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Experimental infection of *Calomys callosus* with atypical strains of *Toxoplasma gondii* shows gender differences in severity of infection

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Abstract There is a significant genetic diversity of *Toxoplasma gondii* in Brazil. Two parasite isolates were recently obtained from chickens in Uberlândia, Minas Gerais state, Brazil, namely, TgChBrUD1 and TgChBrUD2. In this study, we investigated *Calomys callosus* susceptibility to these atypical *T. gondii* strains. Male and female animals were intraperitoneally infected with tachyzoites and monitored to evaluate body weight change, morbidity, and mortality. Immunohistochemical assay and qPCR were performed to determine the parasitism in liver, spleen, and brain. Our data showed that TgChBrUD2-infected males died earlier than TgChBrUD1-infected males and 100 % of mortality was observed after 10 and 12 days of infection, respectively. Also, TgChBrUD1-infected females died earlier than TgChBrUD1-infected males and 100 % of mortality was observed after 9 and 12 days of infection, respectively. Both strains were able to induce a decrease in body weight of males, but only the TgChBrUD1 strain induced an increase in body weight of females. TgChBrUD2-infected females had significantly higher parasite load in both liver and spleen in comparison to TgChBrUD1-infected females, but no significant difference was found between genders or strains when males were infected. There was higher parasitism in the liver than the brain from both males and females infected with either strain. In conclusion, *C. callosus* specimens are susceptible to both *T. gondii* atypical strains with differences between males and females in severity of infection. These findings open new prospects for understanding different aspects of *T. gondii* infection, including reinfection and vertical transmission with these atypical strains when utilizing this experimental model.

Keywords *Toxoplasma gondii* isolates · *Calomys callosus* · Atypical strains

Introduction

The protozoan *Toxoplasma gondii* is an obligate intracellular parasite that infects a wide range of hosts (Cenci-Goga et al. 2011). It causes a relevant zoonotic infection, as it is primarily defined as an opportunistic pathogen in humans (Sibley 2011). Infection with *Toxoplasma gondii* is generally acquired by ingestion of food or water contaminated with oocysts, which are shed with cat feces or consumption of raw or undercooked meat products containing tissue cysts (Elmore et al. 2010). Infection in humans is frequently asymptomatic, but it can lead to severe disease in immunocompromised patients and congenitally infected individuals (Dubey 2010).

A recent study showed high genetic diversity of *Toxoplasma gondii* and the isolates collected worldwide can be roughly grouped into six clades (Su et al. 2012). The genetic differences among these genotypes at genome sequence level are generally low, but there are differences in the virulence phenotypes for mice. Type I strains are highly virulent and infection with a single parasite results in the death of mice. In contrast, types II and III are relatively avirulent, frequently resulting in chronic infections in mice (Howe and Sibley 1995). A fourth clonal lineage, referred as type 12, has

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recently been described in North America, where it is commonly found in wildlife. Type 12 strains in mice revealed that some of them express intermediate or high levels of acute virulence (Khan et al. 2011a). On the other hand, an entirely distinct pattern is seen in South America, where an abundance of atypical (non-clonal) strain types have been found and show markedly greater diversity and divergence within and between groups (Khan et al. 2007, 2011b; Shwab et al. 2013).

In Brazil, a higher genetic diversity was identified and the three most common genotypes were #6, #8, and #11, previously designated as type BrI, BrIII, and BrII, respectively (Pena et al. 2008; Dubey et al. 2012). Analysis of mortality rates in infected mice indicated that type BrI is highly virulent, type BrIII is nonvirulent, whereas type BrII lineages are intermediately virulent (Pena et al. 2008). Two parasite isolates were recently obtained from chickens in Uberlândia city and they were named TgChBrUD1 and TgChBrUD2. The isolate TgChBrUD1 exhibited ToxoDB polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) genotype #11 (also known as type BrII) and the TgChBrUD2 isolate exhibited ToxoDB PCR-RFLP genotype #6 (also known as type BrI Africa 1) (Salomão, personal communication). Recent studies have suggested a relationship between virulence and the genetic makeup of the different strains of *Toxoplasma gondii* present in the environment and in cases of human and animal diseases (Beck et al. 2009; Herrmann et al. 2012; Dubremetz and Lebrun 2012; Su et al. 2012). These phenotypic variations can be important to determine the role of *Toxoplasma gondii* genetic diversity in transmission between species, pathogenicity and immunological response, in addition to implications for diagnosis, treatment, and prevention of toxoplasmosis (Beck et al. 2009; Dubey et al. 2012; Su et al. 2012).

The possibility that the parasite genotype influences the severity of human disease is supported by differences in pathogenicity of parasite strain in animal models (Sibley and Boothroyd 1992). A study with *Toxoplasma gondii* isolates from asymptomatic chickens from Brazil showed that these Brazilian isolates were more pathogenic to mice than isolates from Europe or North America, irrespective of the genotype (Dubey et al. 2006).

Calomys callosus, a rodent of the family Cricetidae widely distributed in Central Brazil, has been described as a reservoir for various infections agents (Borges et al. 1992; Favoreto-Junior et al. 1998). Adapted to laboratory studies, this species has been used as experimental model to study *Toxoplasma gondii* infection (Favoreto-Junior et al. 1998; Ferro et al. 1999, 2002; Franco et al. 2011). Also, our previous studies have demonstrated that *C. callosus* is a useful experimental model for congenital toxoplasmosis (Ferro et al. 1999, 2002; Barbosa et al. 2007; Franco et al. 2011). This rodent was shown to be highly susceptible to infection by *Toxoplasma gondii* RH strain with 100 % mortality after 9 days of infection

(Favoreto-Junior et al. 1998). In addition, this species is able to induce brain tissue cyst formation as well as ocular lesions when infected by *Toxoplasma gondii* ME-49 strain (Pereira et al. 1999).

