Instituto de Biociências

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu

PG-BGA

Resposta imunológica de cordeiros às infecções artificiais por *Haemonchus contortus* e *Haemonchus placei*

Michelle Cardoso dos Santos

Dissertação apresentada ao Instituto de Biociências, Câmpus de Botucatu, UNESP, para obtenção do título de Mestre no Programa de Pós-Graduação em Biologia Geral e Aplicada, Área de concentração Biologia de Parasitas e Microorganismos.

Prof. Titular Dr. Alessandro Francisco Talamini do Amarante
Orientador

BOTUCATU – SP

2013

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Palavras-chave: *Haemonchus contortus*; *Haemonchus placei*; Ovinos; Resposta imunológica.

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Capítulo 2

Lista de abreviaturas

Ac - anticorpo

BCR – Receptor de células B

Ch - Challenge

CP – Crude Protein

D - desafio

ELISA - Enzyme-Linked Immunosorbent Assay

EPG – Eggs per gram of feces

Faostat - Food and agriculture organization of the United Nations

GIN – Gastrointestinal nematodes

Hc – *Haemonchus contortus*

Hp – *Haemonchus placei*

Ig - imunoglobulina

IL – Interleucina

Is – infecção seriada

L3 – larva de terceiro estágio (infectante)

L4 – larva de quarto estágio

L5 – larva de quinto estágio

MHC – Complexo de histocompatibilidade principal

MMC – Mucosal mast cell

NRC – National Research Council

NK – Natural Killer

OPG - Ovos por grama de fezes

PAMP – Padrão molecular associado a patógenos

PBS– Phosphate buffered saline

Pc – Post challenge

PCV – Packed cell volume

Si – Serial infection

Th – Linfócito T helper

TTP – Total plasma protein

RESUMO

A proteção conferida pela resposta imunológica de cordeiros, às infecções seriadas com *Haemonchus contortus* (Hc) e *Haemonchus placei* (Hp) e desafios, por infecções homólogas e heterólogas, com ambas as espécies, foi avaliada. Quarenta e dois cordeiros, com peso corporal inicial médio de 21,3 kg, foram divididos em sete grupos experimentais. No primeiro grupo, 12 cordeiros receberam infecções seriadas (Is) 12 vezes (três vezes por semana) durante quatro semanas, com 500 larvas infectantes (L3) de *H. placei* e foram posteriormente desafiados (D), em dose única, com 4000 L3 de *H. placei* (Grupo IsHp+DHp, n=6) ou com 4000 L3 de *H. contortus* (Grupo IsHp+DHc, n=6). Os animais do segundo grupo (n=12) foram infectados com *H. contortus* da mesma maneira que o grupo 1 (500 L3, três vezes por semana) e foram desafiados com 4000 L3 de *H. contortus* (Grupo IsHc+DHc, n=6) ou com 4000 L3 de *H. placei* (Grupo IsHc+DHp, n=6). O terceiro grupo foi somente desafiado com 4000 L3 de *H. placei* (Grupo DHp, n=6), 4000 L3 de *H. contortus* (Grupo DHc, n=6), ou permaneceram livres de infecções durante todo o período experimental (Grupo Controle, n=6). Análises hematológicas e contagem de ovos por grama de fezes (OPG) foram realizadas semanalmente, o plasma sanguíneo foi armazenado para a realização de análises imunológicas (teste de ELISA). Após a eutanásia dos animais os parasitas foram recuperados, bem como tecido e muco abomasal. Animais que receberam infecções homólogas por *H. placei* (Grupo IsHp+DHp) apresentaram intensa resposta imune com elevados níveis de imunoglobulinas anti-parasita e número de células inflamatórias na mucosa do abomaso. Em adição, esse grupo (IsHp+DHp) apresentou a menor taxa de estabelecimento de parasitas (2,68% de 4000 L3). Estes resultados podem ser associados às infecções em série, que estimularam uma forte resposta imunológica contra a espécie *H. placei*, pois esta apresenta menor adaptabilidade aos ovinos. O oposto foi observado nos animais que receberam somente a infecção desafio com *H. placei* (DHp), apresentando elevada taxa de estabelecimento (25,3%). Contudo, nos

animais que receberam infecções heterólogas, infectados seriadamente com *H. placei* e posteriormente desafiados com *H. contortus* (Grupo IsHp+DHc), a proteção significativa não foi evidenciada (estabelecimento de 19,18%). A infecção em série por *H. contortus*, espécie adaptada aos ovinos, conferiu proteção reduzida contra a infecção desafio, em comparação a infecção por *H.placei*. No grupo que recebeu infecções homólogas por *H. contortus* (IsHc+DHc) houve menor recuperação de parasitas em comparação ao grupo somente desafiado com a mesma espécie (DHc), em média 409 e 914 parasitas, respectivamente. Desta maneira, as infecções seriadas por *H. contortus* conferiram uma proteção de 44,8%. Os cordeiros infectados seriadamente com *H. contortus* não apresentaram forte resposta imunológica contra a infecção, sendo esta observada nos animais infectados em série com *H. placei*, evidenciando a sua baixa adaptabilidade aos ovinos.

Palavras-chaves: especificidade, imunidade, helmintos, ovinos.

