calorie restricted, *ad libitum*], parasite infection

[infected, uninfected] and experiment duration [2, 4, or 6 weeks]) in a 2 x 2 x 3 factorial design (Table 1). I measured a number of dependent variables (differential white blood cell counts, IgA, total IgG1, *H. bakeri*-specific IgG1 and mMCP-1) and sample size ranged from 11 to 15 mice in each of the 24 experimental groups, for a total of 336 animals. Not all dependent variables were tested for each mouse and final sample sizes are provided with each result. All procedures were approved by the California State University San Marcos Institutional Animal Care and Use Committee under protocol numbers 09-001 and 12-003.

Selection of Mouse Strains and Experiment Duration

Behnke *et al*. (2006) observed SJL to be a fast responder strain to *H. polygyr*us, and Ben-Smith *et al*. (2003) also determined that a hybrid mouse strain with a partial SJL background showed rapid expulsion of *H. bakeri*. For these reasons I chose to use the SJL mouse strain as my fast-responding strain. Since the C57BL/6 strain of mice is less effective in eliminating worm burden than numerous other strains (Behnke *et al*., 2006), and only decreased infection by 50% by the time fast responder usually eliminated infection (Morgan *et al*., 2006), the C57BL/6 strain was chosen to contrast against SJL for susceptibility to *H. bakeri*. C57BL/6 are also commonly used in long-term calorie restriction studies (Ferguson *et al*., 2007, Forster *et al*., 2000, Kristan, 2007, Selman and Hempenstall, 2012, Sun *et al*., 2001, Zhang *et al*., 2013). Only male mice were used in the experiment since previous research showed no difference between males and females in response to *H. bakeri* infection during calorie restriction (Kristan, 2007). Because SJL mice can eliminate infection anywhere between two and six weeks (Behnke *et al*., 2006), mice were dissected at two, four and six weeks post infection.

Calorie Restriction and Parasite Infection

CR was chosen to be at 40%, as this food reduction was used commonly by other researchers (Kubo *et al*., 1992; Kristan, 2007; summarized by Kristan, 2008), and because greater food reduction, such as 66%, is considered severe (Esquifino *et al*., 2007). Calorie restricted mice were fed *ad libitum* for the first five days of the experiment to determine their average food intake. The average food intake was then gradually decreased by 10% per week, for four weeks, until it reached 40% CR, making sure that each mouse received at least 2g of food per day to ensure similar protein intake (based on formulation of the diet; CR diet TD92713, AL diet TD92051 by Harlan-Teklad, Madison, WI) since protein deficiency has been linked to susceptibility to *H. bakeri* (Peck *et al*., 1992, Tu *et al*., 2007). The gradual reduction in food availability for CR mice was intended to allow mice to adjust to their new calorie intake. AL mice were fed *ad libitum* for the entire duration of the experiment. Water was provided *ad libitum* in all groups. Since this study strived to establish long term effects of CR, mice were fed CR for six months before *H. bakeri* was administered.

After six months on their experimental diet, mice were inoculated with *H. bakeri* L_3. This step was accomplished by pipetting 300 L_3 suspended in tap water inside the mouse's mouth and ensuring that the mouse then swallowed to complete the infection. L_3 used to infect experimental mice were cultured from eggs obtained from infected, non-experimental animals of the same strain. For the SJL strain, standard deviations of the six cultures used during the experiment ranged between 5-20 worms, while for the C57BL/6 strain the standard deviations of six cultures ranged between 6-24 worms. Infection of experimental mice was confirmed by testing for the presence of parasite eggs in mouse feces.

Differential White Blood Cell Counts

Blood was drawn on the day of dissection from the retroorbital sinus and white blood cell counts were assessed by making 30 µL blood smears using a Diff-Spin (StatSpin: Cat. No. DS02) as described by Wilkinson *et al*. (2001). Blood smears were stained with Wright (Sigma-Aldrich, VS16) and Giemsa stain (Sigma-Aldrich, GS500), and a differential white blood cell count was performed using oil immersion a total magnification of 1000X (Nikon Alphaphot-2 (YS2)) to determine the number of each white blood cell type per 100 white blood cells counted. Out of approximately 30,000 white blood cells counted only 6 were identified as basophils; therefore basophils were excluded from the analysis. In addition, for every 100 white blood cells mice averaged only 1.56 monocytes (± 1.765, standard deviation); therefore monocytes were also excluded from the analysis. Only counts of neutrophils, lymphocytes and eosinophils were analyzed.

Immunoglobulin Measurements

In order to measure the changes in immunoglobulins, blood was collected from the retroorbital sinus on 3 sampling days for each mouse: immediately before infection to establish a baseline, 10 days after infection when adult *H. bakeri* recently entered the lumen and started to reproduce (Bryant, 1973), and on the final day of the experiment (at 2, 4 or 6 weeks post infection). These sampling days are from now on referred to as sampling day 0, sampling day 10, and final sampling day (or Day 0, Day 10 and Final Day for short), respectively, with the understanding that "Final Day" will be different for the different experimental groups. Whole blood was centrifuged at 9875 x g for 15 minutes (Eppendorf Centrifuge 5415C, Eppendorf AG, Hamburg, Germany) and plasma was aliquoted and stored at -80°C for subsequent enzyme-

linked immunosorbent assays (ELISAs) to measure IgA, total IgG1, and *H. bakeri* – specific IgG1. All samples were assayed in duplicate to ensure accuracy. IgA (Immunology Consultants Lab Inc., Portland, OR, USA; kit no. E-90A) and total IgG1 (Enzo Life Sciences Inc., Farmingdale, NY, USA; kit no ADI-900-109) ELISAs were performed according to the manufacturers' instructions. Samples for IgA were diluted 100X – 2000X and read at 450 nm (Biorad Model 680, Biorad Laboratories, Hercules, CA, USA). Samples for total IgG1 were diluted 500X - 240,000X and read at 450 nm with subtraction of 570 nm. For both IgA and total IgG1, optical density (OD) values of standards were plotted against their known concentration (µg/mL) and a standard curve was generated using either a second degree polynomial equation of raw data or a linear equation of natural log (LN) transformed data. Sample concentrations were then determined from the standard curve.

H. bakeri specific IgG1 was measured at the last two sampling days using an adapted ELISA protocol from Johnson & McGuire (2001). Briefly, a high-protein binding 96 well plate (Fisher Scientific, 95029330) was coated with 200 µL/well of adult *H. bakeri* worm homogenate (made in our lab) at concentration of 5 µg/mL of protein (determined by a Bradford assay) and incubated overnight at 4°C. The plate was then washed 3 times for 2 minutes each with PBS 0.05% Tween 20 (Sigma-Aldrich, 08057), and blocked for 1 hour with 100 µL/well of 3% bovine serum albumin (BSA; Sigma-Aldrich, A9418) in PBS 0.05% Tween 20. All experimental plasma samples were diluted at 50X, and pipetted in 100µL duplicates. After 1.5 hour incubation the plate was washed as described above, and 100 µL of 5000 ng/mL horseradish peroxidase conjugated goat anti-mouse IgG1 (Enzo Life Sciences Inc., Farmingdale, NY, USA; CAT no 80-0999) was added. Following a 1.5 hour incubation at room temperature the plate was washed

again as described above, and 100 µL of 3, 3', 5, 5' tetramethylbenzidine (TMB; Pierce Product 34028) substrate was added. The plate was incubated for 30 min at room temperature while mixing at 500 rpm (Heidolph Titramax 100, Heidolph, North America, Elk Grove Village, IL, USA). After this last incubation 100 µL of 2M H_2SO_4 was added to stop the reaction. The OD values were read at absorbance of 450 nm and subtraction of 570 nm. Values are reported in OD because no standard curve was available for comparison.

Mucosal Mast Cell Protease-1 Measurement

Plasma mMCP-1 (eBiosciences, Inc., San Diego, CA, USA; kit no. 88-7503-22) was determined in duplicate by ELISA according to the manufacturer's instructions. Uninfected animals were tested without prior dilution, while dilutions of up to 1000X were required for infected animals. Since the mMCP-1 assay required 100 µL of plasma it was only measured from the final blood draw when sufficient blood was available. Optical density (OD) values of standards were plotted against their known concentration (pg/mL) and a standard curve was generated using a second degree polynomial equation of raw data. Sample concentrations were then determined from the standard curve.

Statistical methods

Each strain was analyzed separately, and independent factors were infection status, experiment duration, and food treatment. Analyses were done using either IBM SPSS (version 20) or R (version 3.0.1) software packages. White blood cell counts were compared using multivariate analysis of variance (MANOVA) followed by ANOVA for each white blood cell type with non-overlapping 95% confidence intervals used for post hoc tests of the ANOVA. Repeated measures analysis of variance (ANOVA) was used to compare IgA, total IgG1 and *H.*

bakeri-specific IgG1 among experimental treatments among the sampling days. Either Mauchly's test of Sphericity or Levene's test of equality of error variances, or both were violated for all three immunoglobulins. Log_{10}, natural log (LN), square root and inverse transformations were attempted, but assumptions were still violated. Therefore, a randomization test with 10^6 permutations was done using R statistical software. Prior to randomization, R statistical software was used to visualize which transformation normalized the dependent variables most and this transformation was used to transform data prior to the randomization test. SJL mMCP-1 was analyzed by ANOVA. For C57 mMCP1, Levene's test of equality of error variances failed; therefore a randomization with 10^6 permutations was done in R software. A regression between infection intensity and each independent variable (except white blood cell count) measured on the Final Day was done in SPSS for factors that were significant in the randomization test or ANOVA if assumptions of regression were met. To test regression assumptions, a histogram of residuals was plotted prior to regression to ensure that residuals were normally distributed and residuals were also plotted against dependent variables to ensure random distribution. Throughout, $P < 0.05$ was considered significant.

Results

Throughout the results section, graphs of group means are presented for each of the twelve experimental groups per strain. When analyses showed significant main effects or interactions, means for those factors or interactions are also presented to better visualize the cause of the significance. Note that when a factor is included in a significant interaction it should not be interpreted separately and in those cases only figures of means for the interactions are presented.

Differential White Blood Cell Counts: C57 Strain

When eosinophils, neutrophils and lymphocytes were examined simultaneously, overall white blood cell counts varied with infection status ($F_{3, 156} = 67.52$, $p < 0.001$) and food treatment ($F_{3, 156} = 4.93$, $p = 0.003$) but not with experiment duration ($F_{6,312} = 1.85$, $p = 0.090$). There were significant interactions between infection status and experiment duration ($F_{6, 312} = 4.73$, $p < 0.001$) and infection status and food treatment ($F_{3,156} = 3.58$, $p = 0.015$), but not between experiment duration and food treatment ($F_{6,312} = 1.23$, $p = 0.293$) or a three-way interaction of all factors ($F_{6,312} = 0.76$, $p = 0.600$). To determine which white blood cells were contributing to the multivariate significance, each white blood cell was examined separately by ANOVA.

Eosinophil counts varied with infection status ($F_{1,158} = 60.01$, $p < 0.001$), but not with experiment duration ($F_{2,158} = 0.96$, $p = 0.386$) or food treatment ($F_{1,158} = 0.59$, $p = 0.444$; Figure 1A). There was a significant interaction between infection status and experiment duration ($F_{2,158} = 5.11$, $p = 0.007$; Figure 2) because, while infected animals always had more eosinophils than uninfected animals (shown by asterisk above each black bar on the graph), the animals that were infected for 4 weeks had a greater increase in eosinophils than animals that were infected for 6 weeks. Uninfected animals at all experiment durations had similar number of eosinophils. There were no significant interactions between infection status and food treatment ($F_{1,158} = 1.10$, $p = 0.296$), experiment duration and food treatment ($F_{2,158} = 1.15$, $p = 0.319$) or among all three factors ($F_{2,158} = 0.14$, $p = 0.871$).

Neutrophil counts varied with infection status ($F_{1,158} = 103.75$, $p < 0.001$) and food treatment ($F_{1,158} = 13.11$, $p < 0.001$), but not with experiment duration ($F_{2,158} = 2.42$, $p = 0.093$;

Figure 1B). There was a significant interaction between infection status and experiment duration ($F_{2,158} = 7.90$, $p = 0.001$; Figure 3A) because, while infected animals always had more neutrophils than uninfected animals, the animals that were infected for 2 weeks had a greater increase in neutrophils than animals that were infected for 6 weeks. Uninfected animals at all experiment durations had similar number of neutrophils. There was also a significant interaction between infection status and food treatment ($F_{1,158} = 7.89$, $p = 0.006$; Figure 3B) because, while infected mice always had more neutrophils than uninfected mice, the increase in neutrophil numbers with infection was greater for CR mice than AL mice. There was no significant interaction between experiment duration and food treatment ($F_{2,158} = 1.13$, $p = 0.325$) or among all three factors ($F_{2,158} = 1.29$, $p = 0.279$).

Lymphocyte counts varied with infection status ($F_{1,158} = 178.93$, $p < 0.001$), experiment duration ($F_{2,158} = 4.24$, $p = 0.016$) and food treatment ($F_{1,158} = 12.98$, $p < 0.001$; Figure 1C). There was a significant interaction between infection status and experiment duration ($F_{2,158} = 10.05$, $p < 0.001$; Figure 4A) because, while infected mice always had fewer lymphocytes than uninfected mice, animals that were infected for 6 weeks had more lymphocytes than animals that were infected for 2 or 4 weeks which did not differ from each other. Uninfected animals at all experiment durations had similar number of lymphocytes. There was also a significant interaction between infection status and food treatment ($F_{1,158} = 10.69$, $p = 0.001$; Figure 4B) because, while infected mice always had fewer lymphocytes than uninfected mice, AL mice had more lymphocytes than CR mice when they were infected with *H. bakeri* but not when they were uninfected. There was no significant interaction between experiment duration and food treatment ($F_{2,158} = 1.50$, $p = 0.226$) or among all three factors ($F_{2,158} = 2.10$, $p = 0.126$).

Differential White Blood Cell Counts: SJL Strain

When eosinophils, neutrophils and lymphocytes were examined simultaneously, overall white blood cell counts varied with infection status ($F_{3, 141} = 18.89$, $p < 0.001$), experiment duration ($F_{6,282} = 2.62$, $p = 0.018$) and food treatment ($F_{3, 141} = 24.66$, $p < 0.001$). There were no significant interactions between infection status and experiment duration ($F_{6, 282} = 1.81$, $p = 0.096$), infection status and food treatment ($F_{3,141} = 1.11$, $p = 0.349$), experiment duration and food treatment ($F_{6,282} = 1.49$, $p = 0.181$) or a three-way interaction of all factors ($F_{6,282} = 1.31$, $p = 0.252$). To determine which white blood cells were contributing to the multivariate significance, each white blood cell was examined separately by ANOVA.

Eosinophil counts (Figure 5A) varied with infection status ($F_{1,143} = 9.05$, $p = 0.003$; Figure 6A) and experiment duration ($F_{2,143} = 3.74$, $p = 0.026$; Figure 6B), but not with food treatment ($F_{1,143} = 0.17$, $p = 0.679$). On average, infected mice had more eosinophils than uninfected mice (Figure 6A). Although there were no non-overlapping confidence intervals among the different experiment durations, mice from the 2 week experiment duration treatment had nearly a significantly greater number of eosinophils than mice from the 4 week experiment duration (95% confidence intervals were 0.0958 - 0.136 and 0.053 - 0.097, respectively), which may have driven the significant result of this factor overall. There were no significant interactions between infection status and infection duration ($F_{2,143} = 2.199$, $p = 0.115$), infection status and food treatment ($F_{1,143} = 0.090$, $p = 0.764$), infection duration and food treatment ($F_{2,143} = 1.77$, $p = 0.174$) or among all three factors ($F_{2,143} = 1.17$, $p = 0.314$).

Neutrophil counts (Figure 5B) varied with infection status ($F_{1,143} = 42.55$, $p < 0.001$; Figure 7A), food treatment ($F_{1,143} = 71.94$, $p < 0.001$; Figure 7B) and experiment duration ($F_{2,143} =$

1.42, p = 0.016; Figure 7C). Infected mice had more neutrophils than uninfected mice, CR mice had more neutrophils than AL mice, and mice from the 4 week experiment duration averaged more neutrophils than mice from the 2 week experiment duration. There were no significant interactions between infection status and experiment duration ($F_{2,143} = 1.84$, p = 0.162), infection status and food treatment ($F_{1,143} = 2.70$, p = 0.102), experiment duration and food treatment ($F_{2,143} = 0.18$, p = 0.832) or among all three factors ($F_{2,143} = 1.73$, p = 0.181).

Lymphocyte counts (Figure 5C) varied with infection status ($F_{1,143} = 48.09$, p < 0.001; Figure 8A) and food treatment ($F_{1,143} = 74.68$, p < 0.001; Figure 8B), but not with experiment duration ($F_{2,143} = 2.80$, p = 0.064). Infected mice had fewer lymphocytes than uninfected mice, and CR mice had fewer lymphocytes than AL mice. There were no significant interactions between infection status and experiment duration ($F_{2,143} = 1.74$, p = 0.180), infection status and food treatment ($F_{1,143} = 2.50$, p = 0.116), experiment duration and food treatment ($F_{2,143} = 0.23$, p = 0.797) or among all three factors ($F_{2,143} = 1.42$, p = 0.244).

Immunoglobulin A (IgA): C57 Strain

Because homogeneity of variances could not be met, I used a repeated measures randomization test of Log_{10} transformed data to examine plasma IgA levels (Figure 9). For the within subject results, IgA production changed among sampling days (F = 62.79, p < 0.001) and there was a significant interaction between infection status and sampling day (F = 5.11, p < 0.001; Figure 10A) because average IgA levels remained the same in uninfected mice at all sampling days, while infected mice averaged greater levels of IgA on Day 10 and the Final Day (i.e., 2, 4 and 6 weeks post-infection) than on Day 0. There was no significant increase in IgA from Day 10 to the Final Day in infected mice, although it was approaching significance (95%

confidence intervals: 1.606-1.722 for Day 10, 1.721-1.838 for Final Day). There was a significant interaction between food treatment and sampling day ($F = 4.13$, $p = 0.029$; Figure 10B) because CR mice had more IgA than AL at each sampling day, but the increase in IgA with CR was more on the Final Day than Day 0. There were no significant interactions between sampling day and experiment duration ($F = 2.06$, $p = 0.209$), between sampling day and experiment duration and infection status ($F = 0.75$, $p = 0.700$), sampling day and infection status and food treatment ($F = 4.20$, $p = 0.105$), sampling day and experiment duration and food treatment ($F = 1.97$, $p = 0.407$) and no four-way interaction ($F = 0.36$, $p = 0.793$).

An examination of between subjects effects of experimental factors showed that IgA was greater for infected than uninfected mice ($F = 18.01$, $p < 0.001$; Figure 11A) and was greater for CR than AL mice ($F = 23.20$, $p < 0.001$; Figure 11B). IgA did not vary with experiment duration ($F = 1.29$, $p = 0.282$) and there was no interaction between infection status and experiment duration ($F = 0.24$, $p = 0.919$), infection status and food treatment ($F < 0.01$, $p = 1$), experiment duration and food treatment ($F = 1.09$, $p = 0.234$) or among all three factors ($F = 0.18$, $p = 0.935$). Regressions of IgA levels and infection intensity did not meet the assumption of randomly distributed residuals for either CR or AL mice regardless of the transformations tested (raw data, $\log_{10}$, square root, inverse); therefore, this test was not performed.

Immunoglobulin A (IgA): SJL Strain

Because homogeneity of variances could not be met, I used a repeated measures randomization test of Log_{10} transformed data to examine plasma IgA levels (Figure 12). For the within subjects results, IgA production did not change among sampling days ($F = 12.23$, $p = 0.212$) but there was a significant interaction between infection status and sampling day ($F =$

12.40, p<0.001; Figure 13A) because IgA levels in uninfected animals remained the same but infected animals averaged more IgA on the Final Day then they did on Day 0 with IgA levels on Day 10 not different either from Day 0 or the Final Day. Further, on Day 0 IgA levels were similar for infected and uninfected mice but on Day 10 and the Final Day infected mice had greater IgA levels than uninfected mice. There was also a significant interaction between food treatment and sampling day ($F = 9.79$, $p < 0.001$; Figure 13B) because AL animals had more IgA on the Final Day than at Day 0 (with Day 10 similar to both Day 0 and the Final Day) while CR animals had similar IgA levels among all sampling days. There were no significant interactions between sampling day and experiment duration ($F = 1.31$, $p = 0.438$), sampling day and infection status and experiment duration ($F = 0.50$, $p = 0.926$), sampling day and infection status and food treatment ($F = 2.80$, $p = 0.204$), sampling day and experiment duration and food treatment ($F = 2.70$, $p = 0.164$), and no four-way interaction ($F = 1.10$, $p = 0.497$).

Regarding between subjects results of experimental factors, infected mice had greater IgA levels than uninfected mice ($F = 12.36$, $p < 0.001$; Figure 14). There were no significant effects of experiment duration ($F = 1.26$, $p = 0.294$), food treatment ($F = 0.28$, $p = 0.607$), or any two-way (infection status and experiment duration ($F = 0.09$, $p = 0.616$), infection status and food treatment ($F = 3.94$, $p = 0.078$), experiment duration and food treatment ($F = 0.13$, $p = 0.687$)) or three-way ($F = 0.78$, $p = 0.225$) interactions. Regressions of IgA levels and infection intensity did not meet the assumption of randomly distributed residuals for any of the transformations tested (raw data, $\log_{10}$, square root, inverse); therefore, this test was not performed.

Total Immunoglobulin G1 (IgG1): C57 Strain

Because homogeneity of variances could not be met, I used a repeated measures randomization test of Log_{10} transformed data to examine plasma total IgG1 levels (Figure 15). For the within subjects effects, total IgG1 varied among sampling days ($F = 646.92$, $p < 0.001$). There were significant two-way interactions between infection status and sampling day ($F = 649.20$, $p < 0.001$) and experiment duration and sampling day ($F = 6.73$, $p = 0.027$) and also a significant three-way interaction among infection status, experiment duration and sampling day ($F = 4.47$, $p = 0.013$; Figure 16A). The three-way interaction occurred because there was no change in total IgG1 for uninfected mice regardless of sampling day and experiment duration, but total IgG1 changed among sampling days in infected mice and the degree of this change depended on the experiment duration. Specifically, at Day 0 all infected mice had total IgG1 levels similar to each other and to uninfected mice. At Day 10 all infected mice had an increase in total IgG1 from Day 0 in a similar fashion; however, for animals that were infected for 4 and 6 weeks the increase from Day 10 to Final day was greater than for animals that were infected for only 2 weeks. There also was a significant three-way interaction among infection status, food treatment and sampling day ($F = 3.47$, $p = 0.037$; Figure 16B). This is because uninfected mice had similar levels of total IgG1 regardless of food treatment and sampling day; however, total IgG1 levels in infected animals increased among sampling days and the increase between Day 10 to the Final Day for AL mice was almost greater than for CR mice (95% confidence intervals were 3.902-4.042 and 3.789-3.927).