Considering that Brazilian isolates are more virulent than those from Europe or North America and that *C. callosus* may be considered a suitable experimental model to study different features of *Toxoplasma gondii* infection, the present study aimed to evaluate the influence of *C. callosus* gender when infected with atypical *Toxoplasma gondii* Brazilian isolates.

Materials and methods

Animals

Calomys callosus specimens were kept under standard conditions on a 12-h light, 12-h dark cycle in a temperature-controlled room (25 ± 2 °C) with food and water ad libitum in the Animal Experimentation Center, Federal University of Uberlândia, Brazil. Animal experiments and procedures were conducted according to institutional guidelines for ethics in animal experimentation.

Parasite strains

Tachyzoites of *Toxoplasma gondii* TgChBrUD1 and TgChBrUD2 strains were obtained initially from peritoneal exudates of previously infected Swiss mice and then maintained by serial passages in human fibroblast (HFF) cells. The cell culture-derived parasites were stained with 0.4 % Trypan blue and viable parasites were counted in a hemocytometric chamber for further experimental infection.

Experimental infections

Males and females ($n=24$) of *C. callosus* were intraperitoneally infected with 100 tachyzoites of the TgChBrUD1 or TgChBrUD2 strain. The animals were monitored to evaluate body weight change, morbidity, and mortality for 15 days. Morbidity was assessed based on the clinical parameters as previously described (Bartley et al. 2006) with modifications as follows: sleek/glossy coat, bright and active (score 0); hunched, starry stiff coat (score 1); and reluctance to move (score 2).

In a second set of experiments, animals from both genders ($n=24$) were infected as described above and euthanized at 8 days post-inoculation (p.i.), when their peritoneal exudates were examined microscopically to confirm *Toxoplasma gondii* infection. Spleen, liver, and brain were collected for further detection of tissue parasitism.

Quantitative real-time PCR

Spleen, liver, and brain harvested from *C. callosus* at 8 days p.i. were frozen in liquid nitrogen. Total DNA was extracted from 20 mg of tissues using Wizard® Genomic DNA Purification Kit (Promega Co., Madison, WI, USA), according to the manufacturer's instructions. Total DNA was quantified by UV spectrophotometry (ND1000 Spectrophotometer; NanoDrop Technologies, Wilmington, Delaware, USA). Real-time PCR was performed with a StepOnePlus® Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) and Go Taq® quantitative real-time PCR (qPCR) Master Mix Kit (Promega Co.), according to the manufacturer's instructions. The reaction conditions followed the protocols of Wahab et al. (2010). The primers (forward, 5′-CACAGAAG GGACAGAAGT-3′ and reverse, 5′-TCGCCTTCATCTAC AGTC-3′) were used for amplification of *Toxoplasma gondii* as described by Homan et al. (2000), and amplify a 529-bp fragment, AF146527 [Genbank]. Positive and negative parasite controls were included in each assay. The reaction was carried out with 200 ng of DNA targets and 100-ng DNA standard curve was concomitantly performed on each reaction in a seven times dilution series.

Immunohistochemical assay

For immunolocalization of the parasites in spleen, liver, and brain tissues, formalin-fixed samples were dehydrated and embedded in paraffin. Sections measuring 4 μm in thickness were placed on glass slides and processed as previously described (Ferro et al. 2002). Briefly, samples were first incubated with 5 % acetic acid to block endogenous alkaline phosphatase and then with 2 % normal goat serum to block nonspecific binding sites. Next, samples were incubated at 4 °C overnight with mouse anti-*Toxoplasma gondii* polyclonal serum (1:100), which was produced by our laboratory by infecting Swiss mice with ME-49 strain, and then with biotinylated goat anti-mouse IgG (1:600) (Sigma-Aldrich, St. Louis, MO, USA). The reaction was amplified by avidin–biotin–alkaline phosphatase system (ABC kit, PK-4000; Vector Laboratories, Inc., Burlingame, USA) and developed with fast red-naphthol (Sigma-Aldrich). Samples were counter-stained with Harris's hematoxylin and examined under light microscopy.