ABSTRACT

The protection conffered by serial infections with *Haemonchus contortus* (Hc) and *Haemonchus placei* (Hp) was evaluated in lambs. Forty two lambs, with initial average body weight of the 21.3 kg, were divided in three experimental groups. In the first group, 12 lambs was serially infected (Si) 12 times (three times a week) for four weeks, with 500 infective larvae (L3) of *H. placei* and then challenged (Ch), in a single dose, with 4000 L3 of *H. placei* (Group SiHp+ChHp, n=6) or with 4000 L3 of *H. contortus* (Group SiHp+ChHc, n=6). The animals of the second group (n=12) were infected with *H. contortus,* in the same way that group 1 (500 L3, three times a week) and then challenged with 4000 L3 of *H. contortus* (Group SiHc+ChHc, n=6) or with 4000 L3 of *H. placei* (Group SiHc+ChHp, n=6). A third group of lambs was single challenged with 4000 L3 of *H. placei* (Group ChHp, n=6), 4000 L3 of *H. contortus* (Group ChHc, n=6), or remained uninfected throughout the trial period (Group Control, n=6). Haematological and fecal eggs counts (FEC) occurred weekely, the blood plasma was stored to immunological exams (ELISA test). After the animals sacrifice, the worms were recoverd, as well as, abomasal tissue and mucus. Animals that received homologous infection with *H. placei* (Group SiHp+ChHp) presented the most intense immune response and number of inflammatory cells in the abomasal mucosa. In adition, this group (SiHp+ChHp) presented the lowest rate of parasite establishment (2.68% of the 4000 L3). These results can be associated to serial infection that stimulated to strong immune respose against *H. placei*, which presents lower adaptability to sheep. The opposite was observed in animals that only received the challenge infection, with *H. placei* (group ChHp), in which the rate of establishment was relatively high (25.3%). However, the animals that received heterologous infection, previously infected serially with *H. placei* and then challenged with *H. contortus* (Group SiHp+ChHc), there was no evidence of significant protection (establishment of 19.18%). The serial infection with *H. contortus*, specie most adapted to sheep, conferred

reduced protection against the challenge compared to *H. placei* infection. The group that received homologous infection with *H. contortus* (SiHc+ChHc) showed lower parasite recovery in comparison to group only challenged with the same specie (ChHc), average 409 and 914 worms, respectively. Thus, the serial infection with *H. contortus* afforded a protection of 44.8%. Lambs serially infected with *H. placei* presented a strong immune response against the parasite, showing its poor adaptation to sheep.

Keywords: helminth, immunity, specificity, sheep.

Capítulo 1

1. Considerações iniciais

No Brasil a produção de carne ovina não é suficiente para suprir a demanda, mesmo com o crescimento do rebanho ao longo dos anos.

No ano de 2010, o rebanho ovino brasileiro era composto por aproximadamente 17,4 milhões de cabeças, com a produção de carne ovina de aproximadamente 81 mil toneladas (Faostat, 2010). Contudo, a produção nacional não é suficiente para atender a demanda do mercado consumidor, assim, faz-se necessária a importação de carne ovina, especialmente do Uruguai, a qual é produzida com menor custo, o que a torna altamente competitiva.

O reduzido índice de produtividade do rebanho ovino no país, em sua maioria, pode ser associado às infecções parasitárias, principalmente decorrente do nematódeo gastrintestinal da espécie *Haemonchus contortus,* devido à sua alta patogenicidade e prevalência no país (Amarante et al., 2009). Visando minimizar perdas no rebanho, tratamentos com anti-helmínticos foram utilizados indiscriminadamente ao longo dos anos, favorecendo o desenvolvimento de populações resistentes às drogas comerciais (Almeida et al., 2010).

Devido a esse fator, estudos tornam-se necessários para a compreensão da biologia, da especificidade do parasita e da influência da resposta imunológica do animal na relação parasita - hospedeiro.

2. Biologia do parasita

Parasitas do gênero *Haemonchus* apresentam ciclo de vida direto, não necessitam de hospedeiro intermediário. Possuem uma fase de vida livre, que ocorre no ambiente e sofre influência direta dos fatores ambientais, e uma fase parasitária, que ocorre no interior do hospedeiro e sua manutenção é influenciada pela resposta imunológica gerada pelo hospedeiro contra a infecção (Oliveira-Sequeira & Amarante, 2001).

A fase de vida livre consiste na eclosão dos ovos, eliminados junto às fezes, e no desenvolvimento da larva até o estágio infectante (terceiro estágio, L3), este desenvolvimento compreende uma série de etapas e processos, sofrendo influência constante das condições climáticas e do microclima da pastagem (O'Connor et al., 2006; Santos et al., 2012). Em adição, a ocorrência de chuvas estimulam o desenvolvimento e a migração das L3 presentes nas fezes para a planta forrageira, e consequentemente, a contaminação dos animais durante o pastejo (Lima, 1998; Rocha et al., 2008; Silva et al, 2008; Santos et al., 2012).

Após a ingestão, as L3 sofrem duas mudas no trato gastrintestinal do hospedeiro, para larva de quarto estágio (L4) e larva de quinto estágio (L5), adulto, e se diferenciam em machos e fêmeas. Os parasitas adultos (L5) são encontrados no abomaso e, após a cópula, uma fêmea de *Haemonchus* spp. libera junto às fezes aproximadamente 5000 ovos/dia (Le Jambre, 1995).

A elevada patogenicidade do gênero *Haemonchus* é associada ao hábito hematófago, promovendo um decréscimo no desempenho produtivo e/ou reprodutivo dos animais, anemia e, em casos extremos, pode levar o hospedeiro a óbito. As principais categorias acometidas pelo parasita são os animais jovens e as fêmeas no período do periparto, devido a menor eficiência de sua reposta imunológica (Gennari et al., 1991; Nishi et al., 2002; Rocha et al., 2004, 2005).

2.1. Especificidade do parasita

Parasitas do gênero *Haemonchus* são encontrados em um conjunto diverso de mamíferos ungulados (Artiodactyla) incluindo 46 espécies de hospedeiros da família Camelidae e da Infraordem Pecora, abrangendo principalmente as famílias Antilocapridae, Giraffidae, Cervídae e Bovidae. São considerados “cosmopolitas” e foram introduzidos nas Américas com os ruminantes que chegaram junto com os colonizadores europeus (Hoberg et al., 2004).

As espécies de *Haemonchus* spp. diferem morfologicamente entre si, as L3 de *H. contortus* e *Haemonchus placei* são identificadas a partir da distância entre a extremidade posterior da larva e o final da cauda da bainha (Fig. 1): os valores médios são de 73,6 µm em *H. contortus* e 99,2 µm em *H. placei* (Santiago, 1968).