For between subjects experimental factors, infected mice had greater total IgG1 than uninfected mice ($F = 604.19$, $p < 0.001$; Figure 17). Total IgG1 did not vary with experiment

duration (F = 2.78, p = 0.0640) or food treatment (F <0.01, p = 1) and there were no interactions between infection status and experiment duration (F = 0.08, p = 0.895), infection status and food treatment (F = 2.91, p = 0.088) experiment duration and food treatment (F = 0.59, p = 0.496) or among all three factors (F = 0.45, p = 0.575). Regressions of total IgG1 levels and infection intensity either did not meet the assumption of randomly distributed residuals or normality, or both for any of the transformations tested (raw data, $\log_{10}$, square root, inverse); therefore, this test was not performed.

Total Immunoglobulin G1 (IgG1): SJL Strain

Because homogeneity of variances could not be met, I used a repeated measures randomization test of Log_{10} transformed data to examine plasma total IgG1 levels (Figure 18). For the within subjects effects, levels of total IgG1 changed among sampling days (F = 179.89, p < 0.001) with significant interactions between infection status and sampling day (F = 192.99, p < 0.001) and between food treatment and sampling day (F = 10.44, p < 0.001). There also was a significant three-way interaction among infection status, food treatment and sampling day (F = 8.57, p < 0.001; Figure 19) because total IgG1 increased among sampling days only in infected mice, and this increase was dependent on food treatment. Specifically, infected AL animals experienced a significant increase in IgG1 from Day 0 to Day 10, as well as from Day 10 to Final Day. However, infected CR animals had a significant increase from Day 0 to Final Day, and from Day 10 to Final Day but not between Day 0 and 10 (although 95% confidence intervals were almost non-overlapping between Day 0 and 10: Day 0 95% confidence intervals: 3.136-3.333, Day 10 95% confidence intervals: 3.32-3.496). Also, AL infected mice on Day 10 and Final Day had more IgG1 than their uninfected controls; however, CR mice did not have significantly more

IgG1 than their uninfected controls on Day 10, although their 95% confidence intervals were almost overlapping (CR infected: 3.32-3.496, CR uninfected: 3.3134- 3.332). Total IgG1 of infected mice was similar for CR and AL mice at each sampling day, but uninfected CR mice had more total IgG1 than uninfected AL mice at each sampling day.

For between subjects results of experimental factors, there was a significant effect of infection status ($F = 85.95$, $p < 0.001$) and food treatment ($F = 12.41$, $p = 0.001$), but no effect of experiment duration ($F = 0.47$, $p = 0.630$). There was a significant interaction between infection status and experiment duration ($F = 3.69$, $p = 0.008$; Figure 20A) because uninfected mice had similar total IgG1 levels regardless of experiment duration but total IgG1 levels of infected mice varied among experiment durations. Specifically, mice infected for two weeks had almost significantly less total IgG1 than mice infected for six weeks (95% confidence intervals were 3.322-3.514 and 3.451-3.646 for mice that were infected for 2 and 6 weeks, respectively) which likely drove the significance of this interaction. Infected mice at four and six weeks had similar total IgG1 levels to each other. There also was a significant interaction between infection status and food treatment ($F = 10.31$, $p = 0.005$; Figure 20B) because uninfected CR mice had more total IgG1 than uninfected AL mice, but when infected the AL and CR mice had similar levels of total IgG1. There was no significant interaction between experiment duration and food treatment ($F = 0.93$, $p = 0.285$) and no three-way interaction ($F = 0.25$, $p = 0.670$). Regressions between infection intensity and total IgG1 did not meet the assumption of randomly distributed residuals; therefore, this test was not performed.

H. bakeri-specific Immunoglobulin G1 (IgG1): C57 Strain

Because homogeneity of variance assumption could not be met, a repeated measures randomization test of $Log_{10}(x+10)$ was performed to examine plasma *H. bakeri*-specific IgG1 levels (Figure 21). For the within subjects effects, *H. bakeri*-specific IgG1 varied among sampling days ($F = 138.91$, $p < 0.001$), and there was a significant interaction between infection status and sampling day ($F = 107.49$, $p < 0.001$) and experiment duration and sampling day ($F = 32.61$, $p < 0.001$). There was a three-way interaction among infection status, experiment duration and sampling day ($F = 25.63$, $p < 0.001$; Figure 22) because levels of *H. bakeri*-specific IgG1 remained close to zero in uninfected mice, while levels in infected mice depended on the experiment duration and sampling day. On Day 10, mice that were infected for 4 weeks had more *H. bakeri*-specific IgG1 than uninfected mice, while mice that were infected for 2 and 6 weeks had similar levels of *H. bakeri*-specific IgG1 as uninfected mice. On the Final Day all infected mice had more *H. bakeri*-specific IgG1 than uninfected mice, but *H. bakeri*-specific IgG1 in infected mice was greater with longer experiment durations. Specifically, *H. bakeri*-specific IgG1 increased from Days 10 to the Final Day when mice were infected for 4 and 6 weeks but not for mice that were infected for only 2 weeks and the increase between Day 10 and the Final Day was greater for mice in the 6 week experiment duration than the 2 or 4 week experiment duration. Further, all infected mice on the Final Day had greater *H. bakeri*-specific IgG1 than uninfected mice, but infected mice at Day 10 had greater levels than uninfected mice only for animals in the 4 week experiment duration. There were no significant interactions between sampling day and food treatment ($F = 1.77$, $p = 0.201$), sampling day and infection status and

food treatment (F = 0.39, p = 0.524), sampling day and experiment duration and food treatment (F = 0.89, p = 0.567) and no four-way interaction (F = 0.22, p = 0.892).

For between subjects results of experimental factors, there was a significant effect of infection status (F = 148.80, p < 0.001) and experiment duration (F = 15.22, p < 0.001), but not food (F = 0.16, p = 0.754). There was a significant interaction between infection status and experiment duration (F = 12.68, p < 0.001; Figure 23) because levels of *H. bakeri*-specific IgG1 were similar in all uninfected mice regardless of experiment duration while *H. bakeri*-specific IgG1 increased with longer experiment durations for infected mice. Specifically, mice that were infected for 4 and 6 weeks had more *H. bakeri*-specific IgG1 than mice that were infected for 2 weeks, but *H. bakeri*-specific IgG1 levels were similar between mice that were infected for 4 and 6 weeks. There were no significant interactions between infection status and food treatment (F = 0.14, p = 0.741) experiment duration and food treatment (F = 1.34, p = 0.388) or among all three factors (F = 1.50, p = 0.240). *H. bakeri*-specific IgG1 was not influenced by the number of worms for mice that were infected for 2 weeks ($F_{1,27}$ = 0.256, p = 0.617, raw data; Figure 24), while all other regressions did not meet the assumption of randomly distributed residuals and were, therefore, not performed.

<u>*H. bakeri*-specific Immunoglobulin G1 (IgG1): SJL Strain</u>

Because homogeneity of variance assumption could not be met, a repeated measures randomization test of $Log_{10}(x+10)$ was performed to examine plasma *H. bakeri*-specific IgG1 levels (Figure 25). For within subjects effects, *H. bakeri*-specific IgG1 varied among sampling days (F = 66.02, p < 0.001) and there was a significant interaction between infection status and sampling day (F = 45.90, p < 0.001) and experiment duration and sampling day (F = 18.26, p <

0.001). There also was a three-way interaction among infection status, experiment duration, and sampling day ($F = 7.25$, $p = 0.014$; Figure 26) because levels of *H. bakeri*-specific IgG1 were similar in uninfected mice regardless of experiment duration or sampling day but levels of *H. bakeri*-specific IgG1 for infected mice depended on the experiment duration and sampling day. Specifically, when sampled on Day 10 post-infection, *H. bakeri*-specific IgG1 levels were similar for infected mice from all experiment durations but on the Final Day *H. bakeri*-specific IgG1 increased with longer experiment duration. Within a single experiment duration, *H. bakeri*-specific IgG1 levels were similar between Day 10 and the Final Day for the 2 week duration but was greater on the Final Day than Day 10 for experiment durations of 4 and 6 weeks. There were no other significant 2-way (sampling day and food treatment ($F = 0.46$, $p = 1$)), 3-way (sampling day and infection status and food treatment ($F = 0.08$, $p = 0.700$) and sampling day and experiment duration and food treatment ($F = 3.54$, $p = 0.123$)) or four-way ($F = 0.60$, $p = 0.683$) interactions.

For between subjects results of experimental factors, *H. bakeri*-specific IgG1 varied with infection status ($F = 91.01$, $p < 0.001$) and experiment duration ($F = 11.30$, $p < 0.001$), but not with food treatment ($F = 1.40$, $p = 0.213$). There was a significant interaction between infection status and experiment duration ($F = 8.11$, $p = 0.001$; Figure 27) because uninfected mice had similar levels of *H. bakeri*-specific IgG1 regardless of experiment duration, while mice that were infected with *H. bakeri* for 6 weeks had more *H. bakeri*-specific IgG1 than mice that were infected for either 2 or 4 weeks. In addition to this, mice that were infected for 4 weeks had almost significantly greater *H. bakeri*-specific levels than mice that were infected for 2 weeks (95% confidence intervals were 1.013-1.019 and 1.007-1.013, respectively). There were no

significant interactions between infection status and food treatment ($F = 2.68$, $p = 0.008$), experiment duration and food treatment ($F = 1.98$, $p = 0.13$) or among all three factors ($F = 0.50$, $p = 0.68$). Regressions between infection intensity and *H. bakeri*-specific IgG1 did not meet the assumption of randomly distributed residuals for any of the experiment durations and were, therefore, not performed.

Mucosal Mast Cell Protease-1 (mMCP-1): C57 Strain

Because homogeneity of variances could not be met, a randomization test of Log_{10} transformed data was performed and 95% confidence intervals were used as a post-hoc evaluation (Figure 28). mMCP1 varied with infection status ($F = 2375.94$, $p < 0.001$), experiment duration ($F = 56.64$, $p < 0.001$), and food treatment ($F = 53.55$, $p < 0.001$). There was a significant interaction between infection status and experiment duration ($F = 106.34$, $p < 0.001$; Figure 29A) because uninfected mice had similar mMCP-1 levels regardless of experiment duration but mMCP1 levels varied among experiment durations for infected mice. Specifically, the highest mMCP1 levels occurred in mice that were infected for 4 weeks; mice that were infected for 6 weeks had 63% lower mMCP-1 levels than mice that were infected for 4 weeks, and mice that were infected for 2 weeks had 97% lower mMCP-1 levels than mice that were infected for 4 weeks and 92% lower than mice infected for 6 weeks. For each experiment duration, infected mice always had higher mMCP-1 levels than uninfected mice. Another significant interaction was that of infection status and food treatment ($F = 39.55$, $p < 0.001$; Figure 29B) because CR mice had greater mMCP1 levels than AL mice when they were uninfected but similar mMCP1 levels when they were infected with *H. bakeri*. Last, there was a significant interaction between experiment duration and food treatment ($F = 16.94$, $p < 0.001$;

Figure 29C). This is because CR mice at 4 and 6 weeks of the experiment had greater mMCP-1 levels than AL mice but mice at the 2 week experiment duration mMCP-1 levels were similar for CR and AL mice. Both AL and CR mice had highest levels of mMCP-1 at 4 weeks of experiment duration, but for CR mice mMCP-1 did not significantly decrease between week 4 and 6. The three-way interaction among infection status, experiment duration and food treatment was not significant ($F = 1.15$, $p = 0.859$).

High levels of mMCP-1 corresponded to low numbers of worms for both CR mice ($F_{1,43} = 15.26$, $p < 0.001$; $\log_{10}$ transformed) and AL mice ($F_{1,41} = 9.71$, $p = 0.003$, $\log_{10}$ transformed) (Figure 30A). After accounting for experiment duration within each food treatment, mice that were infected for 4 weeks in the CR group ($F_{1,13} = 12.31$, $p = 0.004$; $\log_{10}$ transformed) and mice that were infected for 2 weeks in the AL group ($F_{1,12} = 5.97$, $p = 0.031$; $\log_{10}$ transformed) also had fewer worms with greater levels of mMCP-1 (Figure 30B). mMCP-1 for mice that were infected for 6 weeks in the AL group was not influenced by the infection intensity ($F_{1,12} = 1.78$, $p = 0.207$; $\log_{10}$ transformed; Figure 30B). Regressions for CR mice that were infected for 2 and 6 weeks, and for AL mice that were infected for 4 weeks did not meet the assumption of randomly distributed residuals regardless of transformation used (raw data, $\log_{10}$, square root, inverse), therefore, regressions were not done.

<u>Mucosal Mast Cell Protease-1 (mMCP-1): SJL Strain</u>

mMCP1 levels varied with infection status ($F_{1,140} = 298.17$, $p < 0.001$) and food treatment ($F_{1,140} = 51.95$, $p < 0.001$) but not experiment duration ($F_{2,140} = 0.76$, $p = 0.472$; Figure 31). There was a significant interaction between infection status and food treatment ($F_{1,140} = 7.46$, $p = 0.007$; Figure 32A) because even though AL mice had greater mMCP-1 than CR mice in both

infected and uninfected groups this difference was more pronounced in infected mice. There was also a significant interaction between experiment duration and food treatment ($F_{2,140}$ = 3.91, p = 0.022; Figure 32B) because AL and CR mice had similar mMCP1 levels at 4 weeks but CR mice had lower mMCP1 levels than AL mice at both 2 and 6 weeks. There were no significant interactions between infection status and experiment duration ($F_{2,140}$ = 0.22, p = 0.801) or among all three factors ($F_{2,140}$ = 2.88, p = 0.059). Regressions for mMCP-1 did not meet the assumption of randomly distributed residuals and were therefore not done.

Discussion

Many people worldwide suffer from parasite infection (Brooker, 2010). It is estimated that 1 billion people are infected with at least one parasitic worm (helminth) (Wang *et al*., 2008), and that 65,000 deaths occur yearly due to helminth-related complications (Brooker, 2010). Since CR and CR mimetics are investigated as potential therapies in humans to prolong life-span and prevent age-associated disease, greater susceptibility to harmful pathogens during chronic CR must be considered. My experiment examined cellular and immunoglobulin immune responses to study effects of long-term calorie restriction on susceptibility of two strains of laboratory mice to intestinal nematode infection. Overall, I found that both C57 and SJL mice mounted an immune response against *H. bakeri*, but timing of immune responses and effect of CR and experiment duration on immune responses varied between the two strains. Below I discuss each dependent variable with respect to timing (experiment duration) and calorie availability (CR versus AL) and then conclude with a comparison of overall immune function in these two mouse strains.

Differential White Blood Cell Counts

White blood cells (WBCs) are important to the immune response against parasites (Schmidt and Roberts, 2005). Because eosinophils and neutrophils function, in part, during the innate immune response, they may be most important when the parasite first establishes in the host until immunoglobulins build up in sufficient numbers. My study showed that, as expected, both C57 and SJL mice increased production of eosinophils in the presence of *H. bakeri*, but the two strains showed different responses across experiment durations. C57 mice showed an early response to *H. bakeri* infection with a peak in eosinophil numbers after 4 weeks of infection, while infected SJL mice had consistently higher eosinophil numbers at all experiment durations compared to uninfected mice. However, percentage of eosinophils for infected SJL mice ranged from approximately 1-2% whereas percentage of eosinophils for infected C57 mice ranged from approximately 4-6%. This finding indicates that C57 mice may rely more on eosinophil effects than SJL mice despite the constant, albeit lower, eosinophil response seen in SJL mice for all infection durations. Eosinophils and neutrophils are types of WBCs involved in reactive oxygen species (ROS) production (Schmidt and Roberts, 2005) and ROS are used by the host to damage tissue of the intestinal parasite *Heligmosomoides polygyrus* (Ben-Smith *et al*., 2002). While the SJL strain has been postulated to rely more heavily on ROS than some other strains (Ben-Smith *et al*., 2002), ROS in this strain could come from alternative sources (i. e. neutrophils, discussed below) rather than from eosinophils. A determination of actual function of eosinophils during infection for each strain may help elucidate why eosinophilia varied between the two strains. For example, eosinophils contain various enzymes that damage helminths such as major basic protein, eosinophil cationic protein and eosinophil neurotoxin

(reviewed by Abbas *et al*., 2012, Makepeace *et al*., 2012). Eosinophils also contain cytokines, chemokines and growth factors that are used to communicate with the rest of the immune system in order to initiate an anti-helminthic response (Makepeace *et al*., 2012). Since food treatment had no effect on eosinophils in either strain this confirms the previous finding by Kristan (2007) that eosinophilia does not depend on calorie restriction; my results extend this finding to show that eosinophilia does depend on the duration of infection and mouse strain after mice received long-term CR.

Besides eosinophils, neutrophils are also important players in the host response to parasite infections. Upon infection with *N. dubius*, mouse neutrophils enter an "altered" state which enables them to damage the parasite (Penttila *et al*., 1984b). The presence of these altered neutrophils is evident in as little as four days post infection, and is independent of the presence of immunoglobulins. Since altered neutrophils capable of damaging the parasite continue to occur even six weeks after a secondary infection it is obvious that neutrophils may play a role in later stages of infection; however, their relative importance might be greater in earlier stages given that immunoglobulins take time to build in sufficient numbers. My study showed that C57 mice had more neutrophils when infected for 2 weeks than 6 weeks which confirms the role of these cells in earlier stages of infection. An alternative explanation for the decrease in blood neutrophil numbers during later stages of infection in C57 mice is that neutrophils (along with other types of white blood cells) will migrate towards the intestinal tissue as part of a local immune response to *H. bakeri* infection (Makepeace *et al*., 2012). Neutrophils are the first cells to get recruited to the site of inflammation where they can stop apoptosis. Neutrophils can also release lytic enzymes and ROS. ROS can in turn serve to recruit

more neutrophils and other granulocytes out of the blood and to the local site. A future study could be done to compare changes in white blood cell numbers between blood and intestinal tissue to understand the relationship between systemic and local immune responses. By comparison, SJL mice also showed an increase in neutrophils when infected, but neutrophil numbers did not vary with experiment duration. If SJL mice rely on ROS production by neutrophils to continuously damage *H. bakeri*, even when other components of the adaptive immune response (e.g., immunoglobulins) also occur, then high level of neutrophils regardless of infection duration make sense. Continuous production of neutrophils after infection might be the reason why SJL mice are typically more resistant to *H. bakeri* infection (Reynolds *et al*., 2012, Zhong and Dobson, 1996) since neutrophils are key cells in conferring resistance to *N. dubius* infection (Penttila *et al*., 1984a). Interestingly, uninfected SJL mice also showed more neutrophils after 4 weeks of the experiment than after 2 weeks of the experiment which may indicate that neutrophils naturally fluctuate in SJL mice or are related to mouse age.

Since calorie restriction may affect the balance between ROS and antioxidants (Lopez-Torrez *et al*., 2002, Wu *et al*., 2003), it is possible that infected CR C57 mice had to produce more neutrophils than infected AL mice in order to compensate for decreased ROS production by neutrophils on a per cell basis. Unlike C57 mice, SJL mice show that systemic neutrophil responses can become invigorated by CR even in the absence of infection. Therefore, CR may provide some immune boosting effects related to neutrophils that could confer protection against pathogen infection.

Lymphocytes are the most common type of white blood cell in uninfected mice that typically have about 85% lymphocytes, 10% neutrophils, 2% eosinophils and 3% monocytes

(Wilkinson *et al*., 2001). My study also showed lymphocytes to be the most numerous white blood cells in uninfected mice (89% ± 0.6 standard error for C57 and 88% ± 1.1 standard error for SJL) but that lymphocyte percentage decreased with *H. bakeri* infection. It is possible that after *H. bakeri* infection lymphocytes were leaving the blood to go into lymphoid tissues. Naïve T lymphocytes constantly circulate between peripheral blood and secondary lymphoid tissues such as the spleen and mucosal lymphoid tissue (Abbas *et al*., 2012). Naïve T cells that do not encounter an antigen can drain back into the blood, but once an antigen is encountered T cells proliferate into thousands of effector and memory T cells which then migrate to the site of infection and inflammation. Lower percentages of lymphocytes at 2 and 4 weeks after infection than at 6 weeks after infection in C57 mice might be due to migration to and proliferation in lymphoid tissues. Splenic and mesenteric lymph node lymphocyte proliferation has been implicated in exposure to *H. polygyrus in vitro* (Enriquez *et al*., 1987), although only during secondary and tertiary infections. Enriquez *et al*. (1987) might have not observed lymphocyte proliferation after primary infection because they used different strains of mice (B10.BR (H-2k) and B10.M (H-2f) vs. C57BL/6 and SJL in my study) and because they used a different assay (*in vitro* culture with antigen vs. my study used extrapolation from peripheral blood white blood cell count). Future studies should be done to compare differential white blood cell count in lymphoid tissues and gut to the one in peripheral blood to test this hypothesis. Higher percentage of lymphocytes in C57 mice at 6 weeks in comparison to 2 and 4 weeks might indicate that T cells are draining back into peripheral blood for subsequent recruitment to the site of infection, as increased lymphocyte proliferation in peritoneal exudate has been observed before in mice infected with *N. dubius* (Prowse *et al*, 1978). Another explanation might be that

C57 mice started to produce more naïve T cells to make up for the maturation of existing T cells due to *H. bakeri* exposure.

CR has been implicated in preservation of naïve T cells (reviewed by Nikolich-Zugich and Messaoudi, 2005). CR-induced lymphocyte proliferation in lymphoid tissues (reviewed by Pahlavani, 2000 and Pahlavani, 2004), could be the reason why infected C57 mice had less lymphocytes in the blood when they were given CR than when they were fed AL. Since CR SJL mice had less lymphocytes than AL SJL mice regardless of infection, my finding is consistent with SJL being the faster responding strain, as more resistant strains showed greater CR-induced lymphocyte proliferation in lymphoid tissues than susceptible strains which would leave fewer lymphocytes circulating in the blood (Enriquez *et al*., 1987).

While white blood cell proliferation of all white blood cell types may be directly influenced by parasite infection and calorie restriction, it is also possible that results reflect changes in one type of white blood cell which then alters percentages of remaining white blood cells being counted. For example, the increased percentage of neutrophils and eosinophils due to cell proliferation may cause a decreased percentage of lymphocytes. It is also possible that *H. bakeri* and CR had proliferative effects on white blood cells in general, but that the relative effect was higher on neutrophils and eosinophils than on lymphocytes. Implications of *H. bakeri* and CR on C57 and SJL total leukocytes has not been investigated; therefore a future study should be designed to include a total number of white blood cells per unit volume of blood in addition to relative percentages of each white blood cell type.

In summary, I found that *H. bakeri* infection in both C57 and SJL caused an increased percentage of neutrophils and eosinophils in blood with concurrent decreased percentage of

lymphocytes. This might have been due to increased peripheral neutrophil and eosinophil response against *H. bakeri*, migration of lymphocytes into lymphoid tissues and/or the site of infection, or due to increase of total leukocytes with higher percentage increases in neutrophils and eosinophils than in lymphocytes. The overall changes in white blood cells fluctuated in C57 while they remained constant in SJL mice. SJL mice had a higher neutrophil but lower eosinophil response which only partially supported my hypothesis. I also retained my hypothesis that CR would have no effect on eosinophilia. CR had an invigorating response on neutrophils in both strains. Since CR had an invigorating response on neutrophils in SJL even in the absence of infection, this is consistent with the notion that SJL rely heavily on ROS. The reliance could have been so high that SJL increased the number of ROS-producing neutrophils to compensate for the CR attenuation by ROS even in the absence of any immune challenge. Since white blood cells are involved in antigen processing and production of immunoglobulins, changes in white blood cells in the blood may influence the amount of IgA, total IgG1 and *H. bakeri*-specific IgG1 that mice can make during their adaptive immune response.