Statistical analysis

The data were analyzed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA). Differences between groups were analyzed by the Mann–Whitney test. The Kruskal–Wallis test and Dunn multiple post-test were used to compare the parasitism between organs. The Kaplan–Meier analysis was applied to estimate survival rates after infection and survival curves were compared using the log-rank test. Values of $P < 0.05$ were considered as statistically significant.
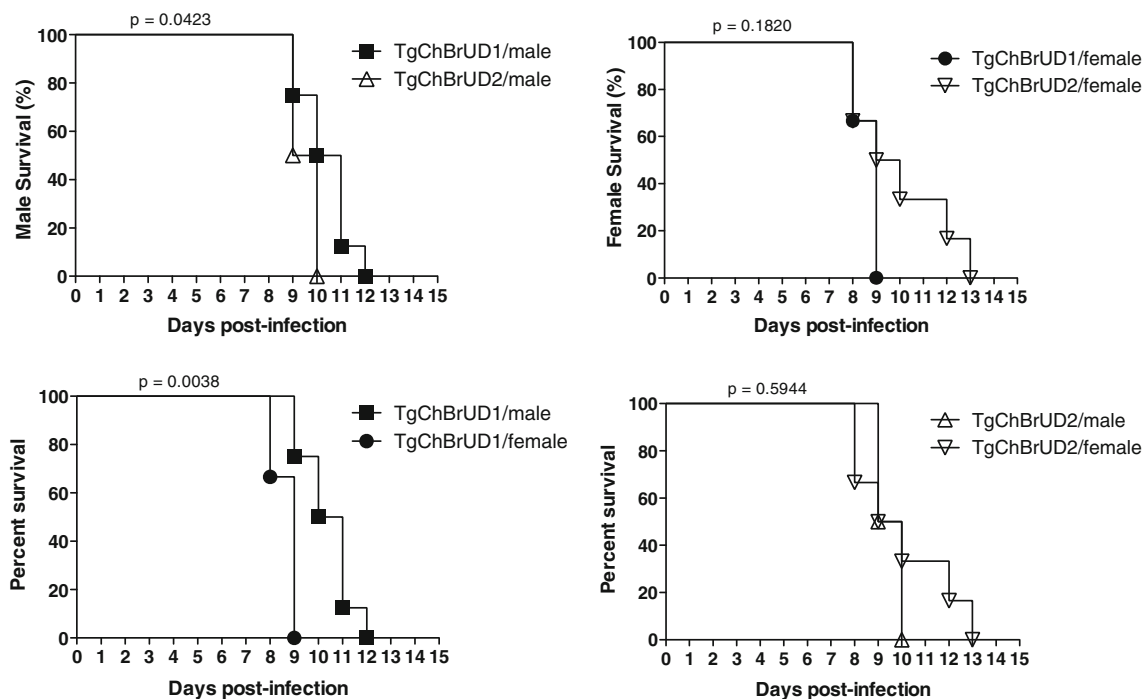
Fig. 1 Survival curves of *C. callosus* after infection with the TgChBrUD1 or TgChBrUD2 strain of *T. gondii*. Animals (six per group) were infected with 100 tachyzoites of the TgChBrUD1 or TgChBrUD2 strain and monitored for 15 days. **a** Comparison between TgChBrUD1- and TgChBrUD2-infected males; **b** Comparison between TgChBrUD1- and TgChBrUD2-infected females; **c** Comparison between TgChBrUD1-infected males and females; **d** Comparison between TgChBrUD2-infected males and females. The *P* values were determined by Log-rank test

Results

Survival rate

Mortality of TgChBrUD1-infected males was detected as early as 9 days, reaching 100 % by 12 days p.i. (Fig. 1a). When males were infected with the TgChBrUD2 strain, the mortality also occurred early (9 days p.i.), but all animals died at 10 days p.i., showing survival times significantly lower as compared to the TgChBrUD1-infected males ($P=0.0423$) (Fig. 1a). On the other hand, the mortality of the infected females was detected as early as 8 days, reaching 100 % by 9 days p.i. with the TgChBrUD1 strain and 13 days p.i. with the TgChBrUD2 strain, but with no significant difference in survival curves ($P=0.1820$) (Fig. 1b).

Comparison between males and females infected with the TgChBrUD1 strain showed that females died earlier than males (median survival 9.0 and 10.5 days, respectively; $P=0.0038$) (Fig. 1c). In contrast, TgChBrUD2-infected males and females showed similar median survival (9.5 days), with no significant difference between survival curves ($P=0.5944$) (Fig. 1d).