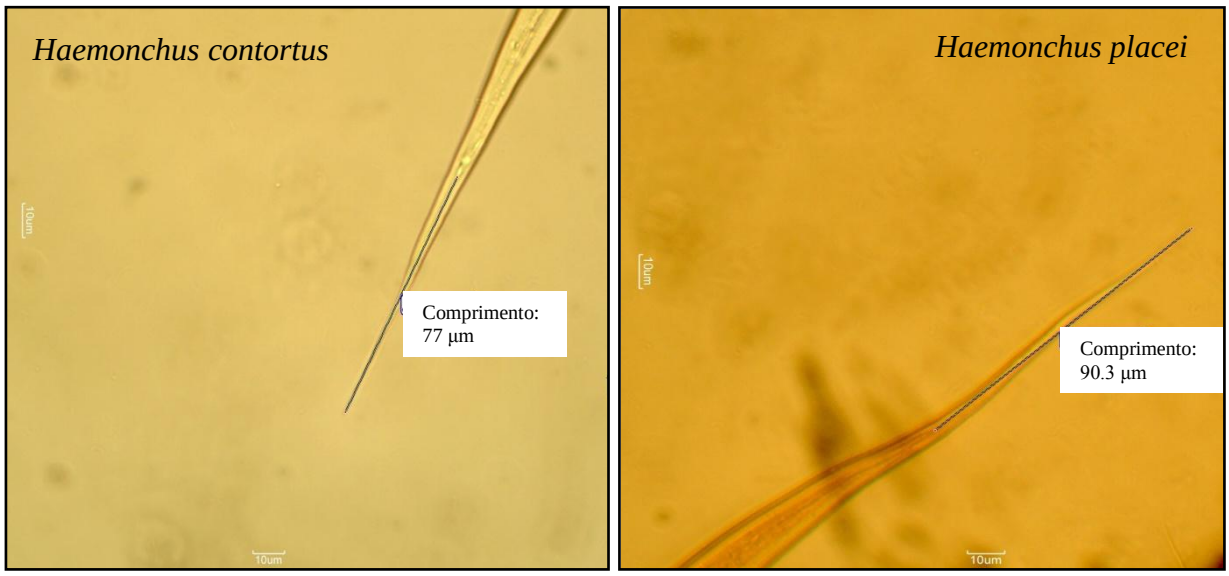
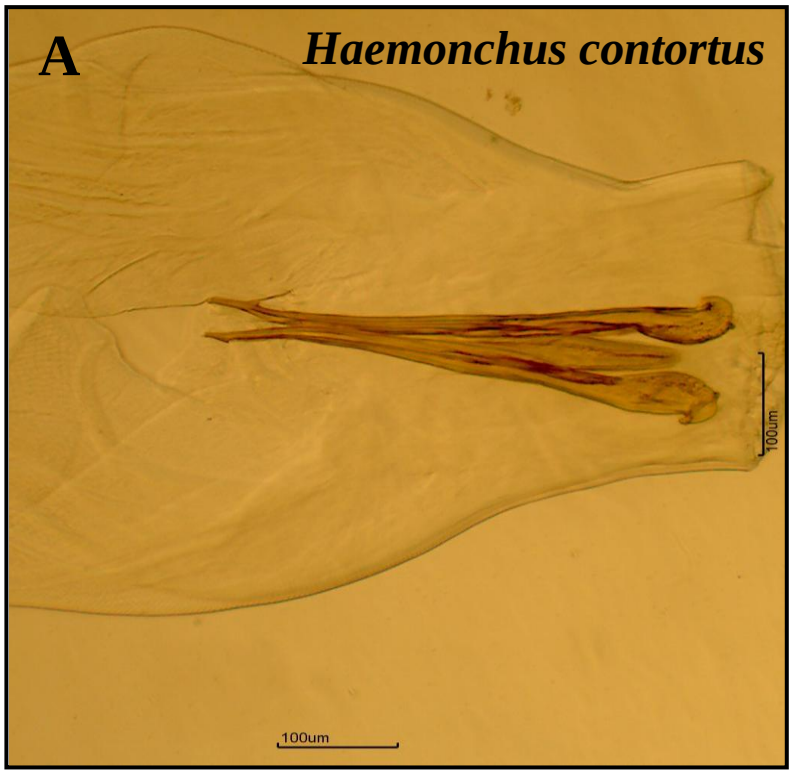


Figura 1. A distância entre a extremidade posterior da larva e o final da cauda da bainha de larvas infectantes (L3) de *Haemonchus contortus* e *Haemonchus placei* são diferentes (aumento 40X). Foto: M.C. Santos, 2011.

Para diferenciar os machos adultos mensura-se o comprimento dos seus espículos e de seus ganchos (Fig. 2 e 3), situados na bolsa copuladora, que usualmente apresentam maiores dimensões em *H. placei* (Amarante et al., 1997; Achi et al., 2003).