Immunoglobulins

Immunoglobulin responses are part of the adaptive immune system which takes longer to develop, but are a potent wing of the immune system. My study showed that both C57 and SJL mice use IgA during early and late stages of *H. bakeri* infection. For example, infected C57 mice had more IgA on Day 10 and the Final Day than on Day 0, with no significant increase from Day 10 to the Final Day (although it was approaching significance). SJL mice also had greater IgA levels from Day 0 to the Final Day, but IgA on Day 10 was not significantly different than IgA on either Day 0 or the Final Day, indicating a more gradual IgA response by SJL than C57 mice,

contrary to my expectations. Since experiment duration was not a significant factor in either strain this suggests that IgA only increases during the first couple of weeks post infection, and then it levels off afterwards with no further increases at 4 and 6 weeks post-infection. Since IgA half-life is only three days (Abbas, *et al*., 2012) the lack of change in IgA levels between different experiment durations indicates that both C57 and SJL mice must continue to produce IgA in later stages of infection in order to maintain similar IgA levels from week 2 through week 6.

The increased levels of IgA in infected mice may coincide with lymphocyte proliferation in lymphoid tissues, although food treatment and experiment duration did not have equal effects on lymphocyte proliferation and IgA. For example, while the percentage of lymphocytes in the blood was lower at weeks 2 and 4 than at week 6 in C57 mice, the experiment duration had no effect on IgA in this mouse strain. While CR mice of C57 strain had less lymphocytes in blood than their AL counterparts (only during infection), IgA was higher in CR mice regardless of infection. In addition for SJL mice, while CR mice always had less lymphocytes than AL mice, food treatment had no effect on IgA. These findings suggest that although experiment duration may affect lymphocyte proliferation in C57 mice and food treatment might affect lymphocyte proliferation in SJL mice, these effects do not further translate into similar changes in IgA levels. Although I did not measure specific populations of lymphocytes directly, it is possible that *H. bakeri* infection increases B cell proliferation, while food treatment and experiment duration affect other lymphocyte populations, such as T cells. An alternative explanation is that experiment duration and food treatment might affect other immunoglobulins instead, such as total and *H. bakeri*-specific IgG1 (discussed later). The findings also suggest that CR might

affect IgA levels in C57 mice through mechanisms other than lymphocyte proliferation. For example, calorie restricted diet can change the gut microbiota (Ley, 2006), which can in turn affect IgA. For example, leaner body type has been associated with higher ratio of *Bacteriodetes* to *Fermicutes* phyla in both humans (Ley, 2006) and mice (Turnbaugh *et al*., 2006). *Bacteriodetes* are the major producers of anti-inflammatory immunomodulators such as short chain fatty acids and glycans (Maslowski and Mackay, 2011). Glycan from *Bacteroides fragilis* increases the production of interleukin 10 (IL10) (Maslowski and Mackay, 2011), and IL10 can in turn upregulate the production of IgA (Friman *et al*., 1996).

Unlike IgA that leveled off on Day 10 in infected C57 mice, total IgG1 not only increased on Day 10, but continued to increase on the Final Day and with longer experiment durations (4 and 6 weeks). In contrast, total IgG1 for SJL mice increased on Day 10 only for AL mice, while CR mice did not show an increase in total IgG1 until the Final Day (although the increase on day 10 was almost significant), and longer experiment durations did not have an effect on total IgG1. This finding suggests that contrary to my expectations, CR attenuated total IgG1 in SJL mice, and that SJL mice mounted a slower total IgG1 response than C57 mice. Since IgG1 half-life is 21-28 days (Abbas *et al*., 2012), similar levels of total IgG1 in SJL mice on the Final Day regardless of experiment duration might mean that total IgG1 production stalls after 2 weeks. Since total IgG1 increased from Day 0 to Day 10 in similar fashion for CR and AL infected C57 mice, and the increase from Day 10 to the Final Day was almost significantly greater in AL mice, it appears that CR also does not have an invigorating effect on C57 mice, which was contrary to my expectations. The lack of total IgG1 invigoration by CR in both strains might be due decreased energy supply available in CR mice to allocate towards immunity. Although

maintaining the immune system requires minimum energy (Klasing (1998) estimated that immune cells and molecules amount to less than 5% of total body mass in chicken), calorie availability may be important in raising the immune response above baseline (i.e. during infection). For example, Derting and Compton (2003) noticed that white footed mice injected with sheep red blood cells had decreased testes and small intestine masses, and therefore presumably diverted energy away from organs of reproduction and toward other physiological processes in order to mount an immune response. It is worth noting that none of the other organ masses changed due to the immune challenge, and resting metabolic rate (RMR) and daily metabolic rate also did not change; therefore in the great scope of things mounting an immune response may not be biologically significant as related to energy expenditure. Additional studies regarding metabolic rates showed that cabbage butterfly pupae raised standard metabolic rate when challenged with a nylon implant (Freitak *et al*., 2003), and resting metabolic rate (RMR) increased after *H. bakeri* infection in two studies by Kristan and Hammond (2000 and 2001), but not in one additional study by Kristan and Hammond (2006). The discrepancy between the studies by Kristan and Hammond may be due to differences in parasite culture infectivities. RMR also increased in juvenile chipmunks infected with bot flies (Careau *et al*., 2010), and the increase in RMR was strongly correlated with the number of bot flies. Infected juvenile chipmunks also lost body mass, although none of these effects were observed in adult chipmunks, presumably because the development part of the life history is more energetically costly than adulthood. The effects of CR on mounting an immune response may be particularly relevant in natural conditions since animals live in a pathogen-rich world (Kristan, 2008), and a prolonged energy restriction can increase susceptibility to opportunistic

pathogens (Lochmiller and Deerenberg, 2000). Although wild mice do not consume fewer calories in nature compared to AL fed laboratory mice (Austad and Kristan, 2003), it is reasonable to expect wild mice may eat more calories if additional food was available. Evolutionary adaption might have resulted in mice that are able to divert limited energy sources away from less crucial immune responses to intestinal nematode, such as total IgG1, into more crucial immune responses potentially related to worm expulsion, such as mMCP-1 (discussed below).

Although total IgG1 may have a role in decreasing the female worm fecundity, protection against repeated *H. polygyrus* infection was only granted by *H. bakeri* specific IgG1 (McCoy *et al*., 2008). I would extend this explanation to include that development of parasite-specific IgG1 occurs during primary infection, but it may not accumulate in great enough numbers in order to provide protection when experimentally transferred to naïve mice prior to their own infection. Indeed, when authors measured IgG1 specific to excretory-secretory (HES) *H. polygyrus* products, they detected HES-specific IgG1 in C57BL/6 mice after 25 days. My study extends this finding to include that *H. bakeri*-specific IgG1 is present as early as 2 weeks after primary infection, and it continues to increase 4 and 6 weeks after primary infection in both C57 and SJL mice. Although McCoy *et al*. (2008) found that *H. polygyrus* HES-specific IgG1 was vital in decreasing worm numbers in secondary infections, I did not find that mice with higher *H. bakeri*-specific IgG1 had lower worm numbers at 2 weeks post infection in C57BL/6 during a primary infection. This is likely because McCoy *et al*. suspected that the main mechanism by which *H. polygyrus* HES-specific IgG1 reduces the adult worm number is by targeting L4 and preventing adult worm establishment in the lumen. Since my study used a primary infection, *H.*

bakeri specific IgG1 was not present when *H. bakeri* was in the larval and early adult stages in my infected mice.

Since *H. bakeri*-specific IgG1 response was similar in C57 and SJL mice in regards to all 3 experimental treatments (infection, food treatment and experiment duration) and in regards to sampling days I rejected my hypothesis that SJL would have a more robust immunoglobulin response than C57. I am postulating that the difference between the fast response in SJL (Behnke *et al*., 2006) and slow response in C57BL/6 (Behnke *et al*., 2006, Morgan *et al*., 2006) observed in previous research might have been observed due to higher levels of *H. bakeri*-specific IgG1 in the fast-responding strains they used, or the differences in strain response to *H. bakeri* might depend on other immune responses.

Since food treatment had no effect on *H. bakeri*-specific IgG1 of either strain I rejected my hypothesis that CR would increase *H. bakeri*-specific IgG1. What is unique about *H. bakeri*-specific IgG1 as opposed to other variables that I measured (white blood cells, IgA, total IgG1 and mMCP-1) is that these other variables are naturally present in the mice prior to infection. As such they are able to be acted upon by the food treatment for the entire duration of the food treatment (6 months). In contrast, *H. bakeri*-specific IgG1 occurs only after infection with *H. bakeri*; and is therefore only able to be acted upon by the food treatment for the duration of the experiment (2, 4 and 6 weeks). It would be exciting to do a future study in which long term calorie restriction is followed by a longer infection duration (> 6 weeks), or repeated infections, in order to establish whether CR can have an invigorating response on *H. bakeri*-specific IgG1 if CR is allowed to act longer on this particular variable.

Another reason for the lack of food treatment effect on *H. bakeri*-specific IgG1 could be that *H. bakeri*-specific IgG1 is present in such small amounts in plasma, and as such it evades the CR effect, or the effect of CR might be so small that it is undetectable with ELISA used in this study. The small amount of *H. bakeri*-specific IgG1 in plasma is evident from dilutions used in ELISAs - samples for *H. bakeri*-specific IgG1 were diluted only 50X, while all plasma samples for total IgG1 were diluted 500-240,000X. In addition, McCoy *et al*. (2008) showed that C57BL/6 mice produced more *H. bakeri*-specific IgG1 on Day 0 and 10 when challenged with a secondary infection than primary infected mice produced on day 10 and 25. Secondary and other subsequent challenge infections might provide a better platform to study the effects of CR on *H. bakeri*-specific IgG1 production since *H. bakeri*-specific IgG1 might be present in larger amounts enabling the study of experimental treatments with such a small effect that could otherwise go undetected (i.e. CR).

In summary, C57 mice showed a more robust IgA and total IgG1 response, while the robustness of *H. bakeri*-specific IgG1 response was similar in both strains with no effects of CR. More specifically, while IgA increased in early stages of infection, this increase was more gradual in SJL mice than C57 mice and no further increases were observed with longer experiment duration in either strain. CR increased IgA response in C57 regardless of infection, but had no effect on IgA in SJL mice. Total IgG1 increased during early stages of infection, but this increase was more robust, and longer lived in C57 than SJL mice. In addition to having a more gradual increase in early stages of infection, SJL mice also showed no further increases with longer infection durations. While total IgG1 initially increased similarly in CR and AL C57 mice, CR actually almost significantly attenuated total IgG1 in later stages. CR also almost

significantly attenuated total IgG1 in SJL mice during early stages of infection, but when SJL mice were uninfected CR actually invigorated total IgG1 response. Overall, my findings showed that, contrary to my hypothesis, non-specific immunoglobulin responses are higher in C57 than in SJL mice, but that *H. bakeri*-specific immunoglobulin response is similar in both strains.

Mucosal Mast Cell Protease-1

Gastrointestinal nematode infections are often accompanied by proliferation of mucosal mast cells and their subsequent activation and release of mucosal mast cell protease-1 (mMCP1) (Miller, 1996). mMCP-1 is thought to target a protein (McDermott *et al*., 2003) or multiple proteins (Andersson *et al*., 2008) in epithelial tight junctions and consequentially increase gut permeability. Given that *H. bakeri* adults reside in intestinal lumen (Bryant, 1973) increased gut permeability may aid the physical expulsion of the worms as related to movement of water across the intestinal tissue. mMCP-1 might be of higher relative importance in primary infections because parasite-specific IgG1 response is slow and it requires specificity. *H. bakeri* has evolved the ability to suppress host mast cell development in some mouse strains (Dehlawi and Wakelin, 1988, Dehlawi *et al*., 1987, Reed *et al*., 1988). However, the fast responding SJL (Dehlawi *et al*., 1987) strain showed a strong mucosal mast cell response after secondary infection but not in a primary infection, and the fast responding SWR (Behnke *et al*., 2003) strain showed a strong mMCP-1 response after both secondary and tertiary infections. My study showed that C57 mice are capable of mounting an mMCP-1 response in as little as 2 weeks after primary infection, although higher mMCP-1 levels were observed after 4 and 6 weeks, with 4 weeks being the highest. These results indicate that mMCP-1 in C57 mice increases after primary infection, but starts subsiding, perhaps due to

increased reliance on adaptive immunity. Another explanation is that mMCP-1 response starts subsiding after worm expulsion begins, since greater mMCP-1 was associated with lower worm numbers after 4 weeks for CR and after 2 weeks for AL C57 mice, while there was no association between mMCP-1 and worm levels for AL C57 mice after 6 weeks. CR seemed to invigorate the immune response as evidenced by higher mMCP-1 in CR uninfected mice than in AL uninfected mice. This invigoration was not enough to show during infection, probably because infection invigorated the mMCP-1 response many times more than CR treatment, therefore obscuring the effect of CR. In SJL mice, CR actually attenuated mMCP-1 response. The reason for this could be that SJL mice rely more heavily on other responses (i.e. neutrophilia) and as a result have even less energy to allocate towards mMCP-1, the protease that might not be as crucial for this strain. Surprisingly, *H. bakeri* infection increased mMCP-1 on average approximately 16 times in SJL mice but by approximately 300 times in C57 mice. This was contrary to my expectations because SJL was characterized as a fast responding strain to *H. bakeri* (Behnke *et al*., 2006) and fast-responding strains have been shown to have strong mMCP-1 responses (Behnke *et al*., 2003). However, my study complements the one by Dehlawi *et al*. (1987) which showed that in response to primary *N. dubius* infection SJL mice increase the number of mucosal mast cells, but this increase is relatively low in comparison to challenge infection. Behnke *et al*. (2003) also showed that a fast responding strain SWR has a relatively low mMCP-1 response to primary infection unlike to secondary and tertiary infection; however SWR mice in this study were challenged weekly with trickle infections, thus preventing the measurement of mMCP-1 after more than one week post primary infection. mMCP-1 results in SWR mice from longer primary infections would be interesting to see for comparison. Lower

mMCP-1 response in SJL mice could be due to a more Th1-biased (Ben-Smith *et al*., 2003) immune response in SJL mice that attenuates the Th2 response, whereas Th2 response is characteristic of C57 mice (Setiawan *et al*., 2007). Since levels of mMCP-1 were not associated with number of worms in AL SJL mice my data further support the notion that SJL mice rely more heavily on other immune responses against *H. bakeri* than mMCP-1.

In summary, both strains showed an increased mMCP-1 response against *H. bakeri*, but C57 had a more robust response that peaked at 4 weeks, while SJL had a much weaker response that remained consistent regardless of the duration of infection. CR invigorated mMCP-1 response in C57, while it attenuated the same response in SJL. Therefore, I rejected my hypothesis that SJL would show a more robust response, while my CR hypothesis remained inconclusive. The fluctuation of mMCP-1 response among 2, 4 and 6 weeks of infection duration in C57 mice could have been possibly due to increased reliance on immunoglobulin response and/or clearance of the worms in later stages of infection.

Conclusions: A Qualitative Comparison of Immune Function in Two Mouse Strains

Although I did not statistically compare responses of the two strains directly, my results are consistent with the idea that SJL may rely more on ROS to combat *H. bakeri* infection than some other mouse strains (Ben-Smith *et al*., 2003) and less on mMCP-1. While non-specific immunoglobulin responses (IgA and total IgG1) were more robust in C57 mice, *H. bakeri*-specific IgG1 response was similar in C57 and SJL mice. The study overall shows that an innate immune responses such as neutrophilia might be more important than adaptive responses in determining the mouse strain responsiveness to primary *H. bakeri* infection, since adaptive immune responses were either slower/less robust in fast responding SJL mice, or similar

between the two strains. mMCP-1 was also less robust in fast-responding SJL, but it could be that mMCP-1 response robustness is greater in fast-responding strains in secondary and other subsequent infections. CR either had no effect or had an attenuating effect on adaptive responses in SJL mice, indicating that this fast responder does not prioritize adaptive immune responses in the situation of limited food availability. In fact, the slower response to *H. bakeri* by C57 mice could potentially be attributed, at least in part, to prioritization of non-specific adaptive responses. This could be detrimental if during restricted food availability mice respond by producing even more non-specific immunity that could take away valuable energy resources for continuous cellular defense against the parasite. Given that wild animals are often repeatedly exposed to the same intestinal parasite species, further work using challenge infections where immune responses rely heavily on parasite specific responses, in the context of calorie restriction, would be informative.

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Table 1. Experimental design of mice of slow responding (C57BL/6) and fast responding (SJL) strains that were kept on either a calorie restricted (CR) or *ad libitum* (AL) food treatment for six months. Mice from each food treatment group were then either infected by oral administration of 300 *Heligmosomoides bakeri* larvae suspended in tap water, or uninfected by oral administration of an equal volume of tap water. Within each infection status, mice were separated into three experiment duration groups.

Mouse strain	Food Treatment	Infection status	Experiment duration (weeks)	Sample Size
C57BL/6	CR	Infected	2	15
			4	15
			6	15
		Uninfected	2	14
			4	15
			6	15
	AL	Infected	2	14
			4	15
			6	15
		Uninfected	2	11
			4	14
			6	13
SJL	CR	Infected	2	15
			4	14
			6	14
		Uninfected	2	15
			4	15
			6	14
	AL	Infected	2	14
			4	12
			6	13
		Uninfected	2	14
			4	13
			6	12

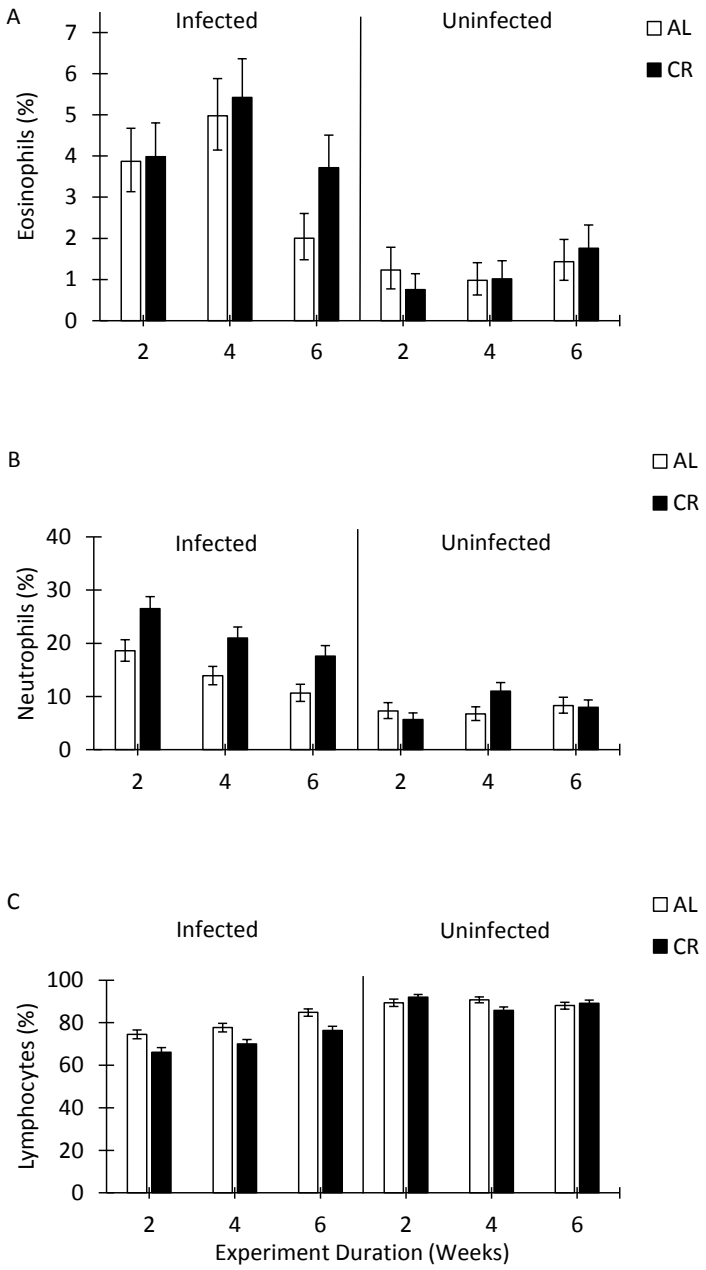


Figure 1. Percentage of eosinophils (A), neutrophils (B) and lymphocytes (C) in *ad libitum* (AL) and calorie restricted (CR) C57BL/6 mice after 2, 4, and 6 weeks of experiment duration after infection with *Heligmosomoides bakeri*. Each value shows the back transformed mean ± 1 standard error of arcsine square root of particular white blood cell percent for 100 cells counted (n=14 infected 2 wk AL, n=15 infected 2 wk CR, n=15 infected 4 wk AL, n=15 infected 4 wk CR, n=14 infected 6 wk AL, n=15 infected 6 wk CR, n=11 uninfected 2 wk AL, n=14 uninfected 2 wk CR, n=14 uninfected 4 wk AL, n=15 uninfected 4 wk CR, n=13 uninfected 6 wk AL, n=15 uninfected 6 wk CR).

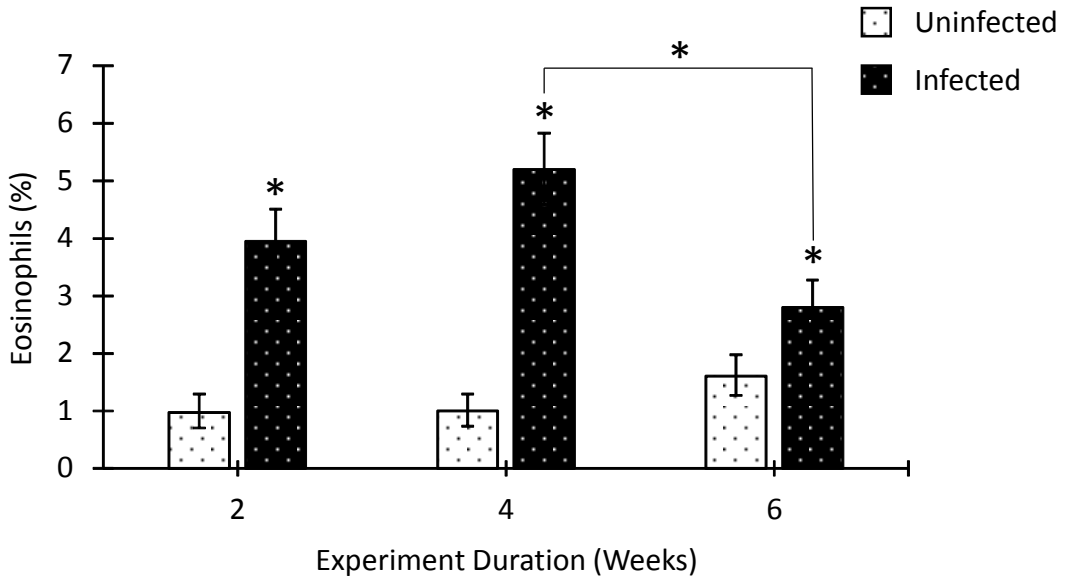


Figure 2. Percentage of eosinophils in C57BL/6 mice after 2, 4, and 6 weeks of experiment duration after infection with *Heligmosomoides bakeri*. Each value shows the back transformed mean ± 1 standard error of arcsine square root of eosinophil percent for 100 cells counted (n=25 uninfected 2 wk, n=29 uninfected 4 wk, n=28 uninfected 6 wk, n=29 infected 2 wk, n=30 infected 4 wk, n=30 infected 6 wk). An asterisk above infected groups signifies a significant difference from the corresponding uninfected group within a particular experiment duration. Lines with an asterisk above connect additional experimental groups that are significantly different from each other. All infected mice had greater eosinophil percentages than all uninfected mice across different experiment durations (not depicted by lines/asterisks on the figure, for clarity).