Body weight change and morbidity

Infected males showed slight body weight changes in the initial days after infection with TgChBrUD1 or TgChBrUD2 strain, but from the sixth day p.i. onwards, TgChBrUD1-infected males started to lose weight, with significantly higher weight loss than the TgChBrUD2-infected males (Fig. 2a). In
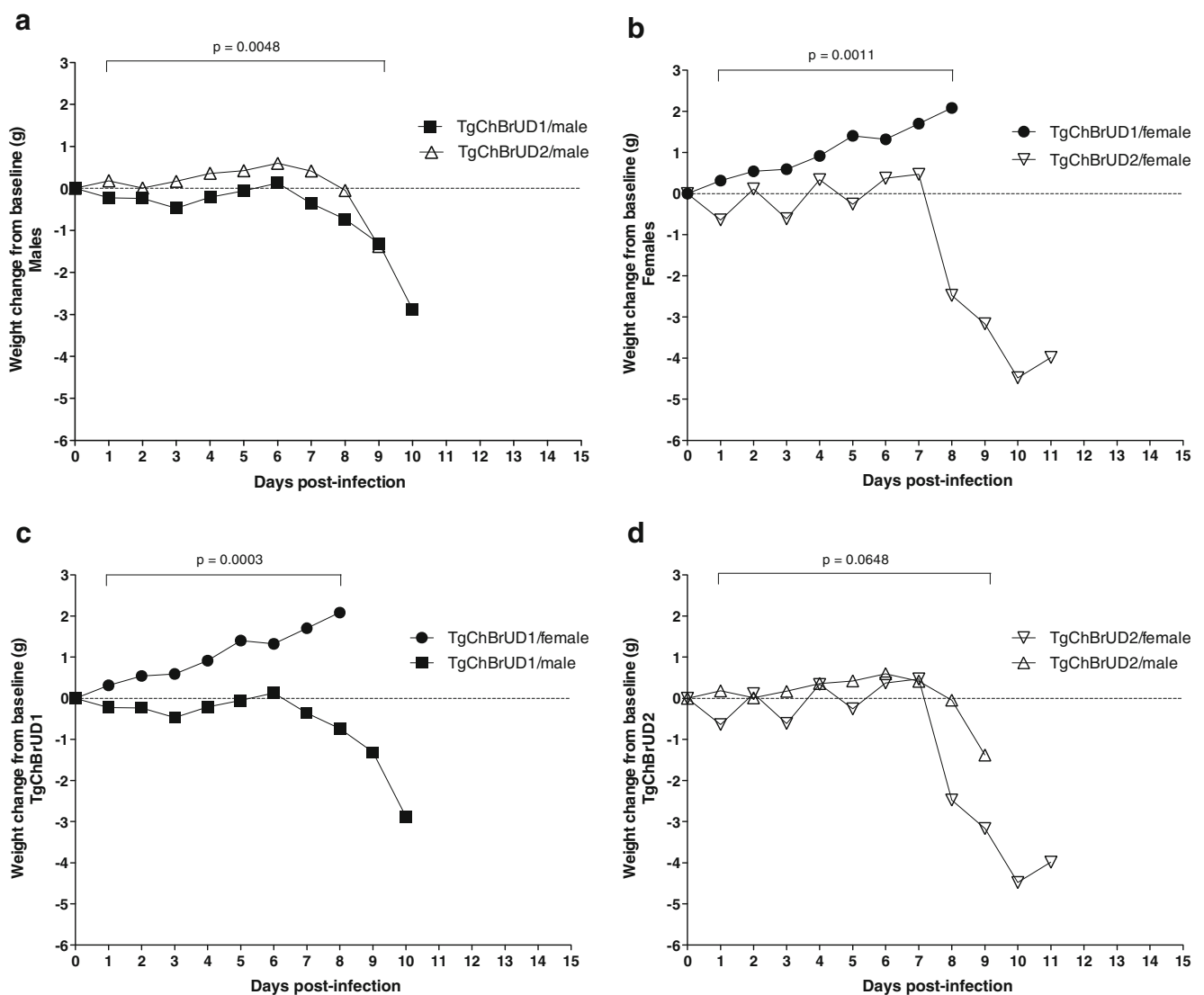
Fig. 2 Body weight change of *C. callosus* after infection with the TgChBrUD1 or TgChBrUD2 strain of *T. gondii*. Animals (six per group) were infected with 100 tachyzoites of the TgChBrUD1 or TgChBrUD2 strain and monitored for 15 days. **a** Comparison between TgChBrUD1- and TgChBrUD2-infected males; **b** Comparison between TgChBrUD1- and TgChBrUD2-infected females; **c** Comparison between TgChBrUD1-infected males and females; **d** Comparison between TgChBrUD2-infected males and females. The *P* values were determined by Mann-Whitney test
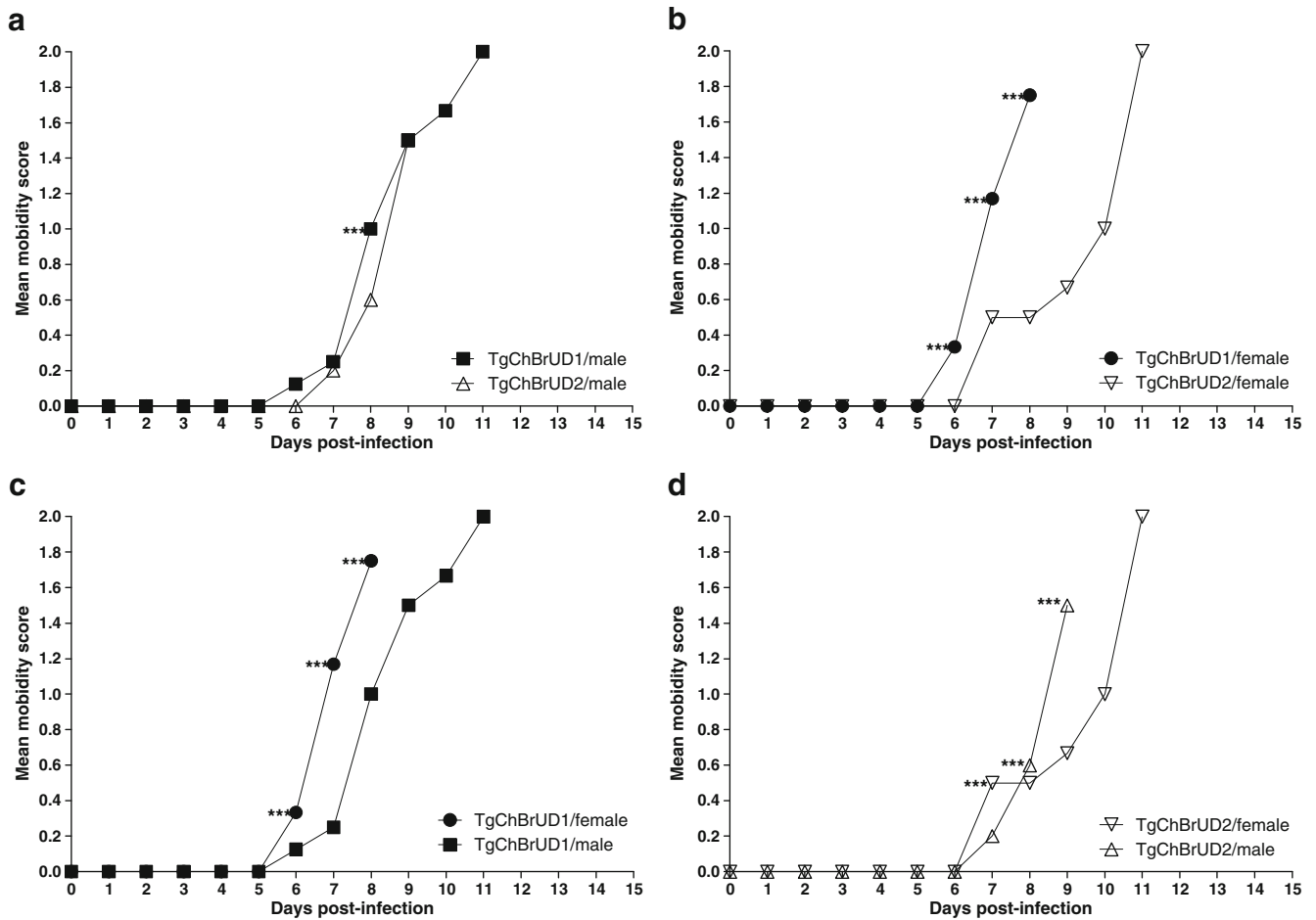
Fig. 3 Effect of *T. gondii* infection with the TgChBrUD1 or TgChBrUD2 strain in clinical parameters of *C. callosus*. Animals (six per group) were infected with 100 tachyzoites of the TgChBrUD1 or TgChBrUD2 strain and monitored for 15 days. **a** Comparison between TgChBrUD1- and TgChBrUD2-infected males; **b** Comparison between TgChBrUD1- and TgChBrUD2-infected females; **c** Comparison between TgChBrUD1-infected males and females; **d** Comparison between TgChBrUD2-infected males and females. **$P<0.01$; ***$P<0.001$ (Mann-Whitney test)