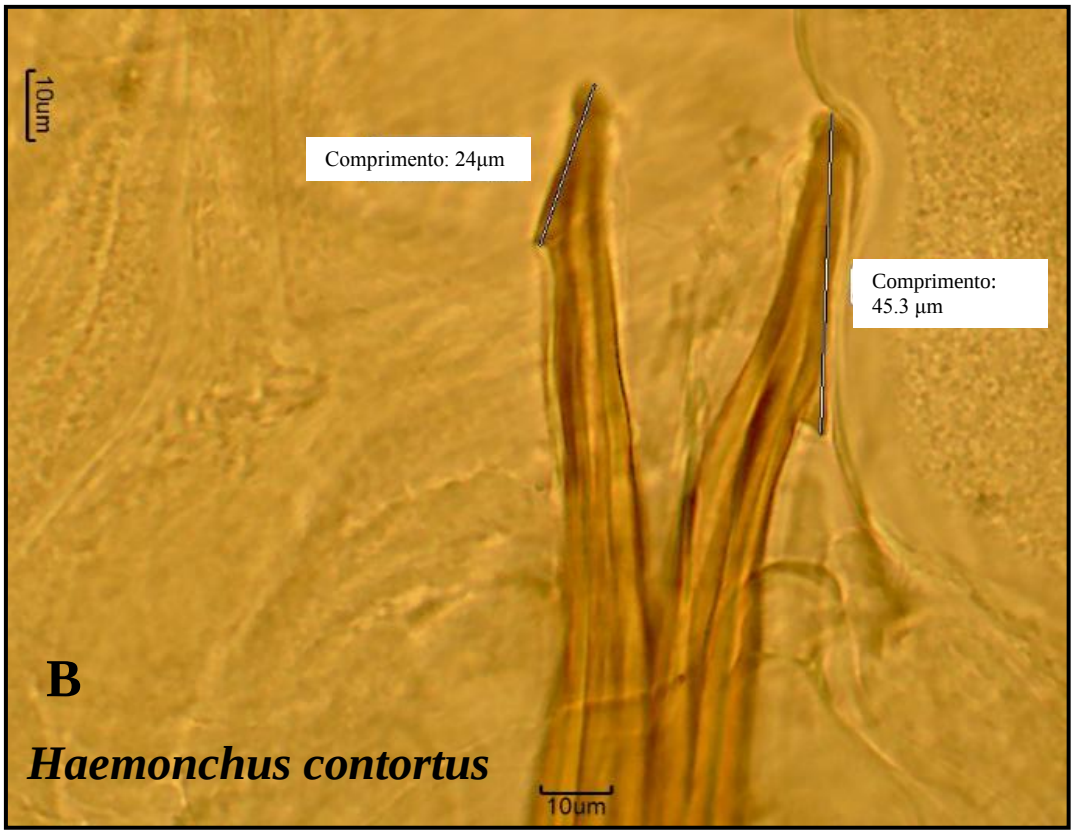
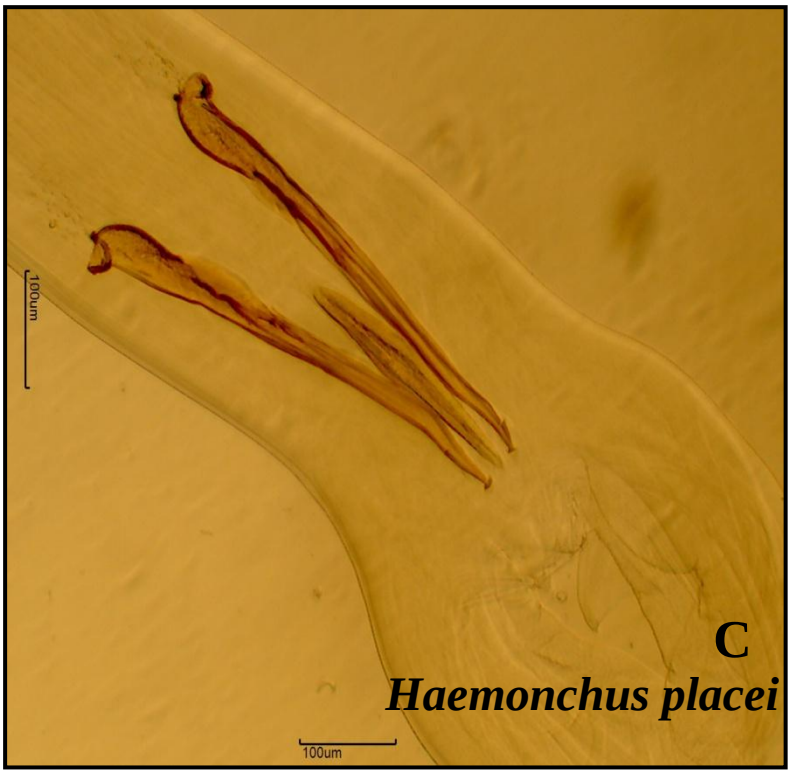


Figura 2. Bolsa copuladora de *Haemonchus contortus* com espículos (A), em aumento de 10X, e seus ganchos (B), em aumento de 40X. Foto: M.C. Santos, 2013.