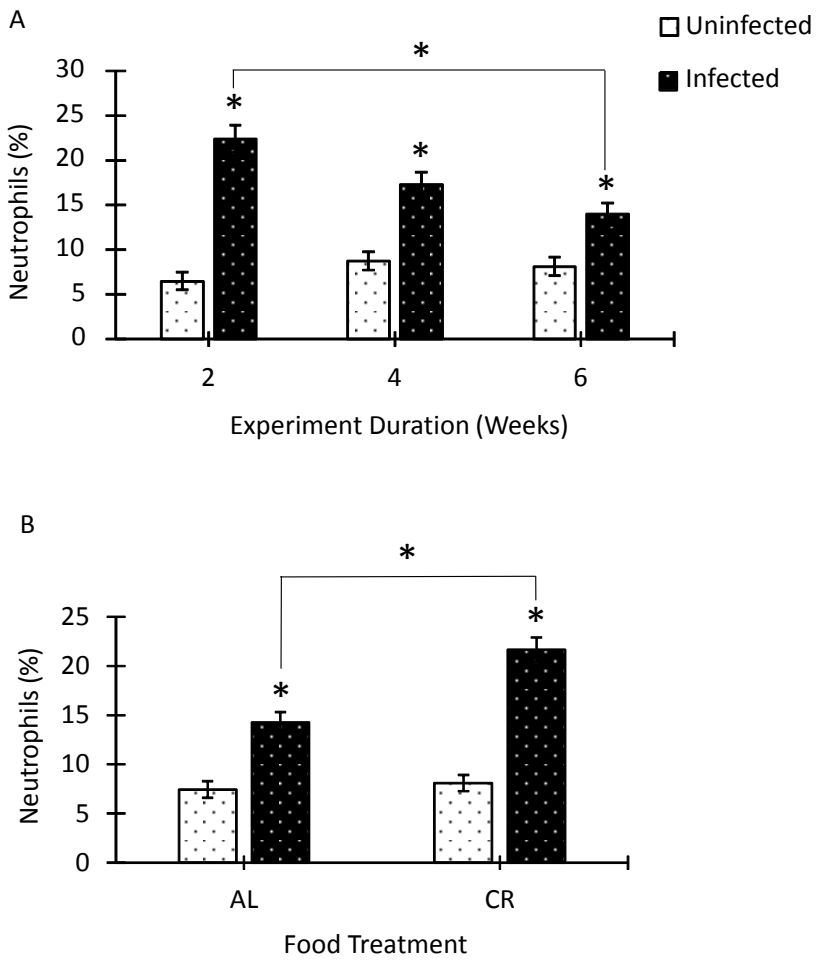


Figure 3. Percentage of neutrophils in C57BL/6 mice after 2, 4, and 6 weeks of experiment duration after infection with *Heligmosomoides bakeri* (A) and in *ad libitum* (AL) and calorie restricted (CR) mice after infection with *Heligmosomoides bakeri* (B). Each value shows the back transformed mean ± 1 standard error of arcsine square root of neutrophil percent for 100 cells counted (n=25 uninfected 2 wk, n=29 uninfected 4 wk, n=28 uninfected 6 wk, n=29 infected 2 wk, n=30 infected 4 wk and n=30 infected 6 wk; n=38 uninfected AL, n=44 uninfected CR, n=44 infected AL, n=45 infected CR). An asterisk above infected groups signifies a significant difference from the corresponding uninfected group. Lines with an asterisk above connect additional experimental groups that are significantly different from each other. All infected mice had greater neutrophils percentages than all uninfected mice across different experiment durations and food treatments (not depicted by lines/asterisks on the figure for clarity).

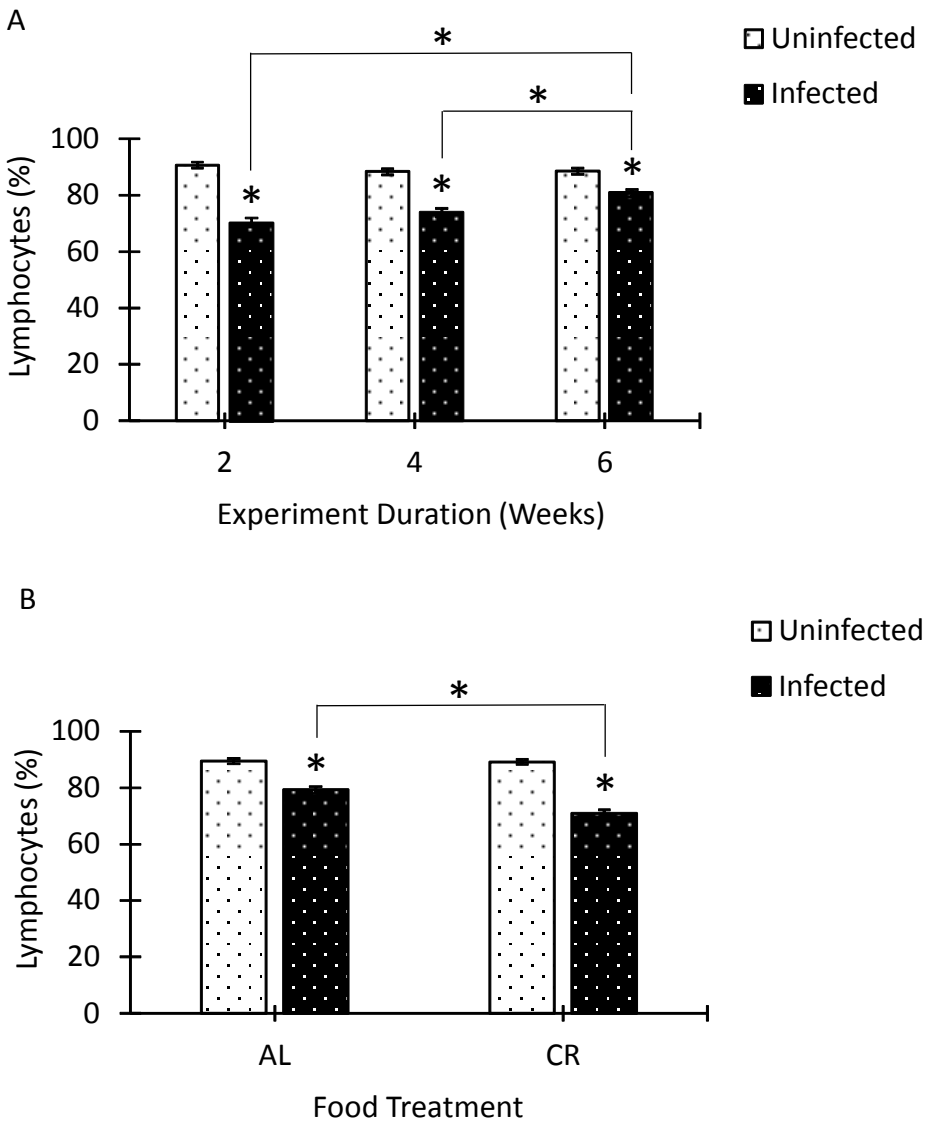


Figure 4. Percentage of lymphocytes in C57BL/6 mice after 2, 4, and 6 weeks of experiment duration after infection with *Heligmosomoides bakeri* (A) and in *ad libitum* (AL) and calorie restricted (CR) mice after infection with *Heligmosomoides bakeri* (B). Each value shows the back transformed mean ± 1 standard error of arcsine square root of lymphocyte percent for 100 cells counted (n=25 uninfected 2 wk, n=29 uninfected 4 wk, n=28 uninfected 6 wk, n=29 infected 2 wk, n=30 infected 4 wk and n=30 infected 6 wk; n=38 uninfected AL, n=44 uninfected CR, n=44 infected AL, n=45 infected CR). An asterisk above infected groups signifies a significant difference from the corresponding uninfected group and lines with an asterisk above connect additional experimental groups that are significantly different from each other. All infected mice had less lymphocytes than all uninfected mice across different experiment durations and food treatments (not depicted by lines/asterisks on the figure for clarity).

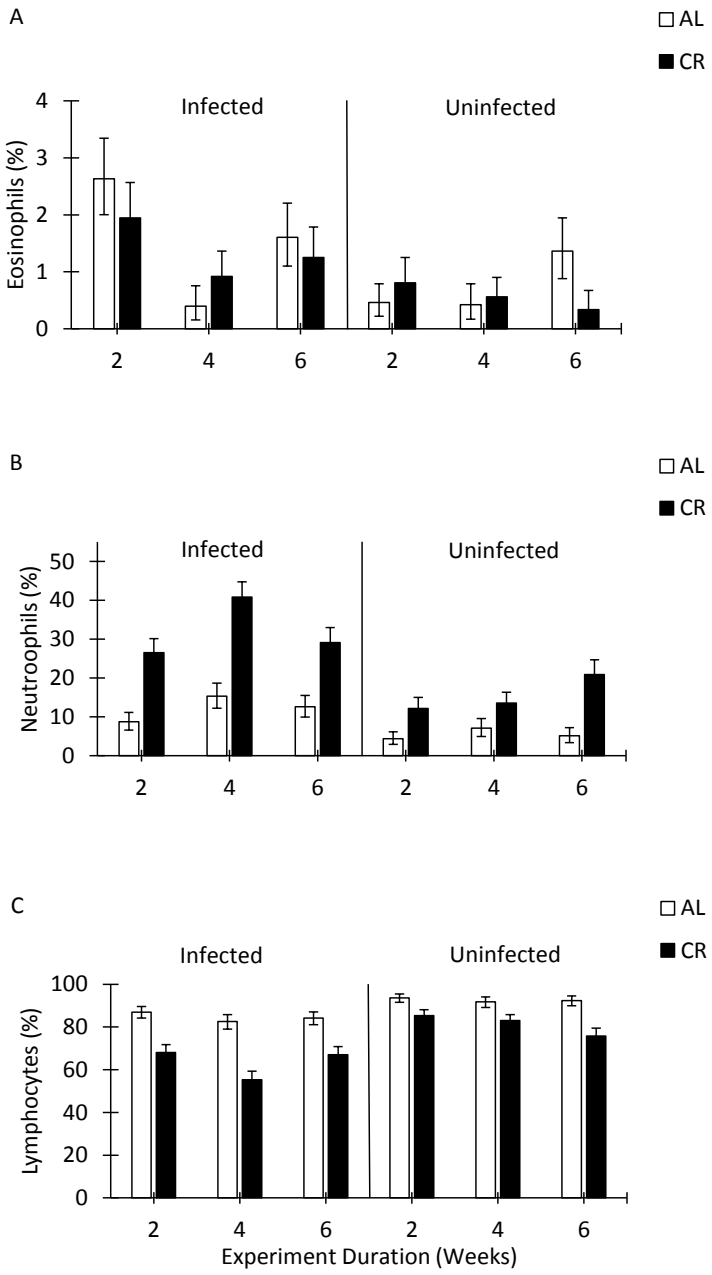


Figure 5. Percentage of eosinophils (A), neutrophils (B) and lymphocytes (C) in *ad libitum* (AL) and calorie restricted (CR) SJL mice after 2, 4 and 6 weeks of experiment duration after infection with *Heligmosomoides bakeri*. Each value shows the back transformed mean ± 1 standard error of arcsine square root of particular white blood cell percent for 100 cells counted (n=14 infected 2 wk AL, n=14 infected 2 wk CR, n=11 infected 4 wk AL, n=14 infected 4 wk CR, n=13 infected 6 wk AL, n=13 infected 6 wk CR, n=14 uninfected 2 wk AL, n=13 uninfected 2 wk CR, n=11 uninfected 4 wk AL, n=15 uninfected 4 wk CR, n=12 uninfected 6 wk AL, n=11 uninfected 6 wk CR).

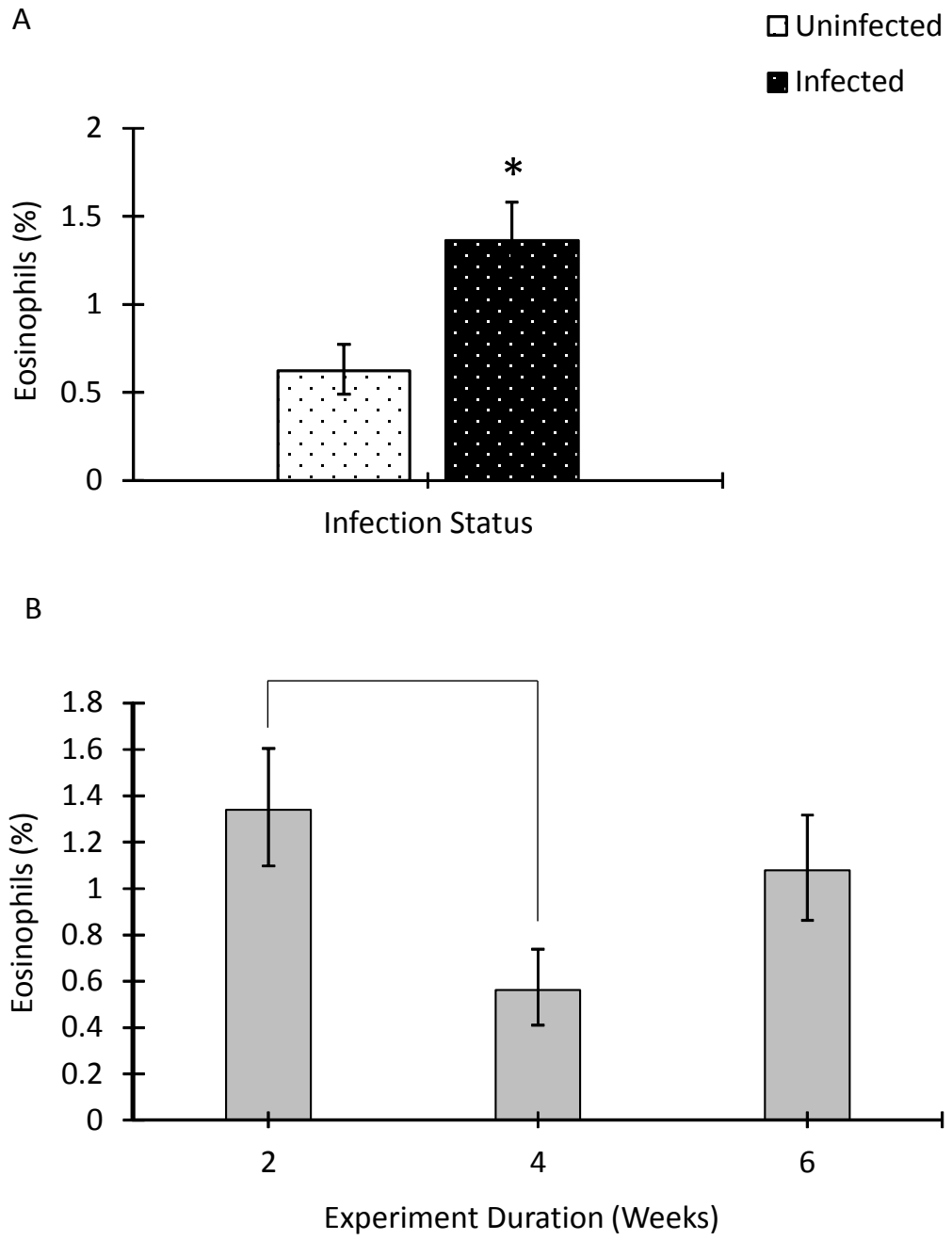


Figure 6. Percentage of eosinophils in SJL mice after infection with *Heligmosomoides bakeri* (A) and after 2, 4 and 6 weeks of experiment duration (B). Each value shows the back transformed mean ± 1 standard error of arcsine square root of eosinophil percent for 100 cells counted (n=83 uninfected, n=82 infected; n=58 2 wk, n=54 4 wk, n=53 6 wk). An asterisk above infected groups signifies a significant difference from the corresponding uninfected group and lines without asterisk connect experimental groups that are almost significantly different from each other (having a 0.001 overlap of the 95% confidence interval).

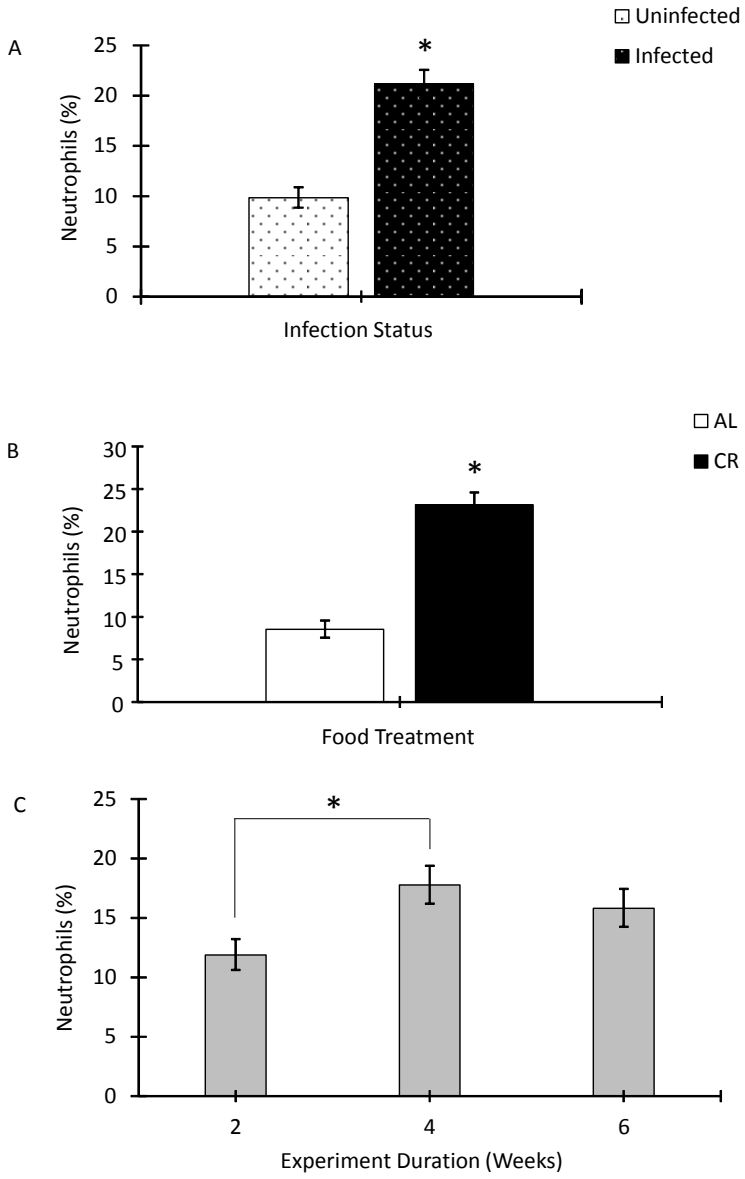


Figure 7. Percentage of neutrophils in SJL mice after infection with *Heligmosomoides bakeri* (A) after either *ad libitum* (AL) or calorie restricted (CR) food treatment (B) and after 2, 4 and 6 weeks of experiment duration (C). Each value shows the back transformed mean ± 1 standard error of arcsine square root of neutrophil percent for 100 cells counted (n=83 uninfected, n=82 infected; n=78 AL, n=87 CR; n=58 2 wk, n=54 4 wk, n=53 6 wk). An asterisk above infected groups signifies a significant difference from the corresponding uninfected group, an asterisk above CR group signifies a significant difference from the corresponding AL group and lines with an asterisk above connect additional experimental groups that are significantly different from each other.

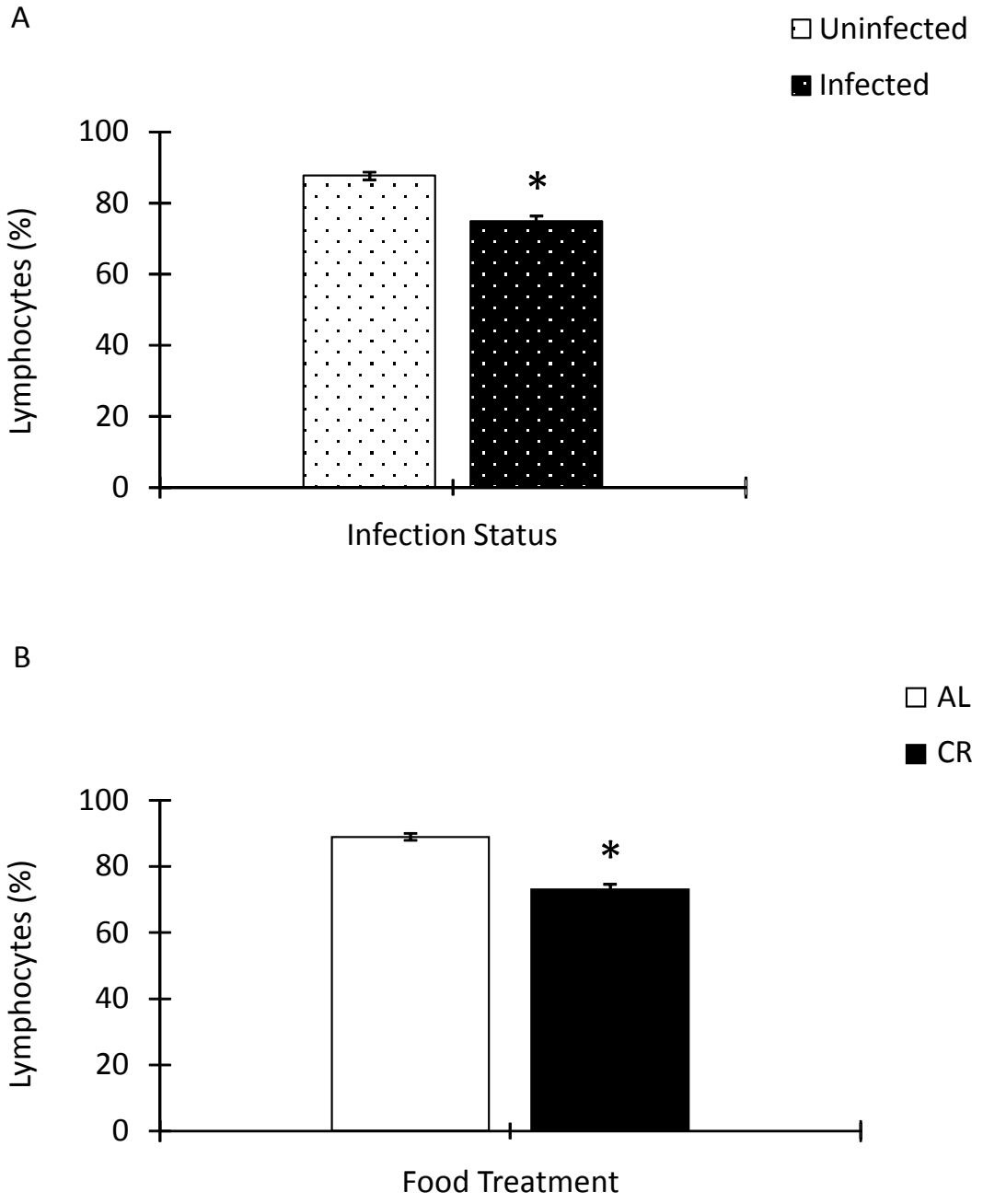


Figure 8. Percentage of lymphocytes in SJL mice after infection with *Heligmosomoides bakeri* (A) and after either *ad libitum* (AL) or calorie restricted (CR) food treatment (B). Each value shows the back transformed mean ± 1 standard error of arcsine square root of lymphocyte percent for 100 cells counted (n=83 uninfected, n=82 infected; n=78 AL, n=87 CR). An asterisk above the infected group signifies a significant difference from the uninfected group and an asterisk above the CR group signifies a significant difference from the AL group.