contrast, TgChBrUD1-infected females showed increasing body weight, whereas those infected with TgChBrUD2 had body weight fluctuations until the seventh day p.i. and started to lose weight onwards, with significantly lower weight loss than those infected with the TgChBrUD1 strain (Fig. 2b). Comparison between males and females showed that the TgChBrUD1 strain caused opposite effects in body weight, with weight gain in females and weight loss in males ($P=0.0003$) (Fig. 2c). In contrast, the TgChBrUD2 strain caused weight loss from the eighth day p.i. onwards in both females and males, with a tendency to be higher in females in the eighth and ninth day p.i. (Fig. 2d).

Table 1 Mortality, body weight, and morbidity of males and females *Calomys callosus* infected with TgChBrUD1 or TgChBrUD2 strain of *Toxoplasma gondii*

	TgChBrUD1			TgChBrUD2		
	Mortality (100 %)	Body weight	Morbidity	Mortality (100 %)	Body weight	Morbidity
Male	12 days p.i.	Lose	+++	10 days p.i.*	Lose*	++
Female	9 days p.i.[#]	Gain[#]	++++	13 days p.i.	Lose*	+

p.i. post-inoculation

*Statistically significant differences between strains ($P<0.05$)

[#] Statistically significant differences between gender ($P<0.05$)

+Clinical parameters severity

Males infected with TgChBrUD1 or TgChBrUD2 strain showed no change in morbidity scores in the initial days' p.i., starting to be hunched with starry stiff coat in the sixth and seventh day p.i with the TgChBrUD1 and TgChBrUD2 strains, respectively (Fig. 3a). At 8 days p.i., morbidity scores were higher in TgChBrUD1- than TgChBrUD2-infected males ($P<0.05$) (Fig. 3a). Females infected with TgChBrUD1 showed higher morbidity scores in the sixth, seventh, and eight day p.i. in comparison to females infected with TgChBrUD2 (Fig. 3b). Reluctance to move was observed 1 day before death in animals of all groups. Comparison between males and females showed that the TgChBrUD1 strain induced higher morbidity in females than males, with significant differences in the sixth, seventh, and eight day p.i (Fig. 3c). In contrast, the TgChBrUD2 strain caused higher morbidity in males than females, with significant differences in the seventh, eighth, and ninth day p.i (Fig. 3c).

The mortality, body weight and morbidity data from male and female *C. callosus* infected with TgChBrUD1 and TgChBrUD2 strain of *Toxoplasma gondii* were summarized in Table 1.

Tissue parasitism

The microscopic examination revealed the presence of tachyzoites in the peritoneal fluids of all animals after 8 days of infection with either strain. Tissue parasitism was detected by qPCR in liver, spleen, and brain. The TgChBrUD2-infected females had significantly higher parasite number in liver and spleen in comparison to TgChBrUD1-infected females (Fig. 4a, b), but with no significant difference in brain tissue parasitism (Fig. 4c). Also, no significant difference in liver, spleen, and brain parasitism was observed between TgChBrUD1- and TgChBrUD2-infected males as well as between males and females infected with either strain (Fig. 4a–c). Comparison between organs showed a significantly higher tissue parasitism in the liver compared to the brain of males or females infected with the TgChBrUD1 or TgChBrUD2 strains (Fig. 5a–d). Immunohistochemical assays confirmed the presence of *Toxoplasma gondii* in the liver, spleen, and brain (Fig. 6a–c).