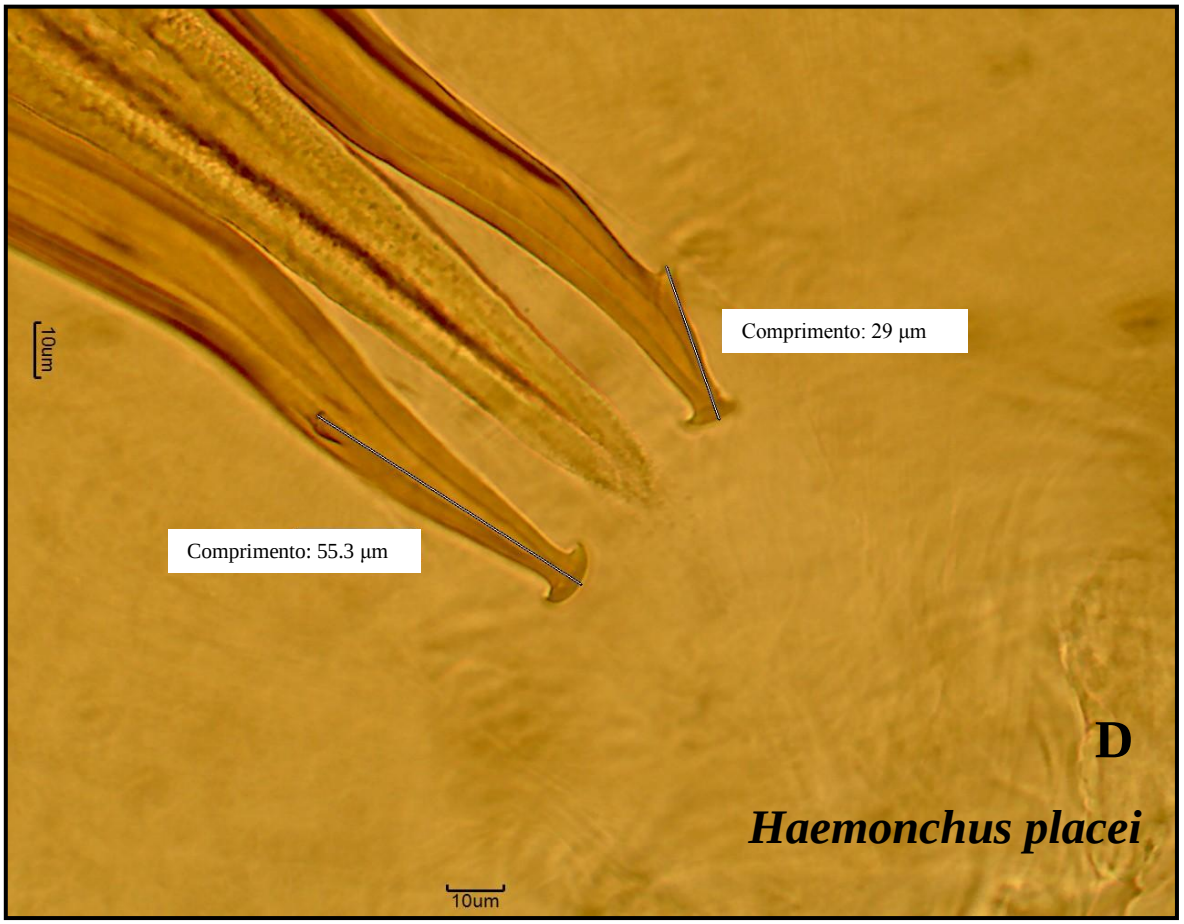


Figura 3. Bolsa copuladora de *Haemonchus placei* com espículos (C), em aumento de 10X, e seus ganchos (D), em aumento de 40X. Foto: M.C. Santos, 2013.

A prevalência de *H. contortus* e a ocorrência de infecções mistas por *H. contortus* e *H. placei* em ovinos foram relatadas em diversos estudos, quando estes animais compartilham pastagens com outros ruminantes. Na África ocidental, em pastagens comumente compartilhadas por dromedários, ovinos, caprinos e zebuínos, 56% do total de exemplares de *H. placei* foram encontrados em ovinos e somente 10% em bovinos zebuínos. Porém, os principais hospedeiros de *H. contortus* foram os pequenos ruminantes (Jacquiet et al., 1998).

Achi et al. (2003), na Costa do Marfim, também observaram que as infecções de *H. contortus* foram específicas para ovinos e caprinos (96,1% e 89,4%, respectivamente) e, em adição, 21,4% dos ovinos apresentaram infecções mistas por *H. placei* + *H. contortus*. Neste mesmo estudo, os autores observaram que a espécie *H. placei* é mais bem-sucedida em *Bos taurus* enquanto *Haemonchus similis* é mais abundante em *Bos indicus* (zebu), com 73,9% de *H. placei* recuperados de taurinos e de 38,6% de *H. similis* recuperados de zebuínos.

Hoberg et al. (2004), ao realizarem análises filogenéticas de 25 caracteres morfológicos e estabelecerem uma lista com 12 espécies válidas de *Haemonchus.* Nesta lista, *H. placei* e *H. similis* foram classificados como parasitas de bovinos, enquanto *H. contortus*

foi classificado como parasita de caprinos e ovinos. Estes resultados corroboram com os dados obtidos por de Khalafalla et al. (2011), no Egito, que ao ao avaliar ovinos abatidos em frigorífico, de janeiro à dezembro de 1999, obtiveram percentual de *H. contortus* superior em relação a *H. placei* (3,5% e 1,7%, respectivamente).

No Brasil, observou-se que as infecções cruzadas, entre nematódeos gastrintestinais de bovinos e ovinos, podem ocorrer quando esses animais compartilham a mesma pastagem, no entanto, com o decorrer do tempo, as duas espécies de hospedeiros aparentemente eliminaram as espécies que não estão bem adaptados. No caso, *H. placei* é mais adaptado a bovinos e *H. contortus* a ovinos (Amarante et al., 1997, Rocha et al., 2008).

Bezerros infectados artificialmente com *H. contortus* e *H. placei* apresentaram menor taxa de estabelecimento para *H. contortus* do que para a infecção com *H. placei* (Bassetto et al., 2011).

Em um estudo recente, em fase final de desenvolvimento (Jorge K. Xavier, processo FAPESP n. 2011/09779-5), observou-se que o estabelecimento da infecção artificial por *H. placei* em ovinos foi possível, com a eliminação de ovos por um prolongado período (Fig. 4).

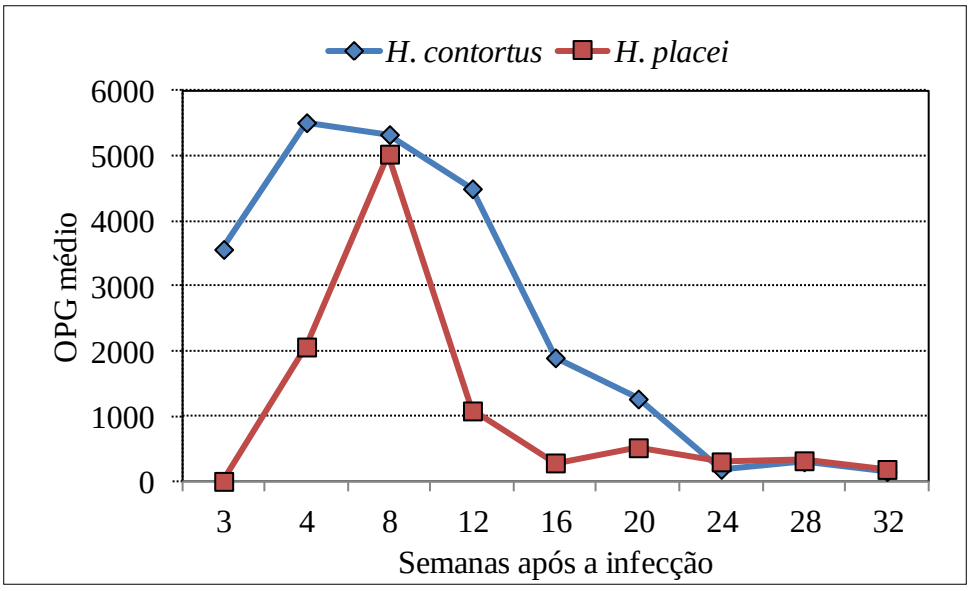


Figura 4. Número médio de ovos por grama de fezes (OPG) após infecção de cordeiros com 4000 L3 de *Haemonchus contortus* ou *Haemonchus placei* (Fonte: Jorge K. Xavier, projeto em andamento, processo FAPESP n. 2011/09779-5).