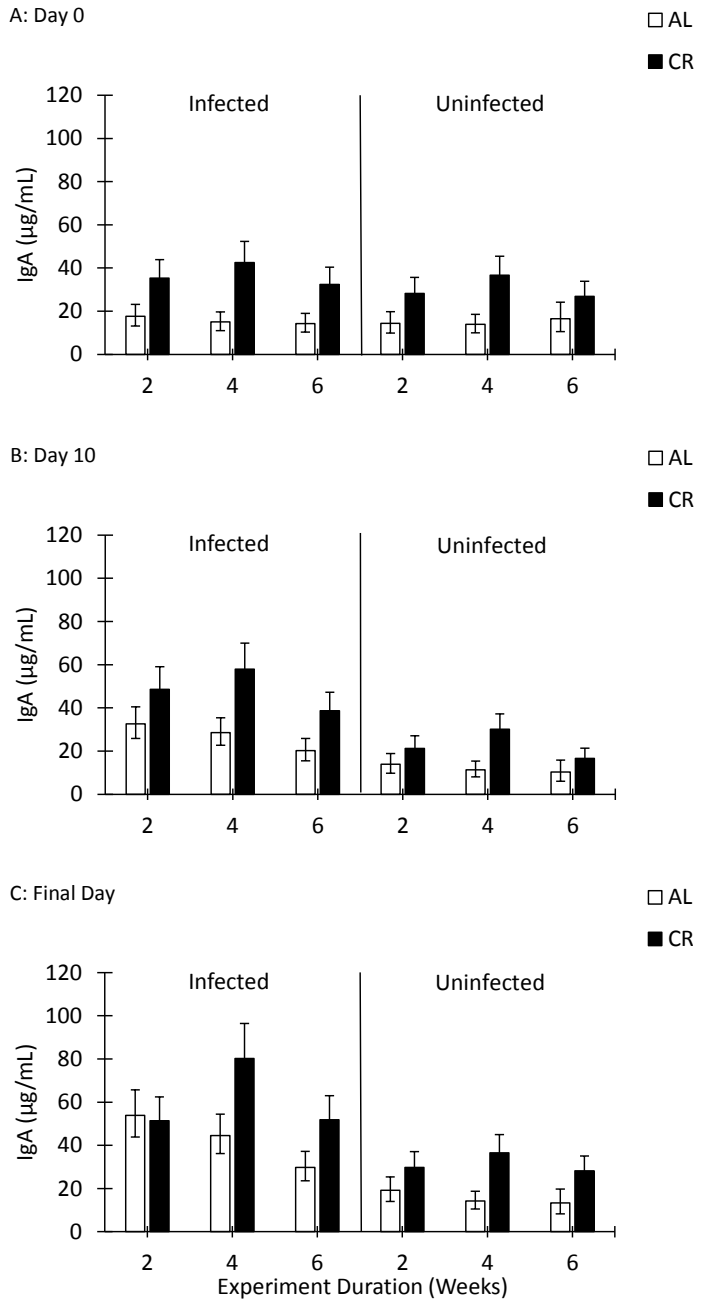


Figure 9. IgA levels on Day 0 (A), Day 10 (B) and Final Day (C) of *ad libitum* (AL) and calorie restricted (CR) C57BL/6 mice after 2, 4 and 6 weeks of experiment duration after infection with *Heligmosomoides bakeri*. Each value shows the back transformed mean ± 1 standard error of $\log_{10}$ of IgA (µg/mL) (n =14 infected 2 wk AL, n=15 infected 2 wk CR, n=15 infected 4 wk AL, n=15 infected 4 wk CR, n=15 infected 6 wk AL, n=15 infected 6 wk CR, n=11 uninfected 2 wk AL, n=14 uninfected 2 wk CR, n=14 uninfected 4 wk AL, n=15 uninfected 4 wk CR, n=13 uninfected 6 wk AL, n=15 uninfected 6 wk CR).

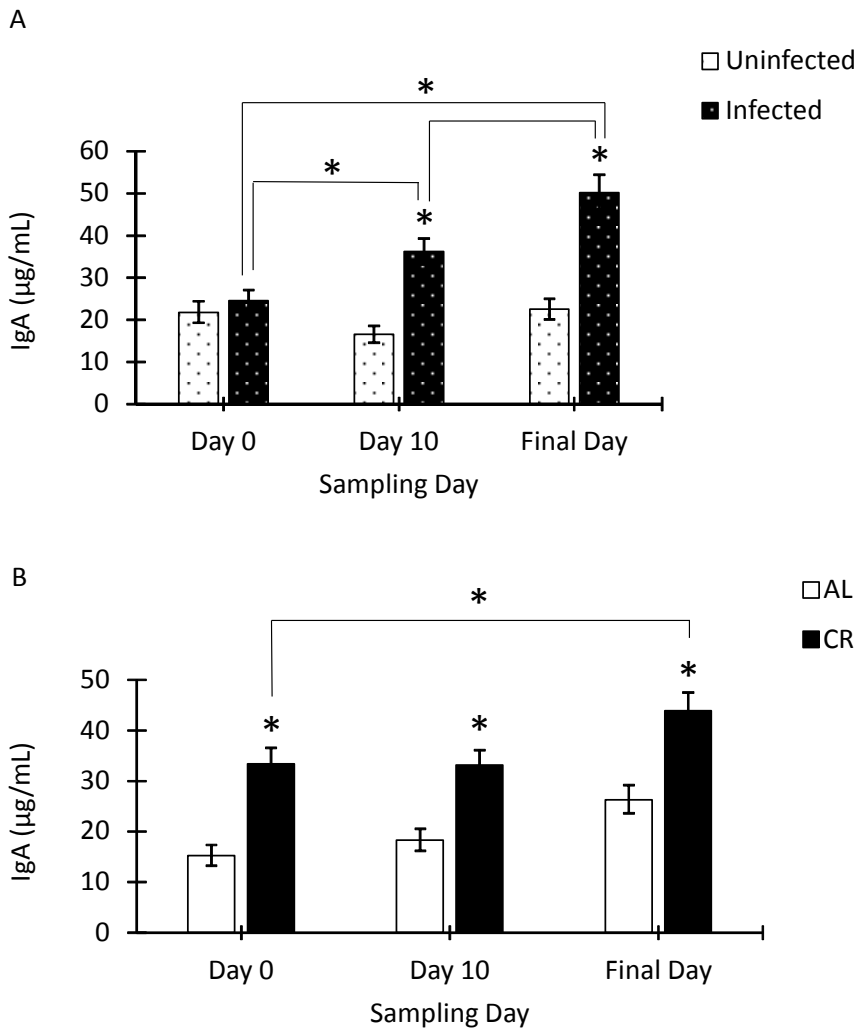


Figure 10. IgA levels on Day 0, Day 10 and Final Day in C57BL/6 mice after infection with *Heligmosomoides bakeri* (A) and after either *ad libitum* (AL) or calorie restricted (CR) food treatment (B). Each value shows the back transformed mean ± 1 standard error of $\log_{10}$ of IgA (µg/mL) (n=82 uninfected, n=89 infected; n=82 AL, n=89 CR). An asterisk above infected groups signifies a significant difference from the corresponding uninfected group. Lines with an asterisk above connect additional experimental groups that are significantly different from each other and lines without an asterisk connect experimental groups that are almost significantly different from each other (having a 0.001 overlap of the 95% confidence interval). On panel A, all infected mice on Day 10 and on the Final day had more IgA than uninfected mice across all sampling days (not depicted by lines/asterisks on the figure for clarity). On panel B, CR mice on the Final Day had more IgA than AL mice across all sampling days, and CR mice on Day 0 and Day 10 had more IgA than AL mice on either Day 0 or Day 10 (not depicted by lines/asterisks on the figure for clarity).

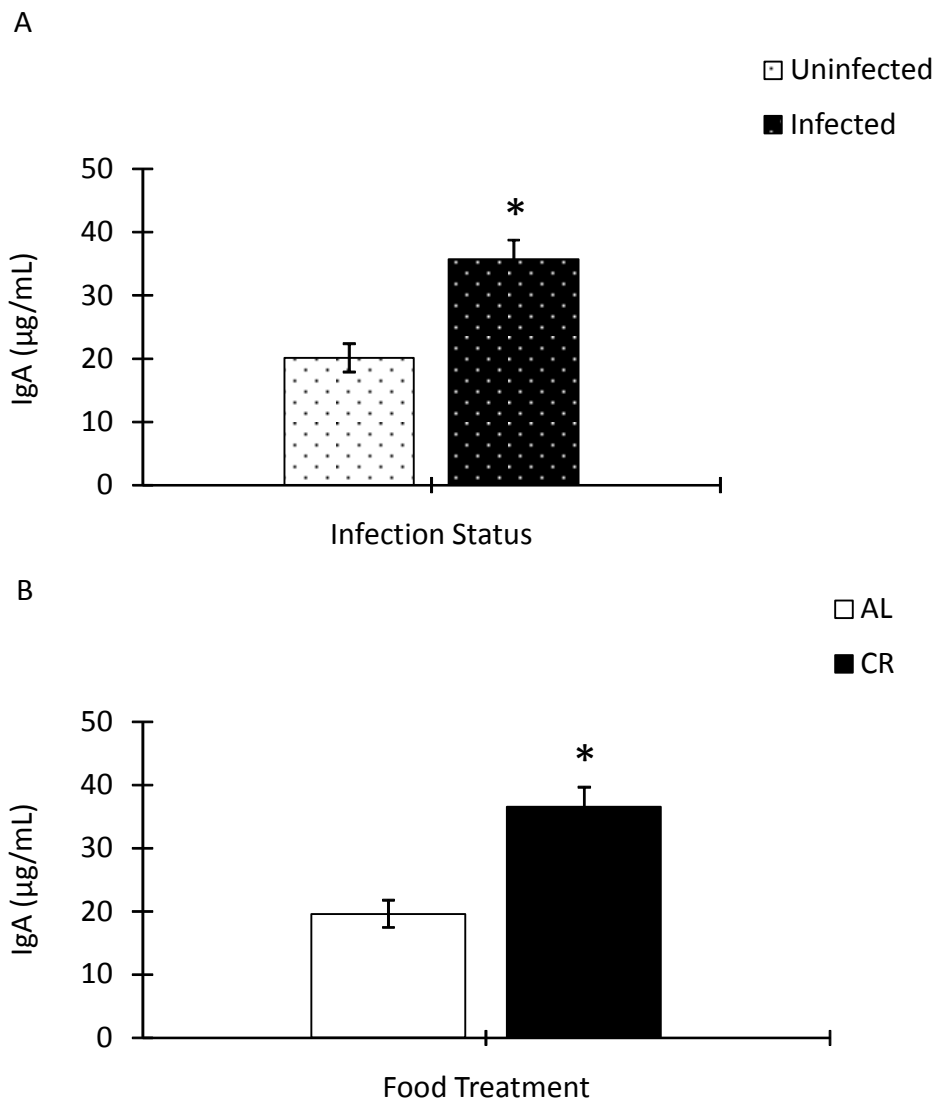


Figure 11. IgA level averages in C57BL/6 mice after infection with *Heligmosomoides bakeri* (A) and after either *ad libitum* (AL) or calorie restricted (CR) food treatment (B). Each value shows the back transformed mean ± 1 standard error of $\log_{10}$ of IgA (µg/mL) (n=82 uninfected, n=89 infected; n=82 AL, n=89 CR). An asterisk above infected groups signifies a significant difference from the corresponding uninfected group and an asterisk above CR groups signifies a significant difference from the corresponding AL group.

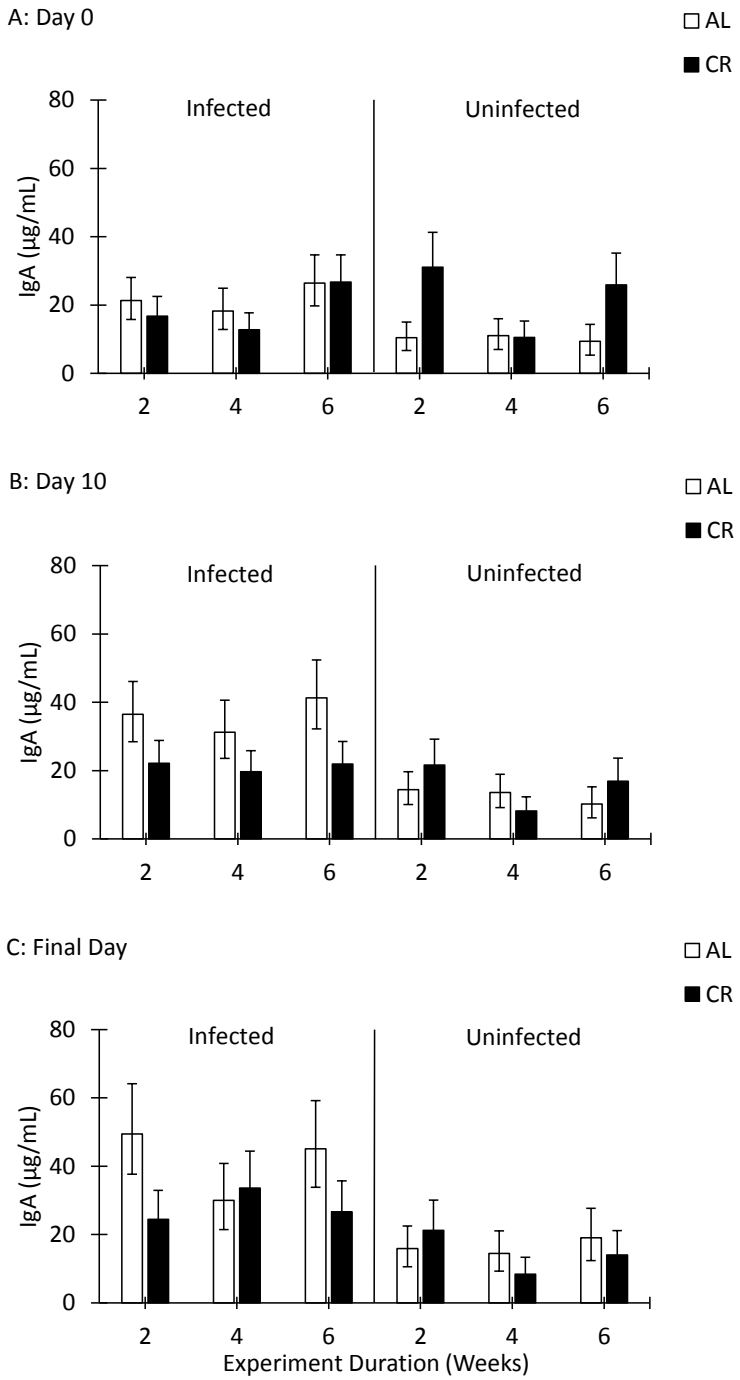


Figure 12. IgA levels on Day 0 (A), Day 10 (B) and Final Day (C) of *ad libitum* (AL) and calorie restricted (CR) SJL mice after 2, 4 and 6 weeks of experiment duration after infection with *Heligmosomoides bakeri*. Each value shows the back transformed mean ± 1 standard error of $\log_{10}$ of IgA (µg/mL) (n=14 infected 2 wk AL, n=15 infected 2 wk CR, n=12 infected 4 wk AL, n=14 infected 4 wk CR, n=13 infected 6 wk AL, n=14 infected 6 wk CR, n=14 uninfected 2 wk AL, n=15 uninfected 2 wk CR, n=13 uninfected 4 wk AL, n=15 uninfected 4 wk CR, n=12 uninfected 6 wk AL, n=14 uninfected 6 wk CR).

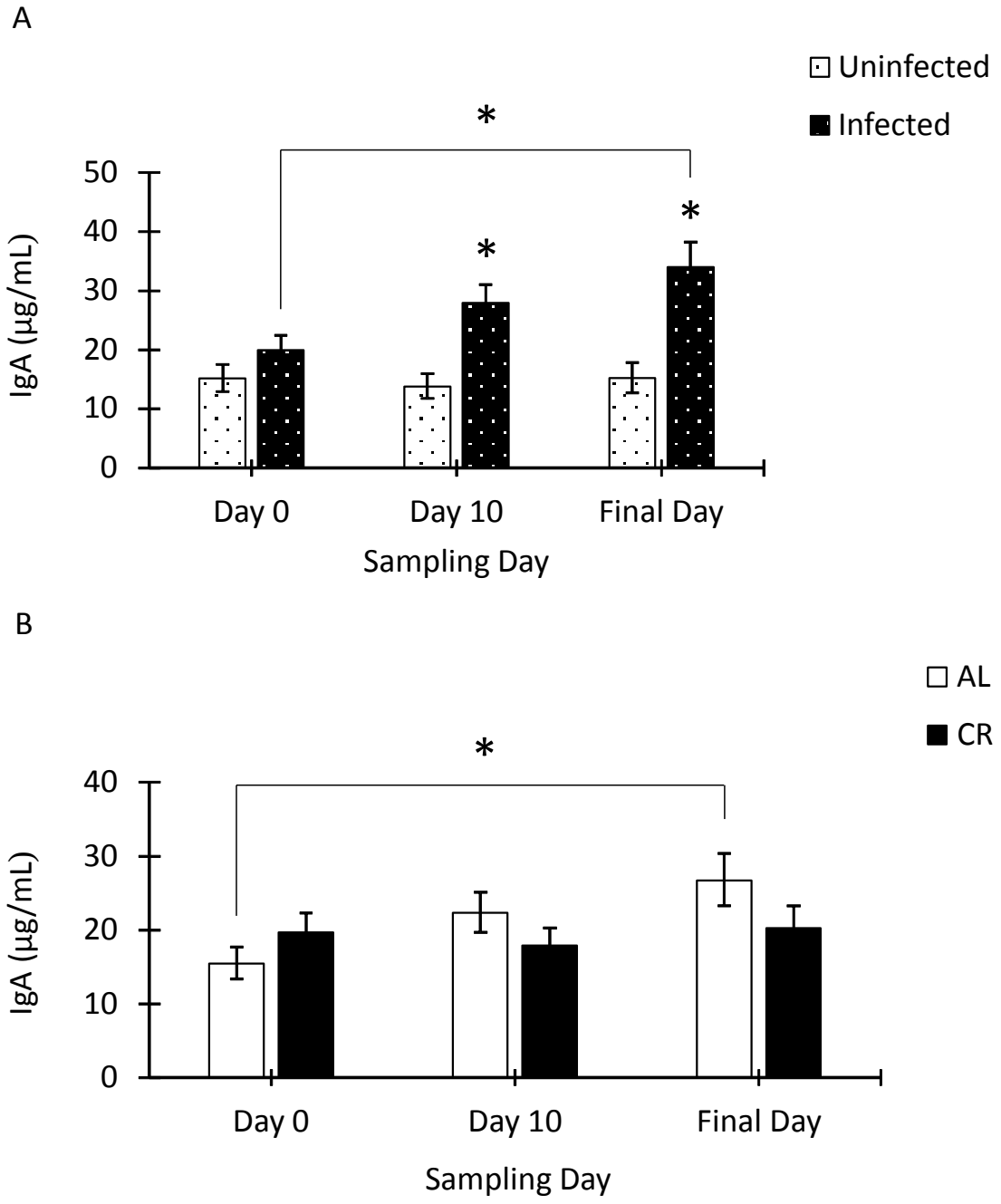


Figure 13. IgA levels on Day 0, Day 10 and Final Day in SJL mice after infection with *Heligmosomoides bakeri* (A) and after either *ad libitum* (AL) or calorie restricted (CR) food treatment (B). Each value shows the back transformed mean ± 1 standard error of $\log_{10}$ of IgA (µg/mL) (n=83 uninfected, n=82 infected; n=78 AL, n=87 CR). An asterisk above infected groups signifies a significant difference from the corresponding uninfected group and lines with an asterisk above connect additional experimental groups that are significantly different from each other. On panel A, infected mice on Day 10 and on the Final day had more IgA than uninfected mice across all sampling days (not depicted by lines/asterisks on the figure for clarity).

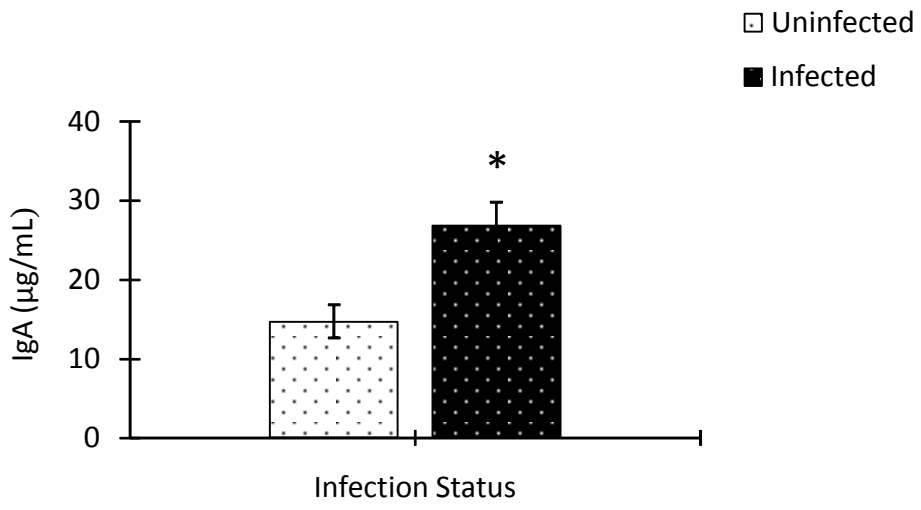


Figure 14. IgA level averages in SJL mice after infection with *Heligmosomoides bakeri*. Each value shows the back transformed mean ± 1 standard error of $\log_{10}$ of IgA (µg/mL) (n=83 uninfected, n=82 infected). Asterisk above the infected group signifies a significant difference from the uninfected group.