Discussion

Toxoplasma gondii has a complex life cycle, involving wide range of intermediate hosts and obligate intracellular stages (Cenci-Goga et al. 2011). Within this host range, susceptibility to infection, as well as the features of acute disease is extremely variable. Differences in susceptibility to infection with *Toxoplasma gondii* in different hosts have been attributed to the parasite stage (tachyzoite or bradyzoite), inoculation route,
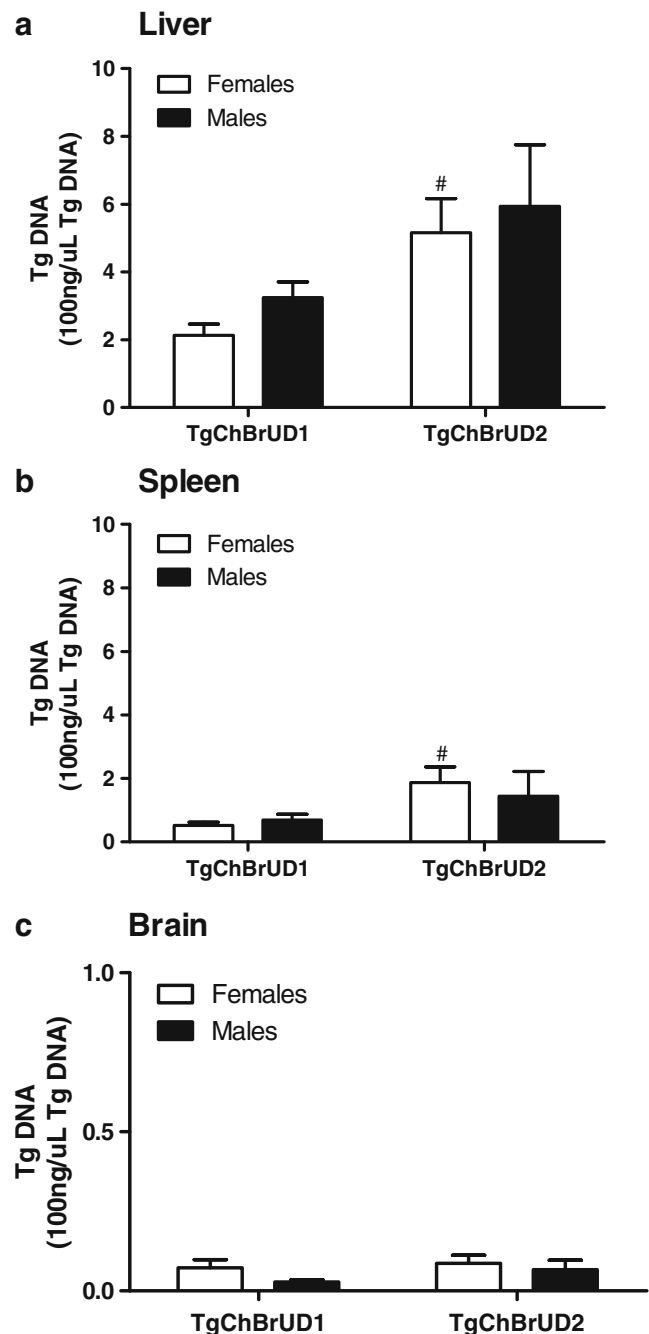
Fig. 4 Parasite burden in the liver (**a**), spleen (**b**), and brain (**c**) tissues from *C. callosus* infected with the TgChBrUD1 or TgChBrUD2 strain of *T. gondii*. Animals (six per group) were infected with 100 tachyzoites of the TgChBrUD1 or TgChBrUD2 strain, euthanized at 8-day post-inoculation and infected tissues were analyzed by real-time PCR. *$P<0.05$ (Mann–Whitney test)

host genetic background, and parasite strain (Munoz et al. 2011; Dubremetz and Lebrun 2012).