3. Resposta imunológica

As infecções helmínticas e a resposta imunológica, correspondente do hospedeiro, são produtos de uma prolongada relação co-evolucionária entre o hospedeiro e o parasita (Anthony et al., 2007).

Ao parasita é vantajoso ludibriar o hospedeiro induzindo-o a desenvolver uma resposta imune ineficiente, buscando um nicho adequado para maturação e propagação, sem matar ou prejudicar o hospedeiro. Reciprocamente, o hospedeiro tem por ideal gerar uma resposta imune eficaz para expulsar o parasita, minimizar seus efeitos nocivos, enquanto não sacrifica sua capacidade de elaborar resposta contra outros patógenos (Anthony et al., 2007).

Após o parasita se alojar na mucosa do hospedeiro, um mecanismo efetivo inicial importante no controle da carga parasitária, e nos fenômenos de autocura, é a inflamação da mucosa (Wakelin, 1978). A inflamação é desencadeada quando o organismo percebe que está sendo atacado, desta maneira, o organismo utiliza de células sentinelas, como por exemplo, mastócitos, macrófagos e as células dendríticas que são ativados quando padrões moleculares associados a patógenos (PAMPs) ou alarminas se ligam a seus receptores. Em resposta eles sintetizam e secretam citocinas e outras moléculas, as quais desencadeiam a inflamação enquanto inicia a ativação da imunidade adquirida (Tizard, 2008).

3.1. Mastócitos

Os mastócitos e os eosinófilos são as principais células efetoras tipicamente associadas com infecções helmínticas (Balic et al., 2000). Os mastócitos possuem citoplasma recoberto por grânulos (lissosomas secretores) e podem localizar-se no tecido conjuntivo, na pele e nas mucosas. E, quando presentes nas mucosas, respondem especificamente à invasão por vermes (Tizard, 2008).

Na sua desgranulação os mastócitos liberam moléculas inflamatórias, presentes nos grânulos, sendo que um dos mecanismos envolvidos nessa etapa está relacionado à imunoglubulina E (Ig E) e antígeno (Tizard, 2008).

3.2. Eosinofilia

Após as infecções por helmintos, o número de eosinófilos pode aumentar dramaticamente (= eosinofilia) no sangue e tecidos, sua liberação da medula óssea é estimulada por eotaxinas, interleucina 5 (IL-5) e quimiocinas, produzidas pelos linfócitos T

helper 2 (Th2) e mastócitos. Os eosinófilos são atraídos aos locais de desgranulação dos mastócitos e ativados, aumentando a sua habilidade para destruir parasitas (Balic et al., 2000; Tizard, 2008).

Estudos *in vitro* demonstram a importância dos eosinófilos no combate aos parasitas e, em muitos casos, os eosinófilos agem mais efetivamente contra os estágios larvais, necessitando da cooperação dos anticorpos, e/ou sistema complemento, para maior eficácia (Meeusen & Balic, 2000).

In vivo os eosinófilos podem danificar e, provavelmente, matar as L3 de *H. contortus* em ovinos infectados artificialmente, contudo, a presença dos eosinófilos no tecido, por si só, não é suficiente e depende da interação com outros fatores microambientais, como a ação de mastócitos intra-epiteliais e IL-4 (Balic et al., 2006).

Terefe et al. (2007), ao avaliarem o potencial de ação dos eosinófilo *in vitro*, observaram que os eosinófilos promoveram redução drástica na motilidade das L3 incubadas com os eosinófilos extraídos do sangue de ovinos infectados artificialmente com *H. contortus*. Após a incubação, as larvas foram transferidas intra-abomaso e a sua maioria não se estabeleceu no hospedeiro.

3.3. Resposta imune e formação de anticorpos

Dois tipos de resposta imonológica são estimuladas no combate à infecção parasitária. A imunidade inata (presente desde o nascimento do indivíduo) envolve células dendríticas, neutrófilos, macrófagos e células *natural killer* (NK), desenvolve-se rapidamente (minutos e/ou horas após o contato) e não apresenta memória, assim, a sua eficiência não é aumentada ao longo do tempo, após reinfecções (Tizard, 2008).

A resposta imune adquirida (ao longo do tempo) possui a capacidade de reconhecer e responder a uma vasta gama de moléculas estranhas, denominadas antígenos, desenvolve-se lentamente (dias e/ou semanas), apresenta memória e assim, possui maior eficiência após reinfecções (Tizard, 2008). Esta resposta é mediada por linfócitos, os quais são denominados de acordo com seu local de maturação. A maturação dos linfócitos T ocorre no Timo enquanto os linfócitos B maturam na bursa de Fabricius, tecido linfóide gastrintestinal ou medula óssea. Após a maturação, os linfócitos são armazenados nos órgãos linfóides secundários: linfonodos, baço, medula óssea e placas de Peyer (Tizard, 2008).

Os linfócitos T atuam em dois tipos de resposta, sendo a resposta do tipo T helper 1 (Th1) tipicamente associada às infecções microbianas, incluindo bactérias e fungos. Em

adição, a proteção contra helmintos é referida como resposta do tipo T helper 2 (Th2), incluindo componentes inatos e adaptativos, sendo caracterizada por elevação dos níveis de citocinas IL-4, IL-5, IL-9, IL13 e IL-21, eosinófilos, mastócitos, basófilos, hiperplasia das células globulares e produção imunoglobulinas (Ig). Contudo, o mecanismo de eliminação dos vermes difere para cada espécie de parasita (Anthony et al., 2007; Maizels et al., 2009; Saddiqi et al., 2011).