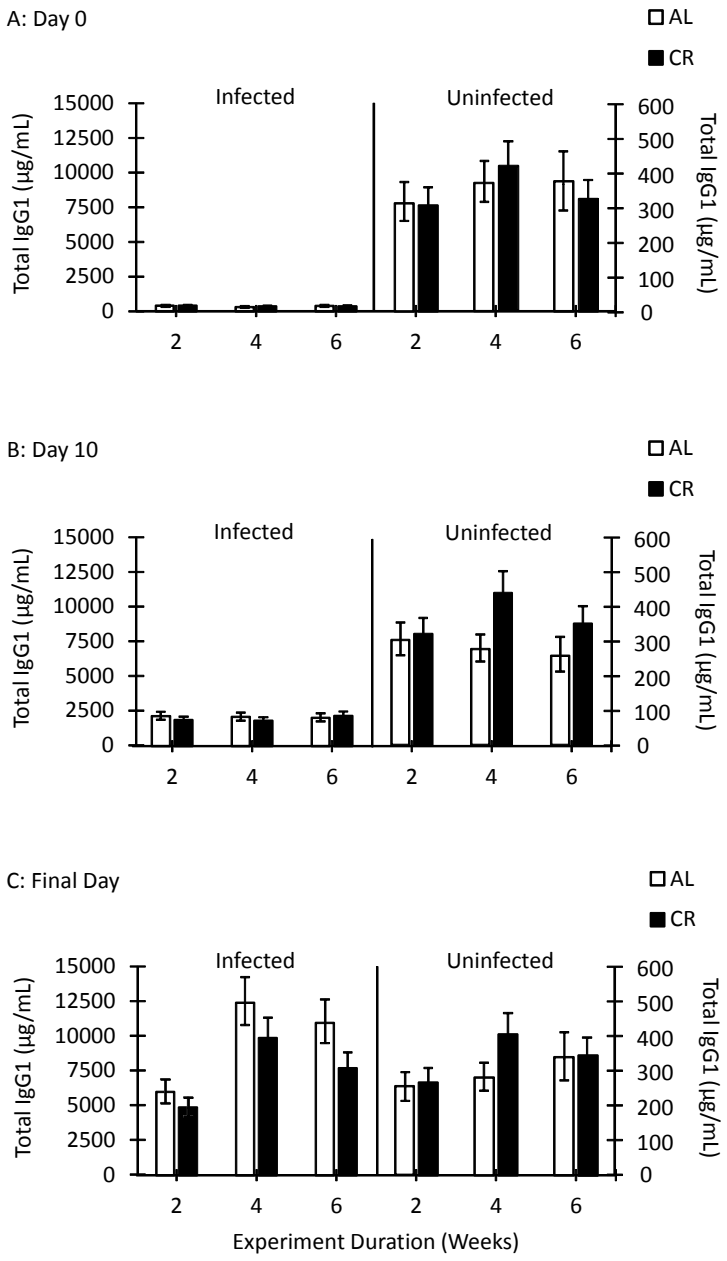
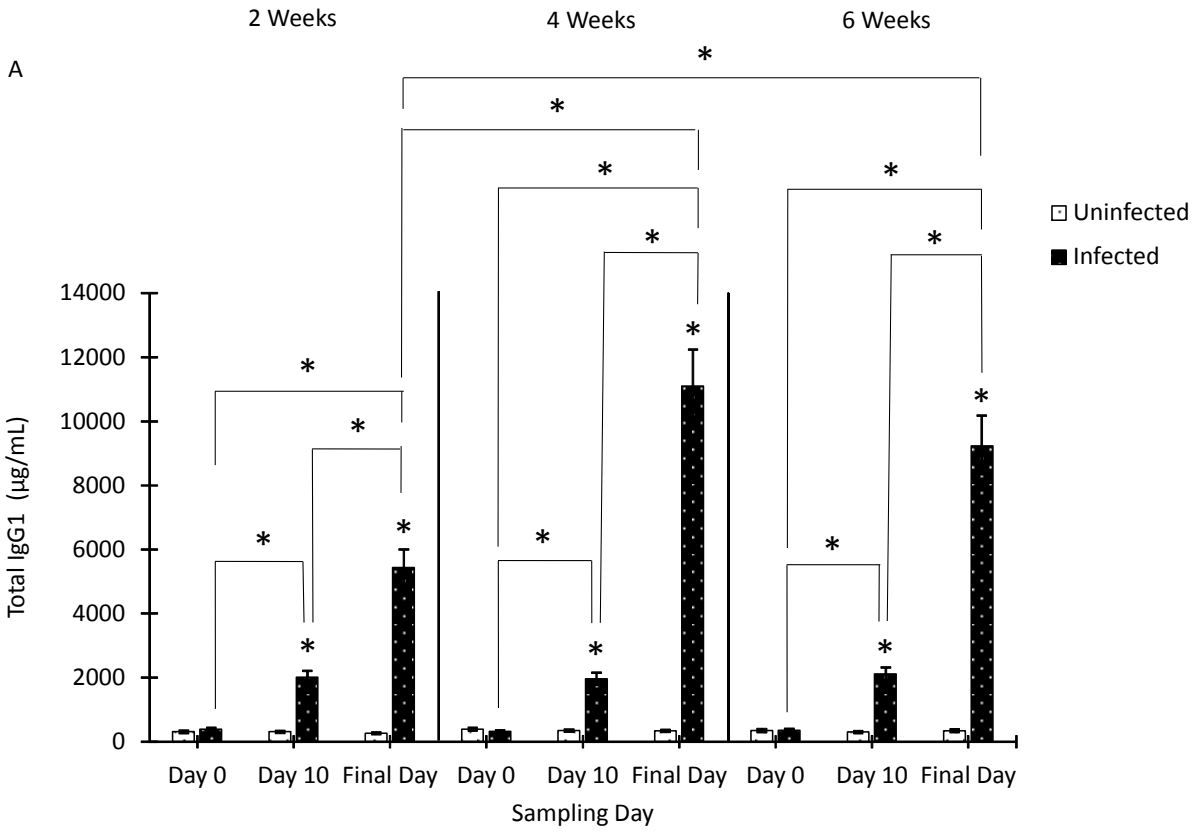


Figure 15. Total IgG1 levels on Day 0 (A), Day 10 (B) and Final Day (C) of *ad libitum* (AL) and calorie restricted (CR) C57BL/6 mice after 2, 4 and 6 weeks of experiment duration with *Heligmosomoides bakeri*. Each value shows the back transformed mean ± 1 standard error of $\log_{10}$ of total IgG1 (µg/mL). (n=14 infected 2 wk AL, n=15 infected 2 wk CR, n=15 infected 4 wk AL, n=15 infected 4 wk CR, n=15 infected 6 wk AL, n=15 infected 6 wk CR, n=11 uninfected 2 wk AL, n=14 uninfected 2 wk CR, n=14 uninfected 4 wk AL, n=15 uninfected 4 wk CR, n=13 uninfected 6 wk AL, n=15 uninfected 6 wk CR). Infected mice are shown by the left axis (µg/mL) and uninfected mice are shown by right axis (µg/mL)


A
2 Weeks
4 Weeks
6 Weeks
Uninfected
Infected
Total IgG1 (µg/mL)
14000
12000
10000
8000
6000
4000
2000
0
Day 0
Day 10
Final Day
Sampling Day

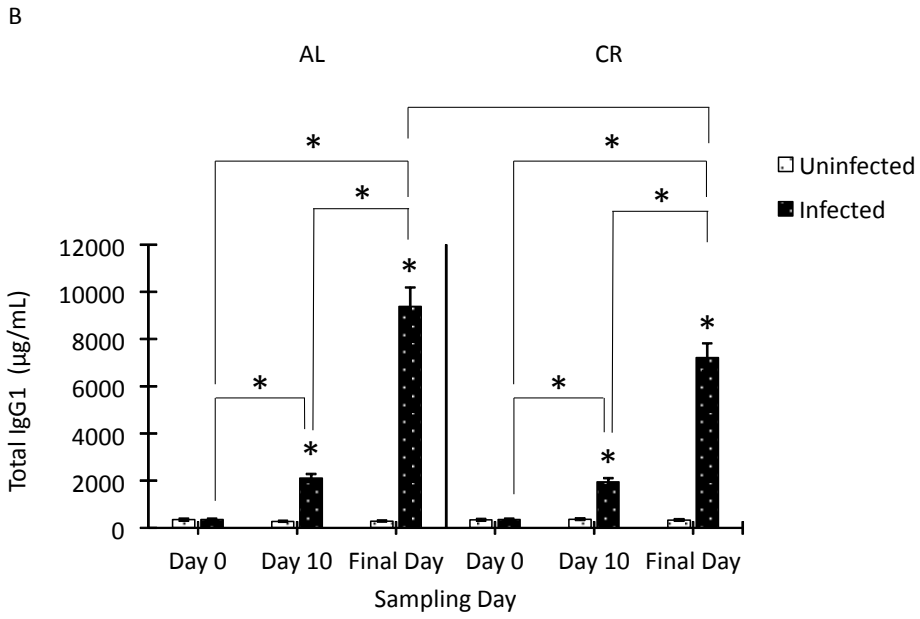

B
AL
CR
Uninfected
Infected
Total IgG1 (µg/mL)
12000
10000
8000
6000
4000
2000
0
Day 0
Day 10
Final Day
Sampling Day

Figure 16. Total IgG1 levels on Day 0, Day 10 and Final Day in C57BL/6 mice after 2, 4 and 6 weeks of experiment duration after infection with *Heligmosomoides bakeri* (A) and either *ad libitum* (AL) or calorie restricted (CR) food treatment and infection with *Heligmosomoides bakeri* (B). Each value shows the back transformed mean ± 1 standard error of $\log_{10}$ of total IgG1 (μg/mL) (n=25 uninfected 2 wk, n=29 uninfected 4 wk, n=28 uninfected 6 wk, n=29 infected 2 wk, n=30 infected 4 wk and n=30 infected 6 wk; n =38 uninfected AL, n=44 uninfected CR, n=44 infected AL, n=45 infected CR). An asterisk above infected groups signifies a significant difference from the corresponding uninfected group and an asterisk above CR groups signifies a significant difference from the corresponding AL group. Lines with an asterisk above connect additional experimental groups that are significantly different from each other and lines without an asterisk connect experimental groups that are almost significantly different from each other (having a 0.02 overlap of the 95% confidence interval). On panel A, all infected mice on Day 10 and on the Final Day had more total IgG1 than uninfected mice across all sampling days and all experiment durations; all infected mice on the Final Day had more total IgG1 than all infected mice on Day 10 across all experiment durations (not depicted by lines/asterisks on the figure for clarity). On panel B, all infected mice on Day 10 and on the Final Day had more total IgG1 than uninfected mice across all sampling days and all food treatments; all infected mice on the Final Day had more total IgG1 than all infected mice on Day 10 across all food treatments (not depicted by lines/asterisks on the figure for clarity).

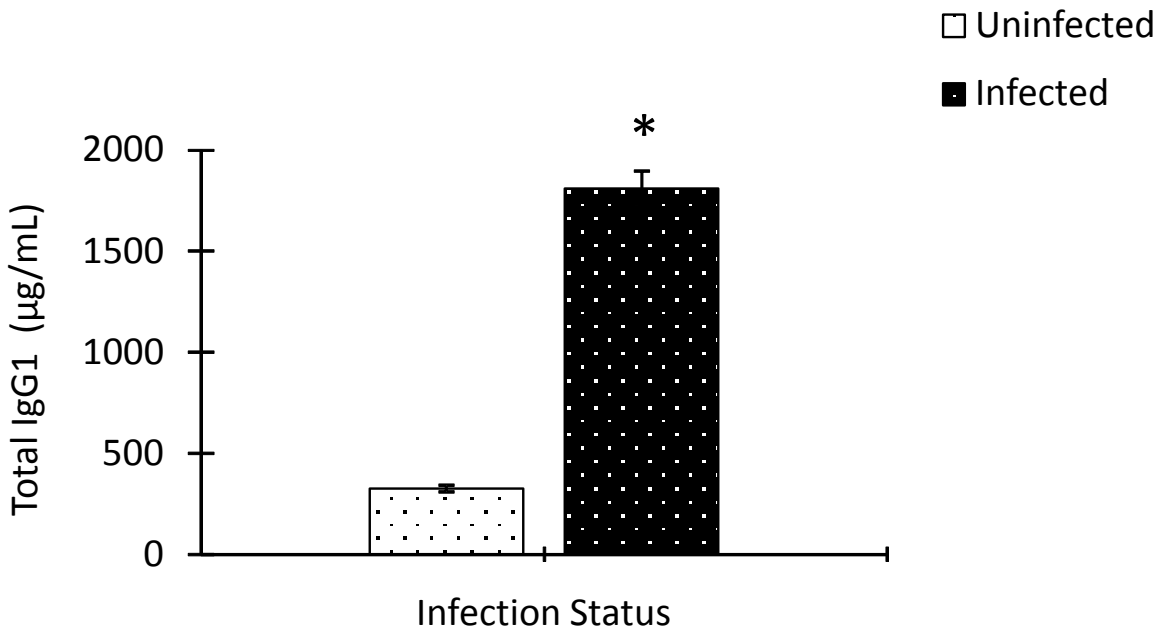


Figure 17. Total IgG1 level averages in C57BL/6 mice after infection with *Heligmosomoides bakeri*. Each value shows the back transformed mean ± 1 standard error of $\log_{10}$ of total IgG1 (µg/mL) (µg/mL) (n=82 uninfected, n=89 infected). An asterisk above the infected group signifies a significant difference from the uninfected group.

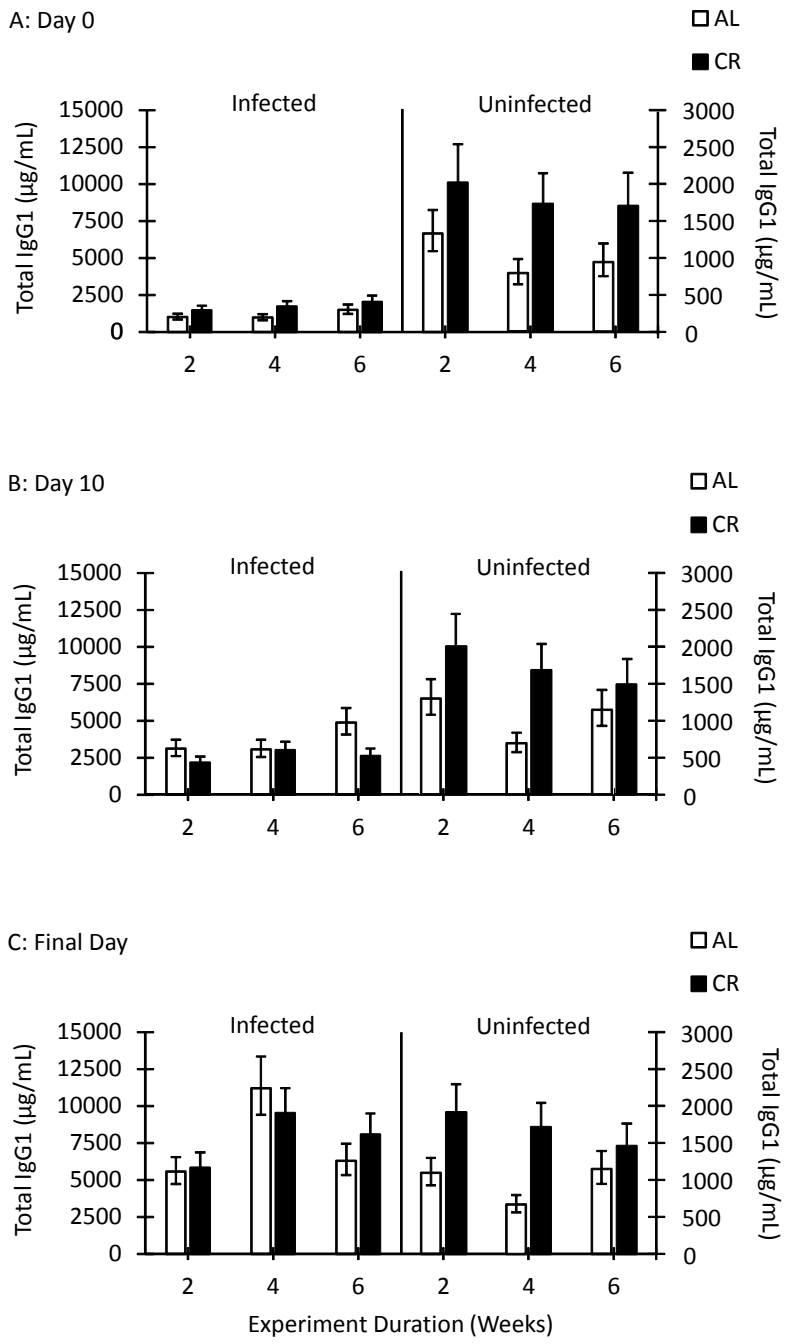


Figure 18. Total IgG1 levels on Day 0 (A), Day 10 (B) and Final Day (C) of *ad libitum* (AL) and calorie restricted (CR) SJL mice after 2, 4 and 6 weeks of experiment duration with *Heligmosomoides bakeri*. Each value shows the back transformed mean ± 1 standard error of $\log_{10}$ of total IgG1 (µg/mL) (n=14 infected 2 wk AL, n=15 infected 2 wk CR, n=12 infected 4 wk AL, n=14 infected 4 wk CR, n=13 infected 6 wk AL, n=14 infected 6 wk CR, n=14 uninfected 2 wk AL, n=15 uninfected 2 wk CR, n=13 uninfected 4 wk AL, n=15 uninfected 4 wk CR, n=12 uninfected 6 wk AL, n=14 uninfected 6 wk CR).

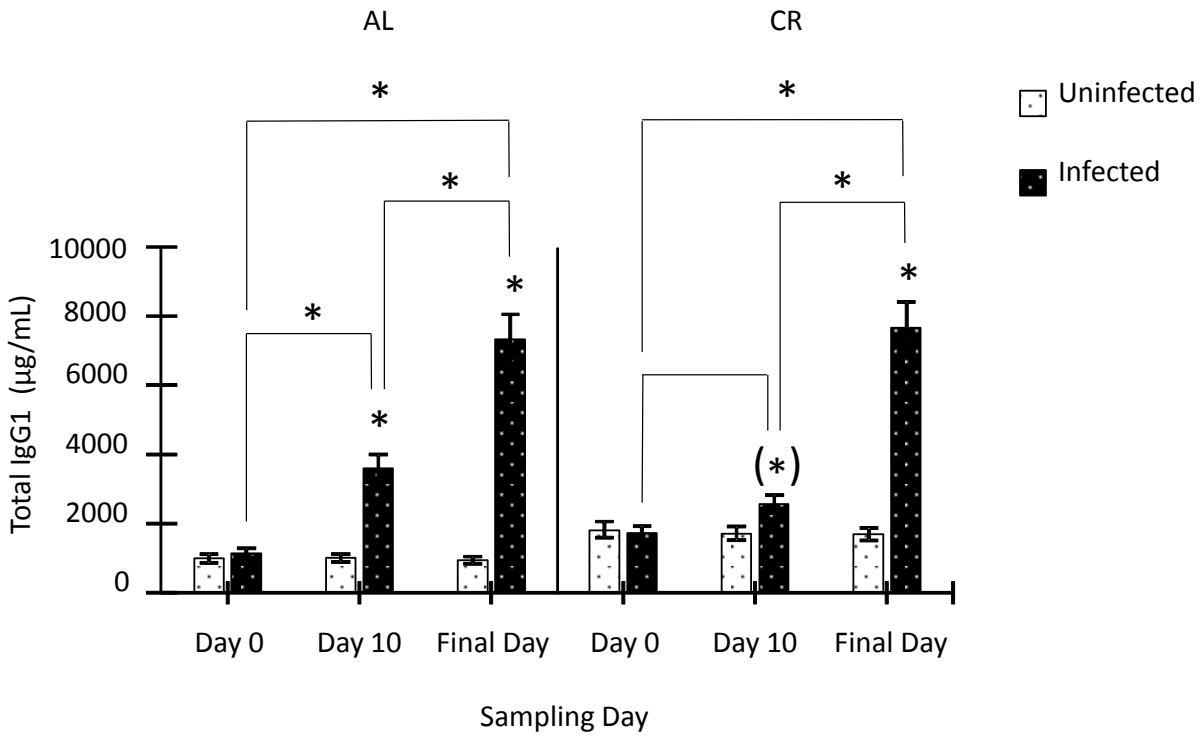


Figure 19. Total IgG1 levels on Day 0, Day 10 and Final Day in SJL mice after either *ad libitum* (AL) or calorie restricted (CR) food treatment and infection with *Heligmosomoides bakeri*. Each value shows the back transformed mean ± 1 standard error of $\log_{10}$ of total IgG1 (µg/mL) (n=39 uninfected AL, n=44 uninfected CR, n=39 infected Al, n=43 infected CR). An asterisk above infected groups signifies a significant difference from the corresponding uninfected group. Lines with an asterisk above connect additional experimental groups that are significantly different from each other. An asterisk in parentheses above infected groups and lines without an asterisk signifies an almost significant difference, (having a 0.01 overlap of the 95% confidence interval). AL infected mice on Day 10 and the Final Day had more total IgG1 than all uninfected mice across different sampling days and food treatments; CR infected mice on Day 10 had more total IgG1 than AL infected mice across different sampling days, and CR infected mice on the Final Day had more total IgG1 than all uninfected mice across different sampling days and food treatments (not depicted by lines/asterisks on the figure for clarity).

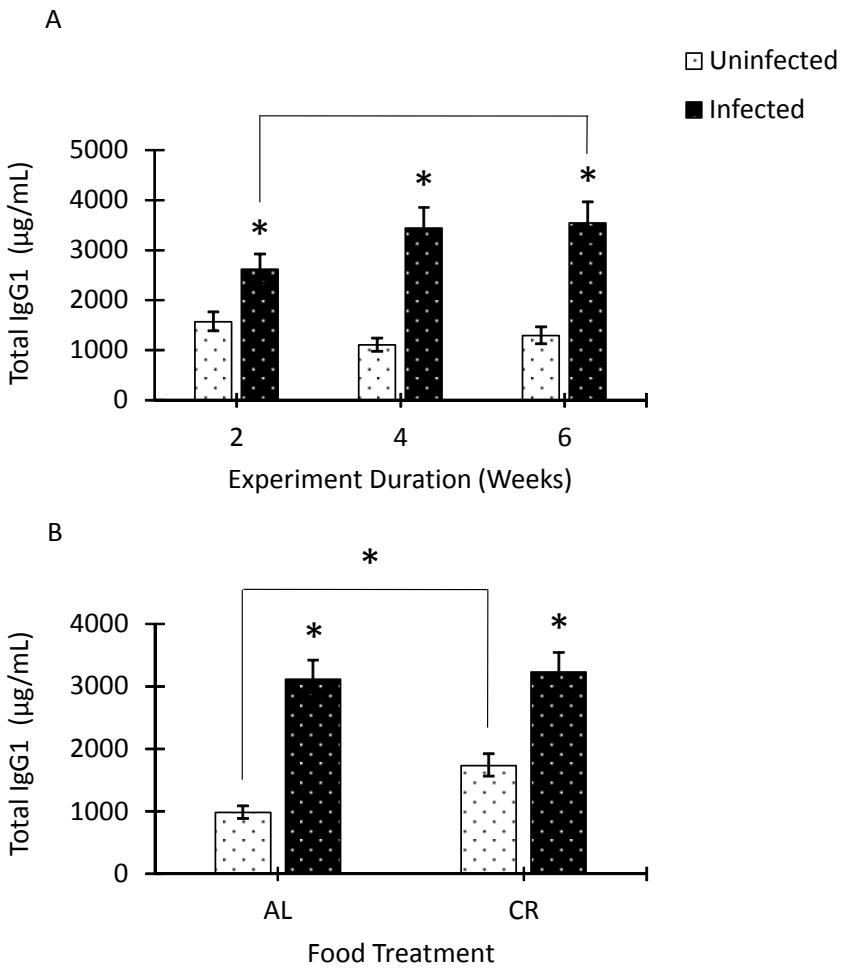


Figure 20. Total IgG1 level averages in SJL mice after 2, 4 and 6 weeks of experiment duration after infection with *Heligmosomoides bakeri* (A) and after either *ad libitum* (AL) or calorie restricted (CR) food treatment and infection with *Heligmosomoides bakeri* (B). Each value shows the back transformed mean ± 1 standard error of $\log_{10}$ of total IgG1 (µg/mL) (n=29 uninfected 2 weeks, n=28 uninfected 4 weeks, n=26 uninfected 6 weeks, n=29 infected 2 weeks, n=26 infected 4 weeks, n=27 infected 6 weeks; n=39 uninfected AL, n=44 uninfected CR, n=39 infected AL, n=43 infected CR). An asterisk above infected groups signifies a significant difference from the corresponding uninfected group and an asterisk above CR groups signifies a significant difference from the corresponding AL group. Lines with an asterisk above connect additional experimental groups that are significantly different from each other and lines without asterisk connect experimental groups that are almost significantly different from each other (having a 0.07 overlap of the 95% confidence interval). On panel A, all infected mice had more total IgG1 than all uninfected mice across different experiment durations and on panel B, all infected mice had more total IgG1 than all uninfected mice across both food treatments (not depicted by lines/asterisks on the figure, for clarity).