In the present study, we observed that *C. callosus* are susceptible to both TgChBrUD1 and TgChBrUD2 atypical strains, but significant differences in the severity of infection were observed between males and females. Our data showed
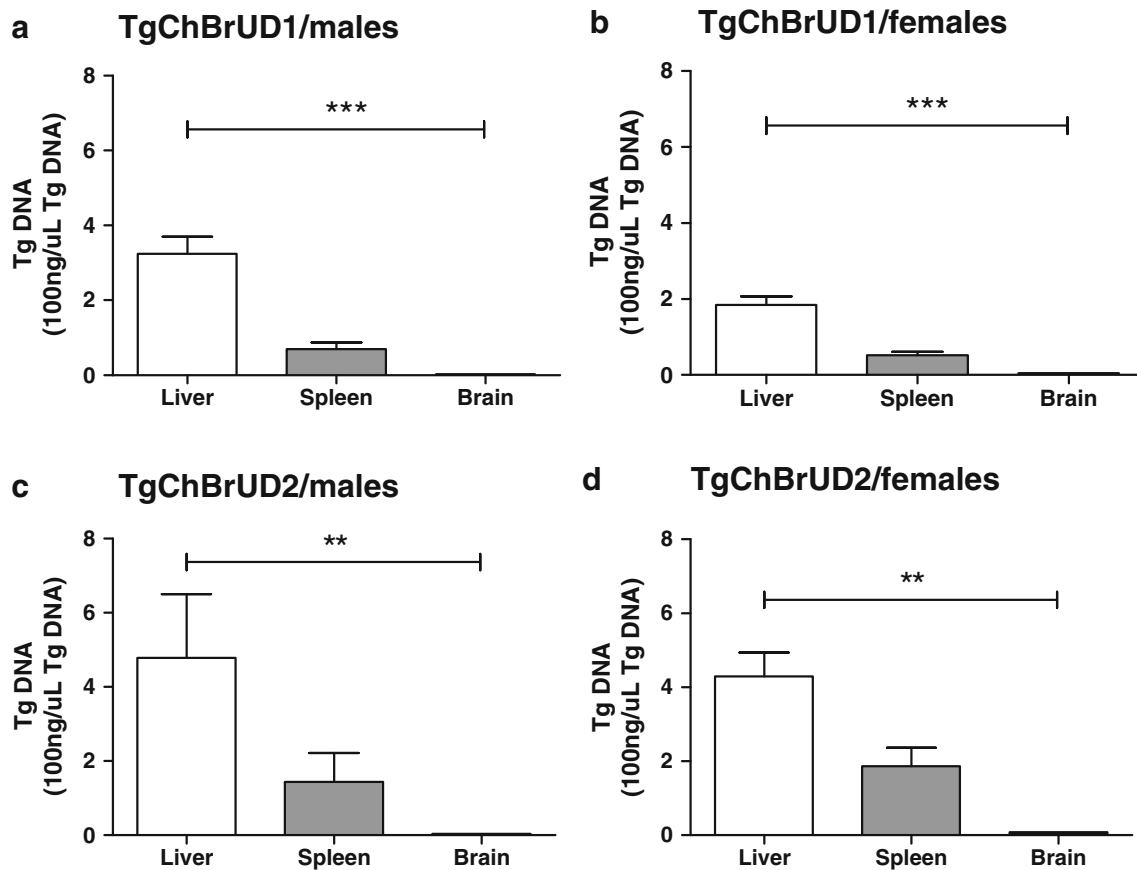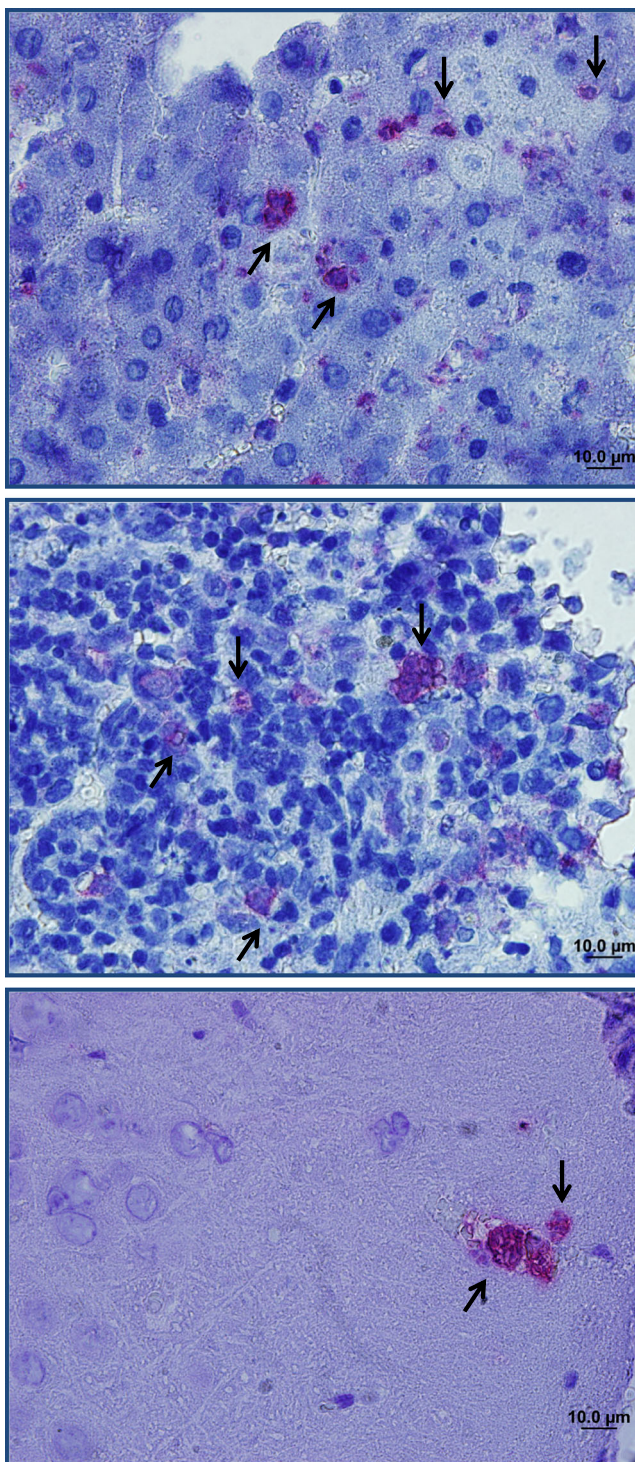
Fig. 5 Comparative parasite burden in the liver, spleen, and brain tissues from *C. callosus* infected with the TgChBrUD1 or TgChBrUD2 strain of *T. gondii*. Animals (six per group) were infected with 100 tachyzoites of the TgChBrUD1 or TgChBrUD2 strain, euthanized at 8-day post- inoculation and infected tissues were analyzed by real-time PCR. **a** TgChBrUD1-infected males; **b** TgChBrUD1-infected females; **c** TgChBrUD2-infected males; **d** TgChBrUD2-infected females. *$P<0.05$ (Kruskal–Wallis test)