Outra classe de citocina com importante papel e associada à resposta tipo Th2 é a IL-17 que pode promover a diferenciação das células Th2 e expulsão do nematódeo (Anthony et al., 2007).

Os linfócitos B, quando encontram um antígeno, podem-se ligar a seus receptores para serem co-estimulados e são eficazes células apresentadoras de antígenos. Após a ligação do antígeno, os receptores de célula B (BCRs) podem ser internalizados, e degradados, ou transportado a um compartimento intracelular, onde recém-sintetizadas moléculas de classe II, do complexo de histocompatibilidade principal (MHC), junto aos fragmentos de antígenos interagem e formam complexos. Desta forma, os linfócitos T são ativados e co-estimulam o linfócito B, permitindo sua total ativação (Tizard, 2008).

Quando liberados nos fluídos corpóreos os BCRs são denominados anticorpos (Ac) ou Ig. Outra fonte de imunoglobulinas são os plasmócitos, uma progênie do linfócito B modificado para secretar quantidades imensas de imunoglobulinas (Tizard, 2008).

As imunoglobulinas diferem em tamanho, carga elétrica, aminoácidos e carboidratos. As principais classes de imunoglobulinas são: IgA, IgD, IgE, IgG e IgM. As respostas de IgA são tipicamente associadas às infecções por nematódeos gastrointestinais, entretanto, são proeminentemente observadas nos locais de infecção das mucosas (Balic et al., 2000; Tizard, 2008).

Em ovelhas, foi observado aumento na proliferação de linfócitos secretores de IgA, Ig1, Ig2 e IgM no abomaso dos animais infectados com *H. contortus* em comparação com o grupo controle (Gill et al., 1992). Em adição, nos cordeiros parasitados os níveis de IgA anti-*Haemonchus* na mucosa abomasal foram inversamente proporcionais a carga parasitária e OPG, indicando que a resposta imunológica pode prejudicar o desenvolvimento e/ou a fecundidade do parasita (Amarante et al., 2005; Silva, 2010).

Em bezerros artificialmente infectados com *H. placei*, observou-se redução significativa no OPG após a segunda infecção artificial e a manutenção dos níveis séricos de IgG foram elevados nos animais parasitados (Nishi et al., 2002).

3.4. Proteção contra o estabelecimento e mecanismo de expulsão dos helmintos

O parasita utiliza-se de mecanismos para ludibriar a resposta imunológica do hospedeiro, visando a sua sobrevivência. Parasitas no quarto estágio (L4) podem adotar de um mecanismo de defesa denominado hipobiose (desenvolvimento interrompido) e esse fenômeno pode ser induzido com o aumento da resistência do hospedeiro (Balic et al., 2000).

Entretanto, quando a resposta imunológica contra os parasitos adultos é eficaz pode-se observar a redução no crescimento, na fecundidade das fêmeas e de mudanças na morfologia do parasita, bem como, a expulsão da população de nematódeos pelo hospedeiro. A expulsão do parasita torna-se mais eficiente em função da imunidade adquirida, em consequência de repetidas infecções do hospedeiro ao longo de sua vida (Miller, 1984; Balic et. al., 2000).

Estímulos constantes leves (durante o hábito de pastejo) podem contribuir com o desenvolvimento da imunidade do animal, esta também pode ser estimulada artificialmente, como por exemplo, cordeiros infectados seriadamente (durante 15 semanas), apresentaram nas semanas iniciais (1ª e 4ª semana) elevada taxa de estabelecimento da infecção (45% das larvas administradas), porém, ao longo do tempo houve um decréscimo gradativo na taxa de estabelecimento dos parasitas (Barger et al., 1985).

Cordeiros primariamente infectados com 200 L3/kg peso vivo (PV) de *H. contortus* e desafiados com o dobro da dose, apresentaram resposta protetora contra a infecção desafio (Gómez-Muñoz et al., 1999). Corroborando com Emery et al. (2000), ao avaliarem cordeiros neonatos imunizados desde o nascimento, durante quatro semanas, observou-se o desenvolvimento de proteção significativa, contra a infecção desafio, na sexta e sétima semana após o nascimento.

Porém, ovinos não apresentaram redução na taxa de estabelecimento dos parasitas e alteração na fecundidade das fêmeas após a administração (em dose única) de diferentes quantidades de L3 de *H. contortus* (Barger & Le Jambre, 1988; Coyne et al., 1991).

4. Desempenho do hospedeiro parasitado

A resposta imune eficiente, contra infecções helmínticas, gera um custo ao metabolismo do animal. Estima-se que a manutenção da imunidade contra nematódeos gastrintestinais em ovinos implica em perdas de 15% na produtividade (Greer, 2008).

Ovelhas em pastejo, tratadas com anti-helmíntico e infectadas artificialmente com 3000 L3 de *H. contortus*, ao longo de 12 semanas, apresentram redução média de 1,5 kg no peso final, em comparação ao grupo controle não infectado (Barger & Cox, 1984).

Contudo, no rebanho deve-se considerar a resposta imunológica de cada indivíduo, pois dentro de uma raça específica alguns animais, relativamente resistentes e/ou tolerantes, apresentam a resposta imune generalizada e local mais eficiente, quando comparados com animais susceptíveis (Windon, 1996; Saddiqi et al., 2011).

No Brasil, ovelhas pertencentes ao mesmo rebanho foram divididas em dois grupos: resistentes e susceptíveis às infecções parasitárias, mantidas em pastagens sob a mesma condição de pastejo, observou-se que as ovelhas resistentes apresentaram peso superior, ao final do experimento, com diferença média de 6 kg entre os grupos (Bassetto et al., 2009).