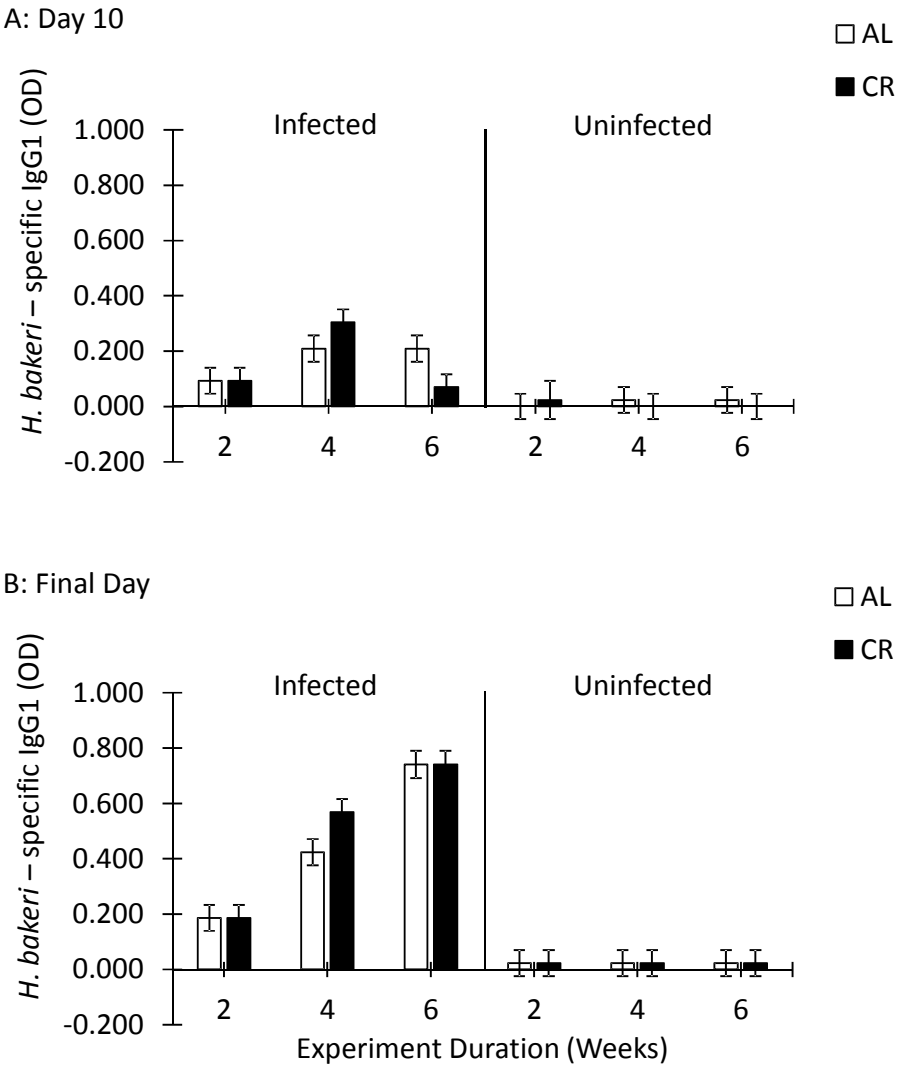


Figure 21. *H. bakeri*-specific IgG1 levels on Day 10 (A) and Final Day (B) of *ad libitum* (AL) and calorie restricted (CR) C57BL/6 mice after 2, 4 and 6 weeks of experiment duration after infection with *Heligmosomoides bakeri*. Each value shows the back transformed mean ± 1 standard error of $\log_{10}(x+10)$ of *H. bakeri*-specific IgG1 570 OD value at a 50X dilution (n=14 infected 2 wk AL, n=15 infected 2 wk CR, n=15 infected 4 wk AL, n=15 infected 4 wk CR, n=15 infected 6 wk AL, n=15 infected 6 wk CR, n=11 uninfected 2 wk AL, n=14 uninfected 2 wk CR, n=14 uninfected 4 wk AL, n=15 uninfected 4 wk CR, n=13 uninfected 6 wk AL, n=15 uninfected 6 wk CR).

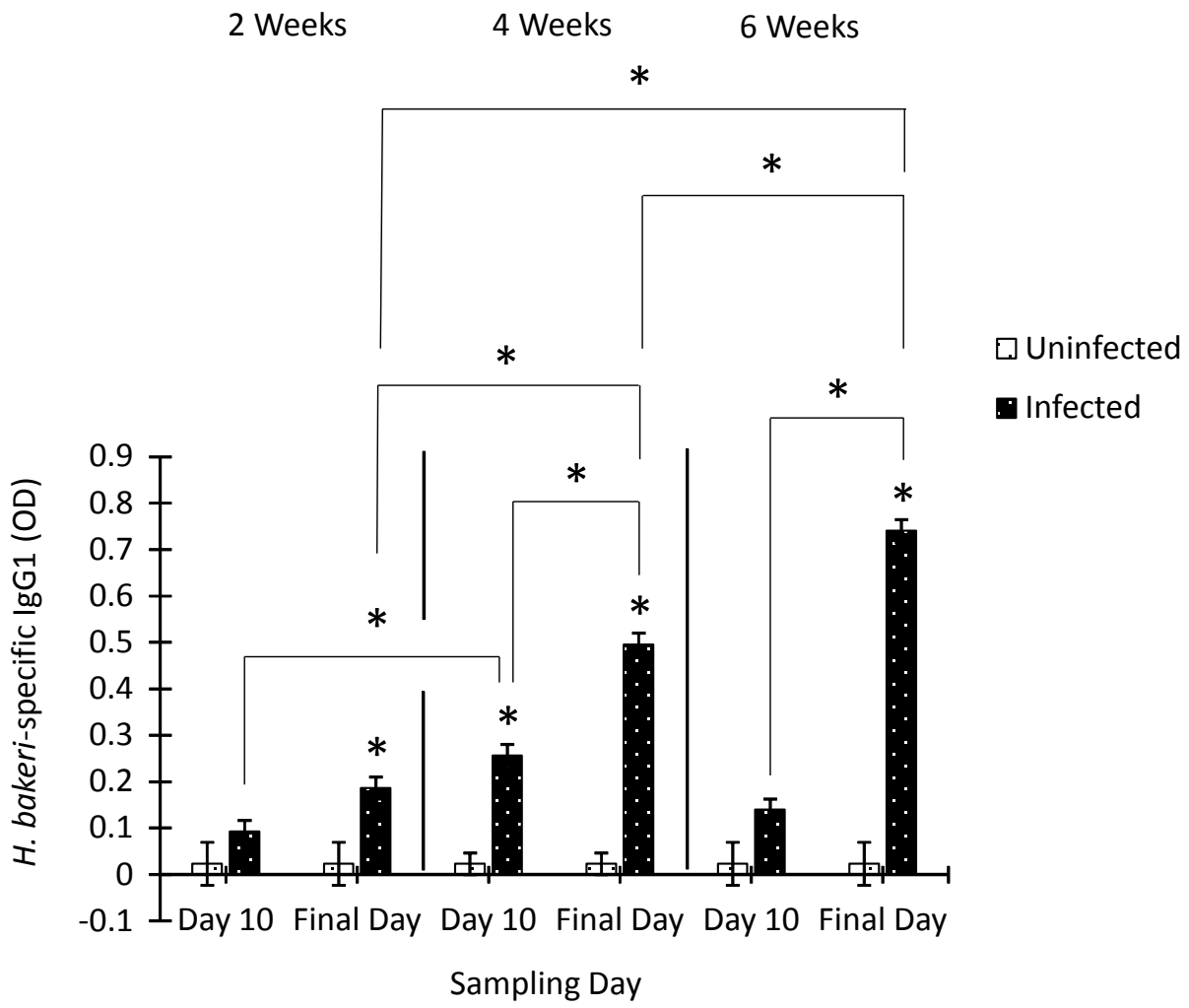


Figure 22. *H. bakeri*-specific levels on Day 10 and Final Day in C57BL/6 mice after 2, 4 and 6 weeks of experiment duration after infection with *Heligmosomoides bakeri*. Each value shows the back transformed mean ± 1 standard error of $\log_{10}(x+10)$ of *H. bakeri*-specific IgG1 570 OD value at a 50X dilution (n=25 uninfected 2 wk, n=29 uninfected 4 wk, n=28 uninfected 6 wk, n=29 infected 2 wk, n=30 infected 4 wk and n=30 infected 6 wk). An asterisk above infected groups signifies a significant difference from the corresponding uninfected group. Lines with an asterisk above connect additional experimental groups that are significantly different from each other. Infected mice from the four week experiment duration group had more *H. bakeri*-specific IgG1 on Day 10 than all uninfected mice across different sampling days and different experiment durations; all infected mice had more *H. bakeri*-specific IgG1 on the Final Day than all uninfected mice across different sampling days and experiment durations (not depicted by lines/asterisks on the figure for clarity).

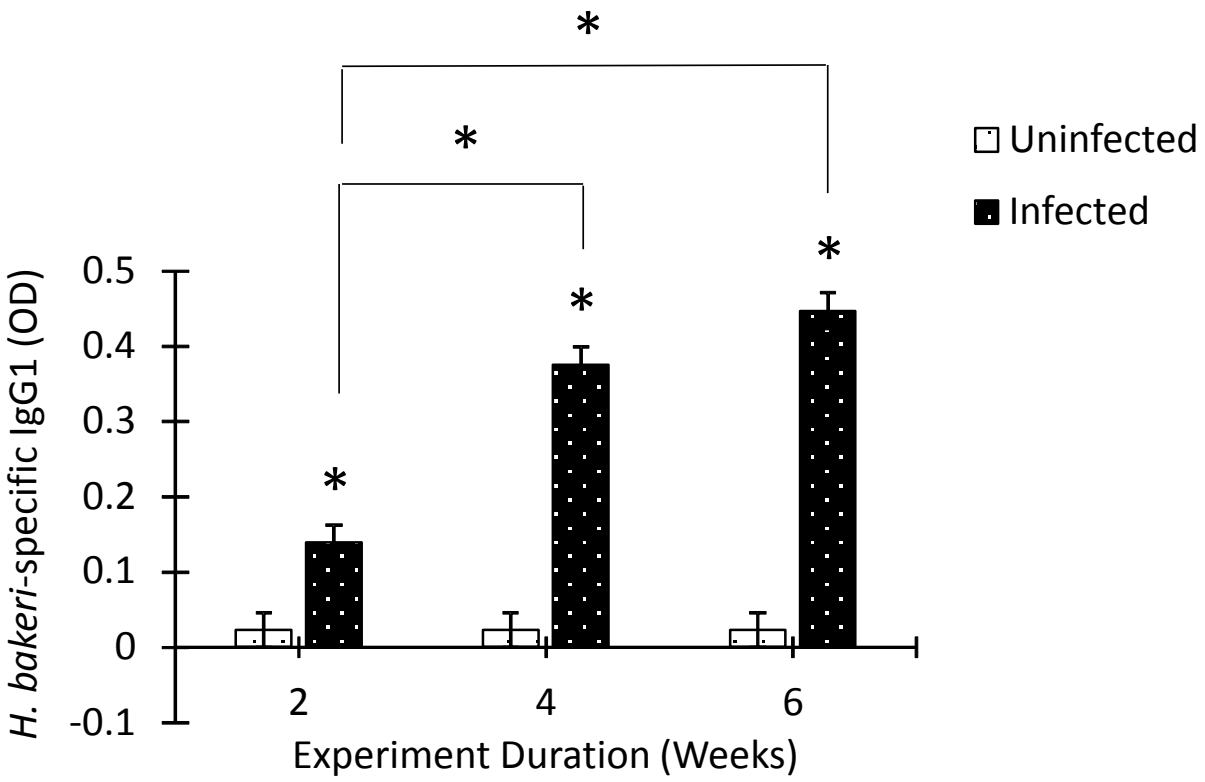


Figure 23. *H. bakeri*-specific level averages in C57BL/6 mice after 2, 4 and 6 weeks of experiment duration after infection with *Heligmosomoides bakeri*. Each value shows the back transformed mean ± 1 standard error of $\log_{10}(x+10)$ of *H. bakeri*-specific IgG1 570 OD value at a 50X dilution (n=25 uninfected 2 wk, n=29 uninfected 4 wk, n=28 uninfected 6 wk, n=29 infected 2 wk, n=30 infected 4 wk and n=30 infected 6 wk). An asterisk above infected groups signifies a significant difference from the corresponding uninfected group. Lines with an asterisk above connect additional experimental groups that are significantly different from each other. All infected mice had more *H. bakeri*-specific IgG1 than all uninfected mice across different experiment durations (not depicted by lines/asterisks on the figure for clarity).

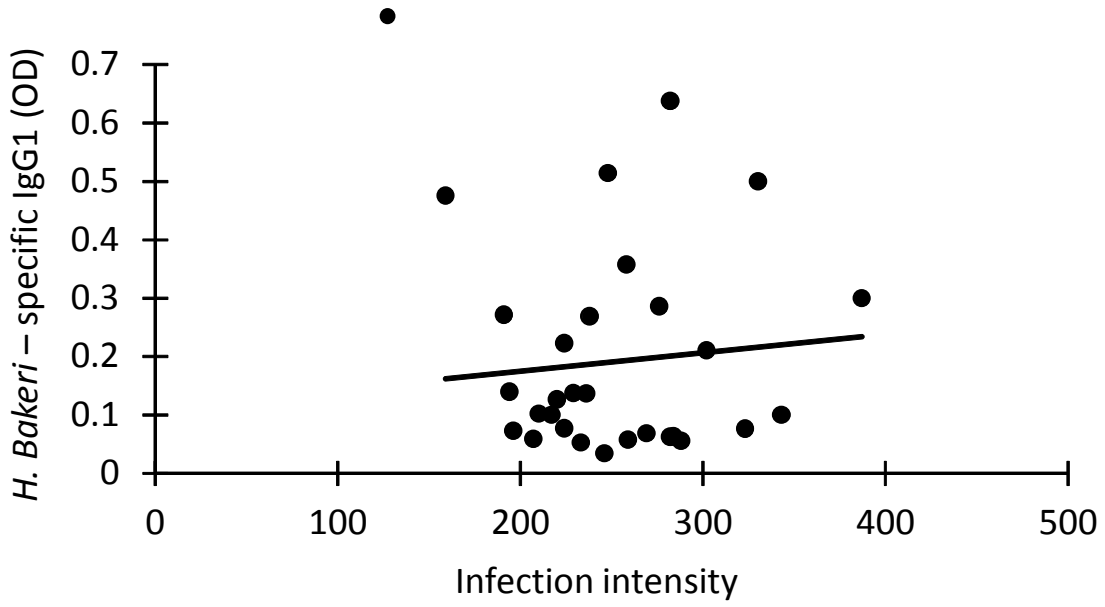


Figure 24. Regression of infection intensity against *H. bakeri* – specific IgG1 ($y = -0.0003x + 0.1124$, $R^2 = 0.0094$) on the Final Day of the experiment for C57BL/6 mice infected with *Heligmosomoides bakeri* for 2 weeks (n=29).

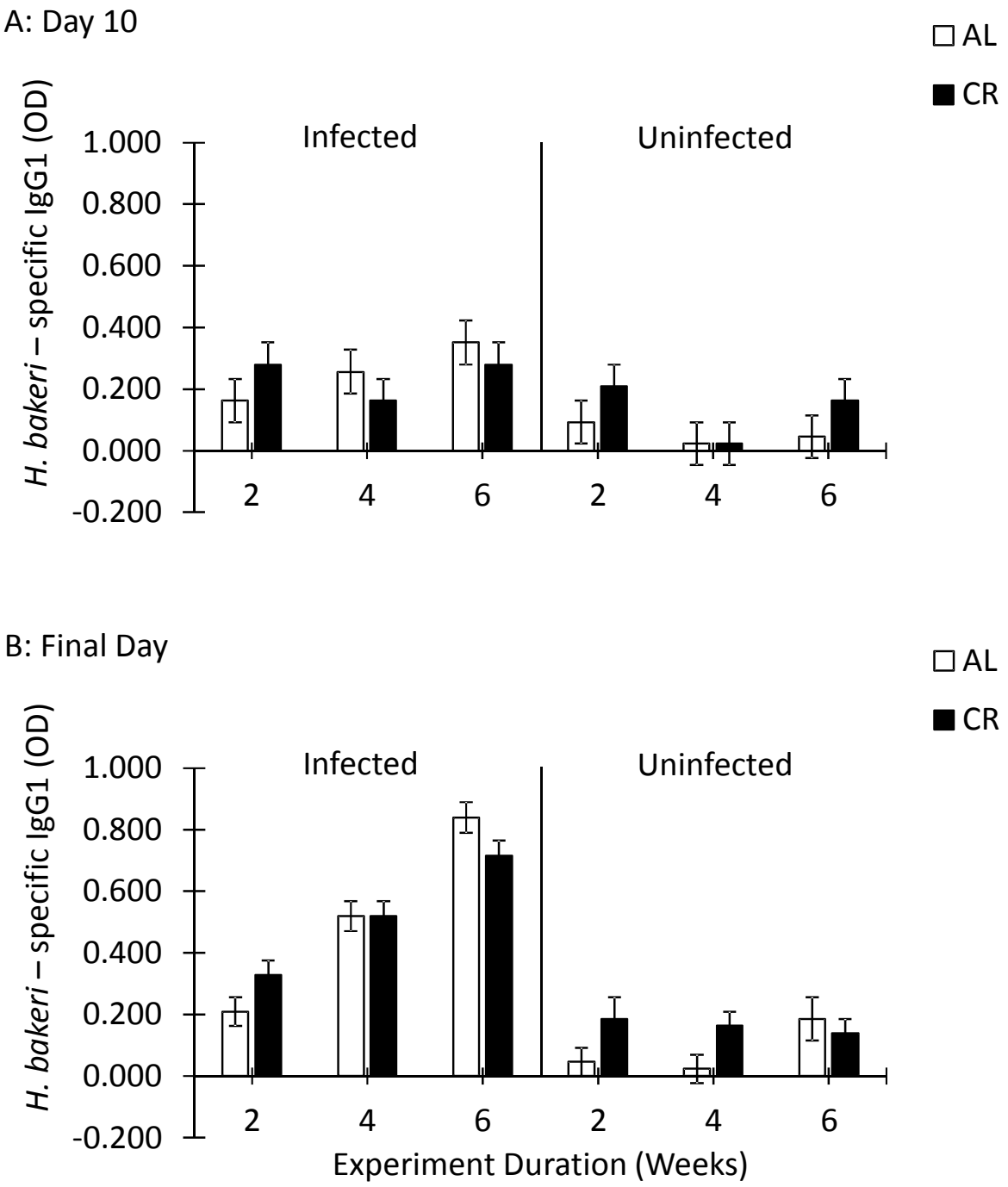


Figure 25. *H. bakeri*-specific IgG1 levels on Day 10 (A) and Final Day (B) of *ad libitum* (AL) and calorie restricted (CR) SJL mice after 2, 4 and 6 weeks of experiment duration after infection with *Heligmosomoides bakeri*. Each value shows the back transformed mean ± 1 standard error of $\log_{10}(x+10)$ of *H. bakeri*-specific IgG1 570 OD value at a 50X dilution (n=14 infected 2 wk AL, n=15 infected 2 wk CR, n=12 infected 4 wk AL, n=14 infected 4 wk CR, n=13 infected 6 wk AL, n=14 infected 6 wk CR, n=14 uninfected 2 wk AL, n=15 uninfected 2 wk CR, n=13 uninfected 4 wk AL, n=15 uninfected 4 wk CR, n=12 uninfected 6 wk AL, n=14 uninfected 6 wk CR).

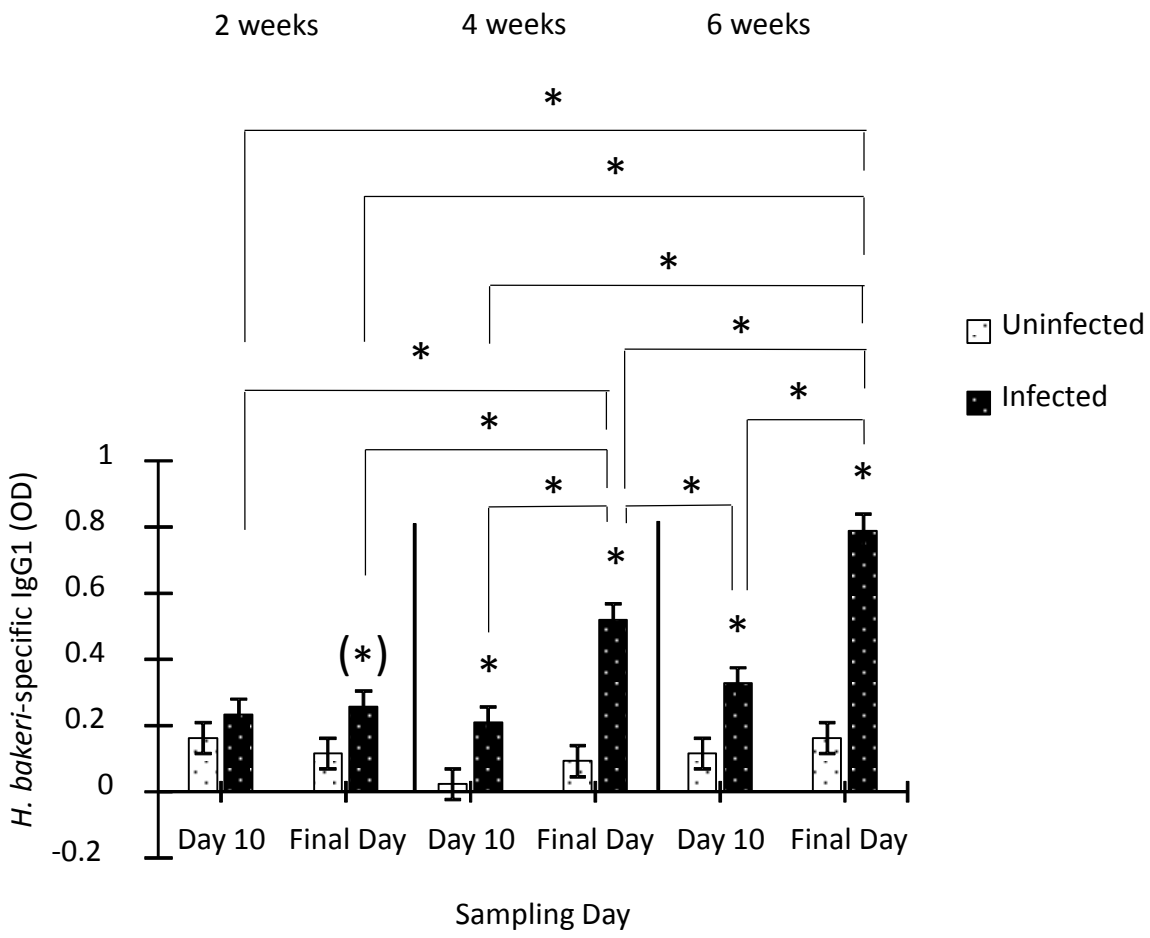


Figure 26. *H. bakeri*-specific levels on Day 10 and Final Day in SJL mice after 2, 4 and 6 weeks of experiment duration after infection with *Heligmosomoides bakeri*. Each value shows the back transformed mean ± 1 standard error of $\log_{10}(x+10)$ of *H. bakeri*-specific IgG1 570 OD value at a 50X dilution (n=29 uninfected 2 wk, n=28 uninfected 4 wk, n=26 uninfected 6 wk, n=29 infected 2 wk, n=26 infected 4 wk, n=27 infected 6 wk). An asterisk above infected groups signifies a significant difference from the corresponding uninfected group and an asterisk in parentheses signifies an almost significant difference from the corresponding uninfected group (having a 0.001 overlap of the 95% confidence interval). Lines with an asterisk above connect additional experimental groups that are significantly different from each other. Infected mice from the four and six week experiment duration groups had more *H. bakeri*-specific IgG1 on the Final Day than all uninfected mice across different sampling days and different experiment durations; all infected mice across all sampling days and experiment durations had more *H. bakeri*-specific IgG1 than uninfected mice from four week experiment duration group on Day 10 (not depicted by lines/asterisks on the figure for clarity).