that infected males and females died during the acute phase, between 9 and 13 days p.i. with either strain. Furthermore, the presence of tachyzoites in peritoneal fluids, weight loss, and several clinical signs were observed after infection. In mice, the acute phase of infection with proliferating tachyzoites lasts approximately 14 to 21 days when infected with intermediate or avirulent strains. In this case, the tachyzoites proliferation is controlled by the host immune response, and a small subpop- ulation that converts to bradyzoites, forming tissue cysts in the musculature and central nervous system, which results in a lifelong chronic infection (Munoz et al. 2011). This is true for *C. callosus* that are susceptible to infection by *Toxoplasma gondii* clonal RH strain, but are resistant to ME-49 clonal strain with formation of tissue cysts (Favoreto-Junior et al. 1998; Pereira et al. 1999). In addition, there are several num- bers of diverse genotypes of *Toxoplasma gondii* that may be associated with severe infections (Beck et al. 2009). Previous studies analyzed the virulence of *Toxoplasma gondii* strains obtained from domestic animals in the states of Minas Gerais and São Paulo in Brazil, and showed higher prevalence of samples with high/intermediate virulence phenotype (Brandão et al. 2006; Pena et al. 2008). Recently, a study reported high

genotypic diversity in parasites isolated from newborns with congenital toxoplasmosis in Brazil (Carneiro et al. 2013).

It is well known that sex steroids regulate a variety of functions such as growth, reproduction, and differentiation. More recently, evidence has accumulated that gender may also play an important role in susceptibility to infectious diseases (de Souza et al. 2001; Roberts et al. 2001; Nava-Castro et al. 2012). The ability of a parasite to differentially affect a female or a male of the same species (sexual dimorphism of an infection) can be due to the regulation of the immune response by sex hormones (Roberts et al. 1995; Walker et al. 1997; do Prado et al. 1997, 1999). Sex hormones can alter susceptibility to parasitic infection by enhancing or suppressing the immune system or by influencing the growth and development of the parasite in the host (Roberts et al. 2001). Many parasites change the concentration of steroid hormones in infected hosts, including *Toxoplasma gondii* (Flegr et al. 2008; Duneau and Ebert 2012). There is abundant evidence that sex steroid hormones affect the course of toxoplasmosis in humans and mice. Studies suggest that sex hormones such as estrogen can influence macrophage activity and modulate IFN-γ levels (Chao et al. 1994). A study with mice showed

Fig. 6 Representative photomicrographs of liver, spleen, and brain tissues from *C. callosus* infected with the TgChBrUD1 or TgChBrUD2 strain of *T. gondii*. *Arrows* indicate parasites inside the parasitophorous vacuoles. Animals (six per group) were infected with 100 tachyzoites of the TgChBrUD1 or TgChBrUD2 strain, euthanized at 8-day post-inoculation and infected tissues were analyzed by immunohistochemical assays. **a** Liver, **b** spleen, and **c** brain tissues from TgChBrUD2-infected females

that infected females die significantly earlier than males, an effect associated with greater number of tachyzoites and

necrosis in small intestine of females. In contrast, testosterone treatment of female mice reduced intestinal parasite numbers and pathology (Liesenfeld et al. 2001). It was also observed that a decrease occurred in testosterone levels for both male and female mice compared with uninfected animals, indicating that the infection induced a change in testosterone metabolism (Kankova et al. 2011). Interestingly, the differences according to gender are dependent on the parasites considered. Studies with *C. callosus* infected with *Trypanosoma cruzi* showed that sexual dimorphism seemed to predict the fate of infection, since males always displayed the highest percentage of positivity. These findings suggest that testosterone can influence the immune response of *C. callosus*, reflecting an enhanced susceptibility of males to *Trypanosoma cruzi* infection (Lourenço et al. 2008). Therefore, orchiectomized *C. callosus* displayed a lesser number of blood parasites when compared to its sham and control counterparts, indicating that steroid gonadal ablation actually influences the immune response, which led animals to become more resistant against *Trypanosoma cruzi* infection (Pinto et al. 2010).

In the present study, mortality of *C. callosus* females was earlier than males when infected with TgChBrUD1, but it was similar in males and females after TgChBrUD2 infection. However, the TgChBrUD2 infection in males caused lower survival than that induced by TgChBrUD1. These findings show that the gender and the strain type influence the infection outcome in *C. callosus*, with the TgChBrUD1 strain more virulent to females and the TgChBrUD2 strain more virulent to males. Moreover, males had weight loss independently of the strain type, even though it was more pronounced with the TgChBrUD1 strain. In contrast, TgChBrUD1-infected females had weight gain whereas those infected with TgChBrUD2 had weight loss. These findings show that the TgChBrUD2 strain induces weight loss in both males and females, but the TgChBrUD1 strain causes opposite effects in body weight according to the *C. callosus* gender. When morbidity scores were evaluated, the TgChBrUD1 strain induced higher morbidity in females than males, whereas the TgChBrUD2 strain caused higher morbidity in males than females. Also, the TgChBrUD1 strain induced higher morbidity than TgChBrUD2 in both males and females. Again, these data indicate a strong dependence of the parasite strain and the gender on the severity of infection in *C. callosus*, with the TgChBrUD1 strain more severe to females and the TgChBrUD2 strain more severe to males. The analysis of tissue parasitism showed higher parasite number in liver and spleen from females infected with TgChBrUD2 than TgChBrUD1, but with no significant difference between genders or strains when males were infected. These findings indicate that the tissue parasitism in the acute phase is influenced by the gender in *C. callosus*, notably with the TgChBrUD2 strain in females.

In conclusion, *C. callosus* specimens are susceptible to both atypical *Toxoplasma gondii* strains with significant

differences between males and females in severity of infection. These findings open new perspectives to understand different aspects of *Toxoplasma* gondii infection in *C. callosus*, including reinfection and vertical transmission with these atypical strains in this experimental model.

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