Figure 27. *H. bakeri*-specific level averages in SJL mice after 2, 4 and 6 weeks of experiment duration after infection with *Heligmosomoides bakeri*. Each value shows the back transformed mean ± 1 standard error of $\log_{10}(x+10)$ of *H. bakeri*-specific IgG1 570 OD value at a 50X dilution (n=29 uninfected 2 wk, n=28 uninfected 4 wk, n=26 uninfected 6 wk, n=29 infected 2 wk, n=26 infected 4 wk, n=27 infected 6 wk). An asterisk above infected groups signifies a significant difference from the corresponding uninfected group. Lines with an asterisk above connect additional experimental groups that are significantly different from each other and lines without an asterisk above connect additional experimental groups that are almost significantly different from each other (having a 0.001 overlap of the 95% confidence interval). Infected mice at four and six week experiment durations had more *H. bakeri*-specific IgG1 than all uninfected mice across different experiment durations and uninfected mice at the two week experiment duration had more *H. bakeri*-specific IgG1 than uninfected mice at the four week experiment duration (not depicted by lines/asterisks on the figure for clarity).

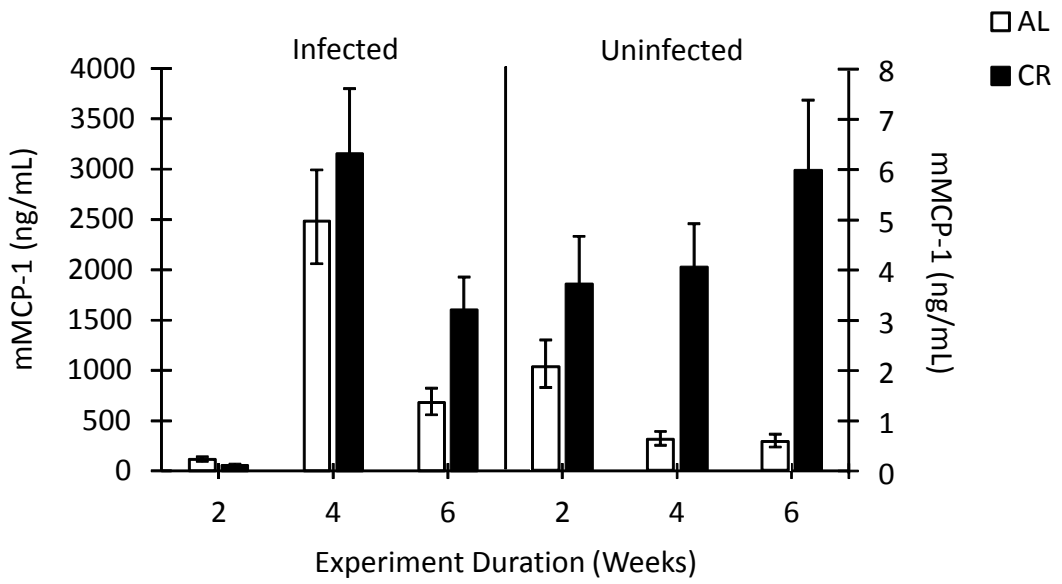


Figure 28. mMCP-1 levels of *ad libitum* (AL) and calorie restricted (CR) C57BL/6 mice after 2, 4 and 6 weeks of experiment duration after infection with *Heligmosomoides bakeri*. Each value shows the back transformed mean ± 1 standard error of $\log_{10}$ of mMCP-1 concentration (ng/mL) (n=14 infected 2 wk AL, n=15 infected 2 wk CR, n=15 infected 4 wk AL, n=15 infected 4 wk CR, n=14 infected 6 wk AL, n=15 infected 6 wk CR, n=10 uninfected 2 wk AL, n=10 uninfected 2 wk CR, n=11 uninfected 4 wk AL, n=14 uninfected 4 wk CR, n=11 uninfected 6 wk AL, n=12 uninfected 6 wk CR). Infected mice are presented on the left y-axis and uninfected mice are presented on the right y-axis.

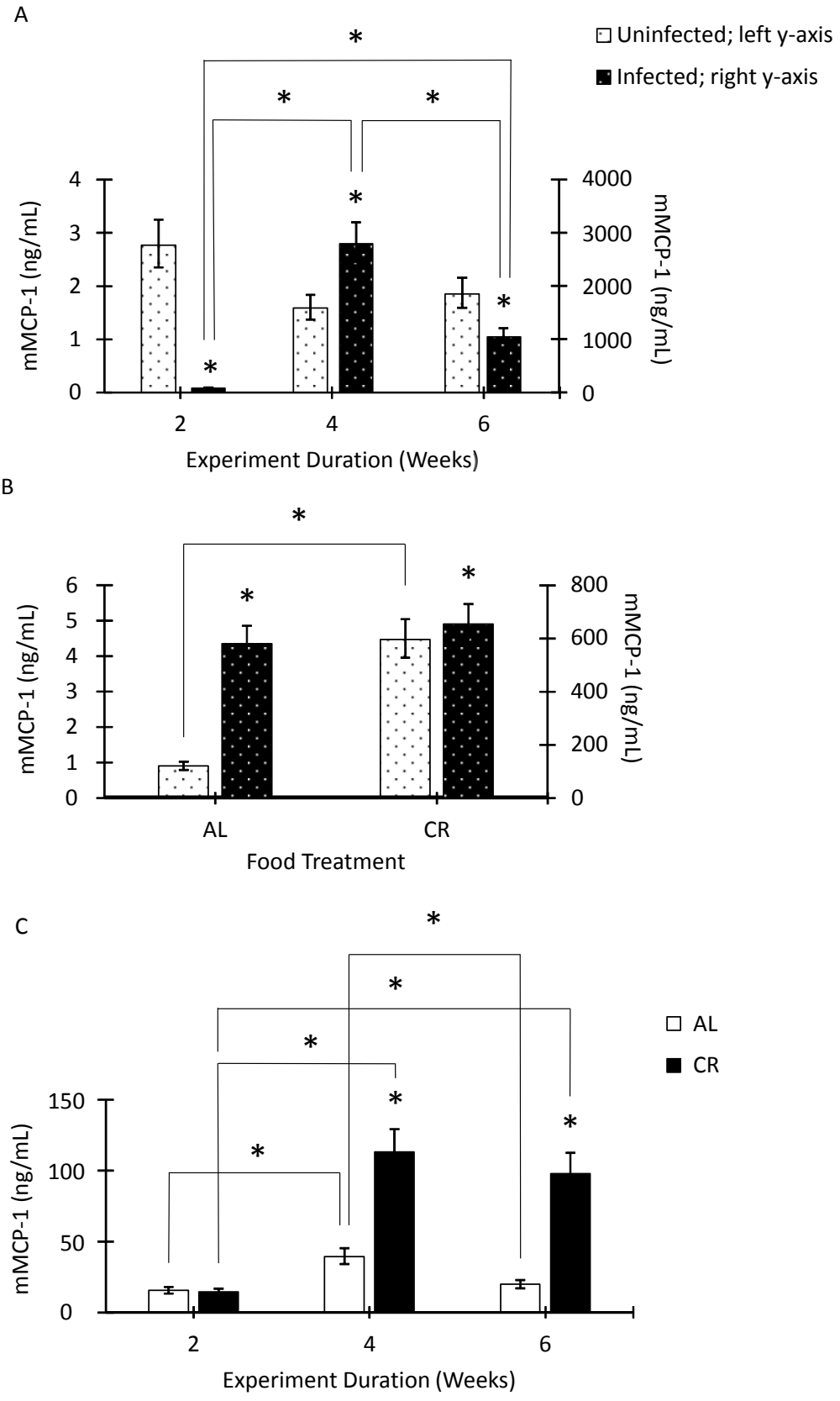

A
Uninfected; left y-axis
Infected; right y-axis
mMCP-1 (ng/mL)
mMCP-1 (ng/mL)
Experiment Duration (Weeks)
B
mMCP-1 (ng/mL)
mMCP-1 (ng/mL)
AL
CR
Food Treatment
C
AL
CR
mMCP-1 (ng/mL)
Experiment Duration (Weeks)

Figure 29. mMCP-1 levels in C57BL/6 mice after 2, 4 and 6 weeks of experiment duration after infection with *Heligmosomoides bakeri* (A), after either *ad libitum* (AL) or calorie restricted (CR) treatment (B) and after either 2, 4 or 6 weeks of experiment duration (C). Each value shows the back transformed mean ± 1 standard error of $\log_{10}$ of mMCP-1 concentration (ng/mL) (n=25 uninfected 2 wk, n=29 uninfected 4 wk, n=28 uninfected 6 wk, n=29 infected 2 wk, n=30 infected 4 wk and n=30 infected 6 wk; n=38 uninfected AL, n=44 uninfected CR, n=44 infected AL, n=45 infected CR; n=25 AL 2 wk, n=29 AL 4 wk, n=28 AL 6 wk, n=29 CR 2 wk, n=30 CR 4 wk, n=30 CR 6 wk). An asterisk above infected groups signifies a significant difference from the corresponding uninfected group and an asterisk above CR groups signifies a significant difference from the corresponding AL group. Lines with an asterisk above connect additional experimental groups that are significantly different from each other. On panel A, all infected mice had more mMCP-1 than all uninfected mice across different experiment durations (not depicted by lines/asterisks on the figure for clarity). On panel B, all infected mice had more mMCP-1 than all uninfected mice across different food treatments (not depicted by lines/asterisks on the figure for clarity). On panel C, CR mice at four and six week experiment durations had more mMCP-1 than all AL mice across different experiment durations (not depicted by lines/asterisks on the figure for clarity).

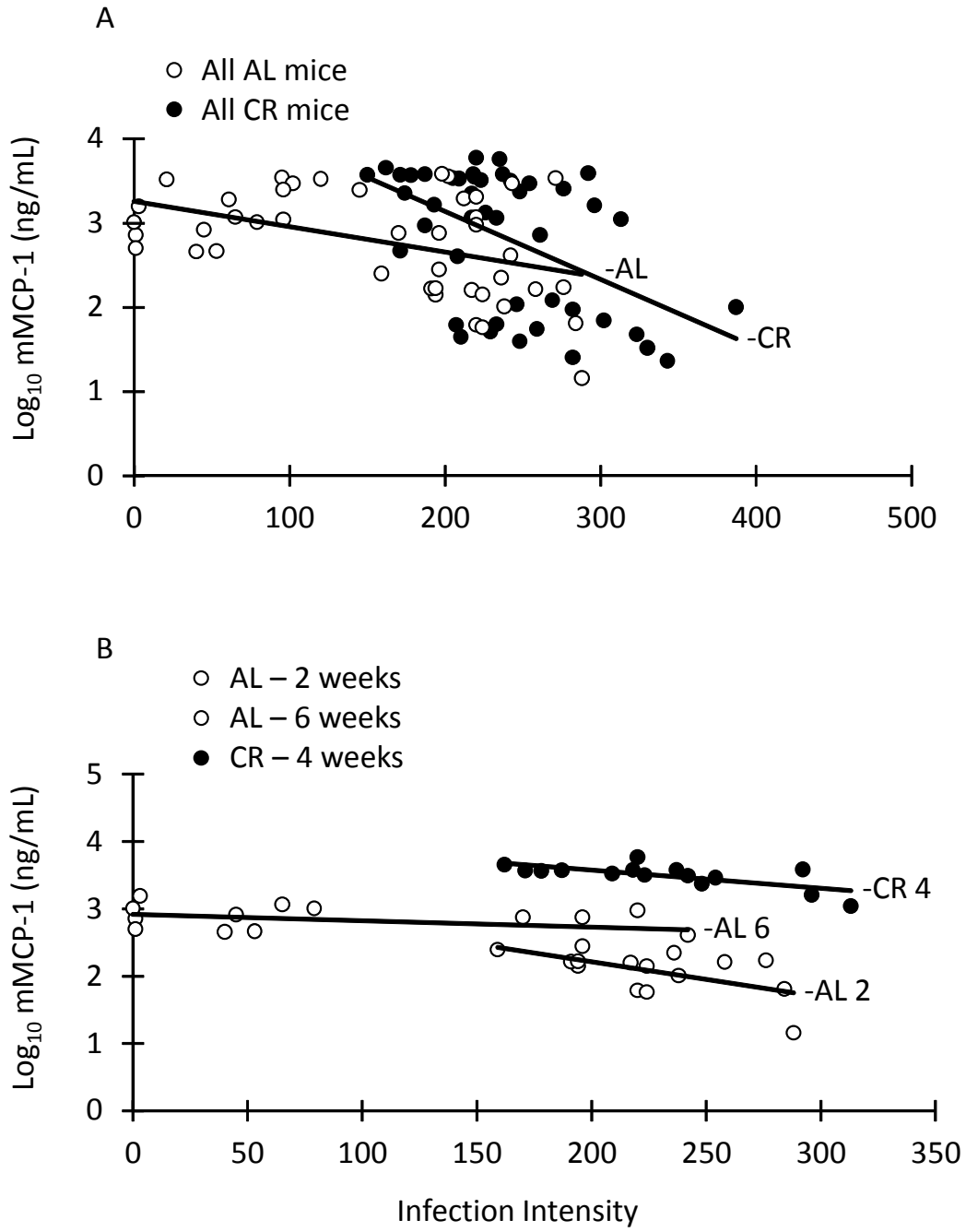


Figure 30. Regression of infection intensity against mMCP-1 on the Final Day of the experiment for all *ad libitum* (AL) ($y = -0.003x + 3.2533$, $R^2 = 0.1915$) and all calorie restricted (CR) ($y = -0.0081x + 4.7506$, $R^2 = 0.2619$) C57BL/6 mice (A), for *ad libitum* (AL) mice at 2 ($y = -0.0052x + 3.249$, $R^2 = 0.3323$) and 6 weeks ($y = -0.001x + 2.9225$, $R^2 = 0.1293$) of experiment duration and calorie restricted (CR) mice at 4 weeks ($y = -0.0027 + 4.1204$, $R^2 = 0.4863$) of experiment duration after infection with *Heligmosomoides bakeri* (B) (n = 43 all AL mice, n = 45 all CR mice, n = 14 AL 2 wk, n = 14 AL 6 wk, n = 15 CR 4 wk).

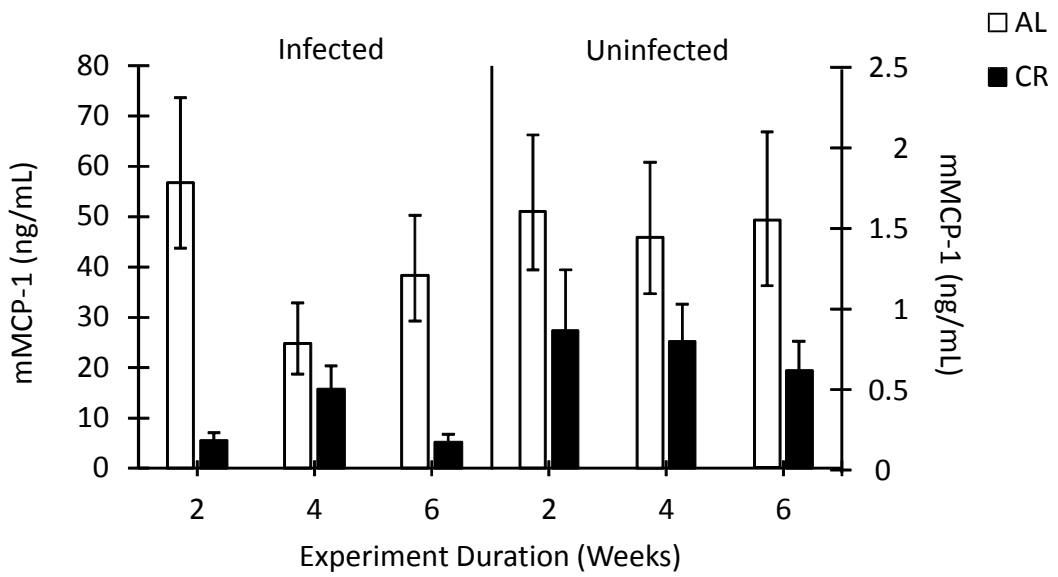


Figure 31. mMCP-1 levels of *ad libitum* (AL) and calorie restricted (CR) SJL mice after 2, 4 and 6 weeks of experiment duration after infection with *Heligmosomoides bakeri*. Each value shows the back transformed mean ± 1 standard error of $\log_{10}$ of mMCP-1 concentration (ng/mL) (n=14 infected 2 wk AL, n=14 infected 2 wk CR, n=12 infected 4 wk AL, n=14 infected 4 wk CR, n=13 infected 6 wk AL, n=14 infected 6 wk CR, n=14 uninfected 2 wk AL, n=7 uninfected 2 wk CR, n=12 uninfected 4 wk AL, n=14 uninfected 4 wk CR, n=10 uninfected 6 wk AL, n=14 uninfected 6 wk CR). Infected mice are presented on the left y-axis (ng/mL), while uninfected mice are presented on the right y-axis (ng/mL).

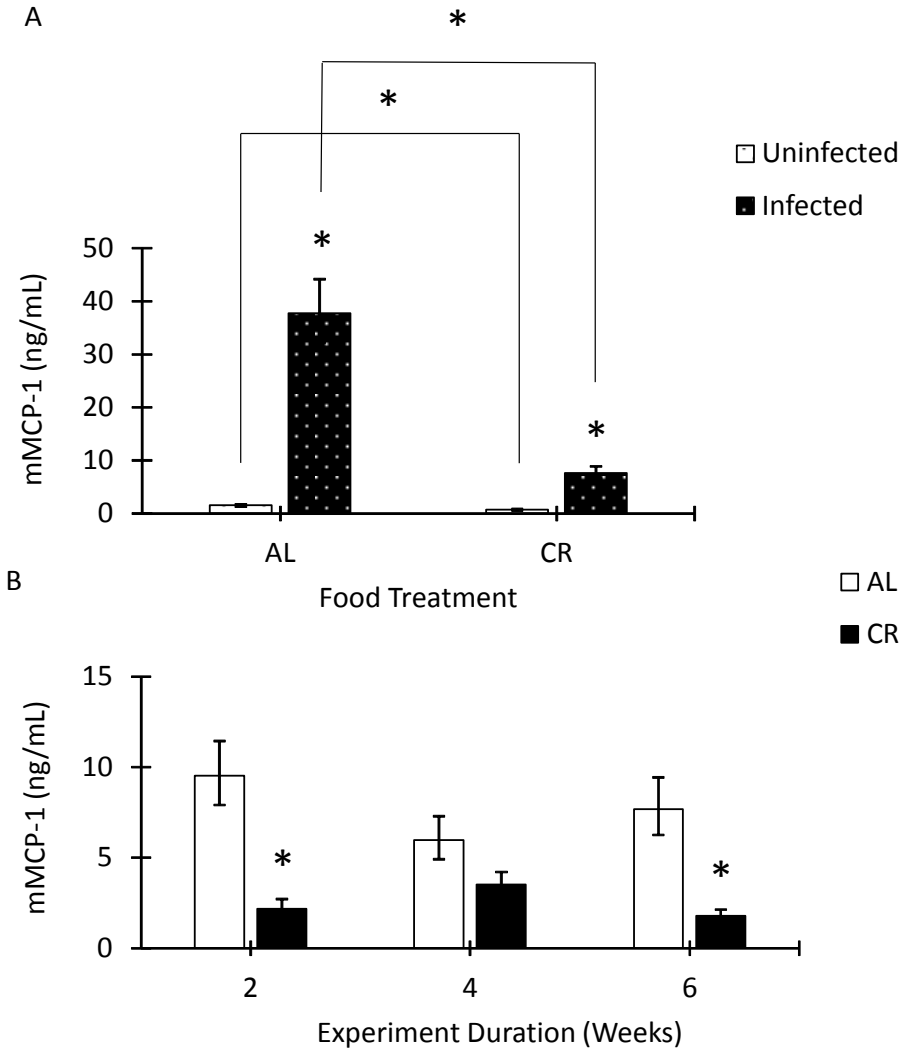


Figure 32. mMCP-1 levels of *ad libitum* (AL) and calorie restricted (CR) SJL mice after infection with *Heligmosomoides bakeri* (A) and after 2, 4 and 6 weeks of experiment duration (B). Each value shows the back transformed mean ± 1 standard error of $\log_{10}$ of mMCP-1 concentration (ng/mL) (n=39 uninfected AL, n=44 uninfected CR, n=39 infected AL, n=28 infected CR; n=28 AL 2 wk, n=25 AL 4 wk, n=25 AL 6 wk, n=30 CR 2 wk, n=29 CR 4 wk, n=28 CR 6 wk). An asterisk above infected groups signifies a significant difference from the corresponding uninfected group and an asterisk above CR groups signifies a significant difference from the corresponding AL group. Lines with an asterisk above connect additional experimental groups that are significantly different from each other. On panel A, all infected mice had more mMCP-1 than all uninfected mice across all food treatments; on panel B, AL mice at the two week experiment duration had more mMCP-1 than CR mice at the six week experiment duration and AL mice at the six week experiment duration had more mMCP-1 than CR mice at the two week experiment duration (not depicted by lines/asterisks on the figure for clarity).

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