Em adição, a resistência parasitária é mais comum em animais adaptados ao meio em que vivem, como por exemplo, raças locais, que se caracterizam por apresentarem grande rusticidade, são consideradas mais resistentes à verminose em comparação aos animais de raças exóticas (Windon, 1996). Assim, a resposta imune eficiente pode ser associada como característica de algumas raças ovinas (Amarante & Amarante, 2003).

No Brasil, destacam-se animais da raça Crioula Lanada (proveniente da região sul do país) e Morada Nova e Santa Inês (raças originárias do nordeste do Brasil). Ao longo dos anos, a raça Santa Inês foi amplamente divulgada e na atualidade é facilmente encontrada em todo o país (Amarante et al., 2004; Bricarello et al., 2004).

Animais da Santa Inês apresentam menor susceptibilidade à infecção por nematódeos. Necessitando de menor número de tratamento com anti-helmínticos, esse fator pode ser associado com a maior efetividade da resposta imune, quando comparada com a resposta imunológica gerada por animais de raças comerciais importadas de outros continentes (Amarante et al., 2004). Contudo, os ovinos da raça Santa Inês apresentam potencial produtivo reduzido, não sendo uma raça economicamente atraente. Já as raças comerciais apresentam elevado potencial produtivo, porém, são altamente susceptíveis às infecções por *H. contortus* (Bueno et al., 2002; Amarante et al., 2004; Rocha et al., 2004, 2005; Bricarello et al., 2005; Silva et al., 2012).

Raças resistentes às infecções helmínticas podem apresentar ganho médio de peso e peso ao abate superior, em relação aos animais de raças susceptíveis, quando criados sob a mesma condição de pastejo. Como por exemplo, no Brasil, ovinos das raças Crioula Lanada e Santa Inês, raça resistente às infecções parasitárias, apresentaram pesos superiores em comparação aos animais das raças Corriedale e Ile de France (Bricarello et al., 2004; Rocha et al., 2005).

No entanto, a resistência conferida pela raça pode ser alterada a partir da dieta oferecida aos animais. Dietas ricas em proteína podem contribuir com a resposta imunológica gerada pelos animais, em resposta a infecção parasitária, proporcionando desempenho satisfatório de raças susceptíveis, apesar de albergarem parasitas. Esse fato foi observado por Silva et al.(2012) em cordeiros criados em pastagens e suplementados, com 3% do peso vivo/dia, com ração comercial (18% de proteína bruta). Os cordeiros da raça Ile de France apresentaram ganho em peso e peso de carcaça quente superior (200g/dia e 16,6 kg (± 0,94), respectivamente), em comparação aos animais da raça Santa Inês (154 g/dia e 11,4 kg (± 0,66), respectivamente). Entretanto, os animais da raça Ile de France apresentaram maiores contagens de OPG e carga parasitária (Silva et al., 2012).

Durante o hábito hematófago os parasitas abomasais podem lesionar a mucosa e, ao reparar a lesão, o organismo utiliza as proteínas da dieta que usualmente seriam destinadas a manutenção, desenvolvimento e reprodução do animal. Além desses fatores, a proteína da dieta pode ser desviada para contribuir com a resposta imune, pois muitos componentes do sistema imunológico, como por exemplo, imunoglobulinas, citocinas e proteases liberadas pelos mastócitos celulares são proteínas *in natura* (Houdijk et al., 2001).

Desta maneira, os ovinos parasitados necessitam de uma quantidade extra de proteína metabolizável, que será destinada para o reparo ou substituição dos tecidos danificados, bem como, para expressar uma resposta imune mais eficiente (Rocha et al., 2011).

5. Considerações finais

A resposta imune produzida pelo hospedeiro após o contato com o parasita é complexa. Vários fatores estão relacionados para uma resposta imunológica eficiente, como por exemplo, genética, categoria do animal, nutrição e fatores intrínsecos do hospedeiro. Em um rebanho, são observadas variações na susceptibilidade de diferentes indivíduos, assim como, o tipo de resposta que será induzida contra o parasitismo, como por exemplo, a resposta do tipo Th1 (em animais susceptíveis às infecções helmínticas) ou, do tipo Th2 (nos animais resistentes) mais eficiente, com consequente eliminação do parasita.

Medidas corretas de manejo e suplementação podem contribuir para a resposta mais eficiente do hospedeiro. Os animais parasitados e suplementados com dieta rica em proteína podem apresentar resposta imunológica adequada, bem como, desempenho produtivo satisfatório com desempenho similiar ao de animais mantidos livres de parasitas.

Para o combate das infecções parasitárias não existe uma fórmula única a ser adotada por todos os tipos de sistemas de produção de ovinos.

Deve-se ressaltar que as ações preventivas, como manejo sanitário e nutricional adequados em associação à utilização de raças adaptadas às condições ambientais, bem como o cruzamento de raças resistentes e susceptíveis, contribuirão no aumento da produtividade, viabilizando economicamente a atividade.

O Capítulo 2 da dissertação intitulado “Immune response to *Haemonchus contortus* and *Haemonchus placei* in young sheep and its role on parasite-host specificity” teve por objetivo avaliar a resposta imune de cordeiros às infecções artificiais, homológas e heterólogas, por parasitas das espécies *Haemonchus contortus* e *Haemonchus placei*.

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Capítulo 2

Immune response to *Haemonchus contortus* and *Haemonchus placei* in young sheep and its role on parasite-host specificity[a]

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Abstract

In this trial, we aimed to evaluate the immune response elicited by *Haemonchus contortus* and *Haemonchus placei* in lambs in order to assess whether the serial infections with both species confer protection against homologous or heterologous challenge. One group of lambs (n=12) was serially infected 12 times (three times a week; on Mondays, Wednesdays and Fridays) for four weeks, with 500 infective larvae (L3) of *H. placei* and then challenged with *H. placei* (n=6) or with *H. contortus* (n=6); lambs of a second group (n=12) were serially infected 12 times with 500 L3 of *H. contortus* and then challenged with *H. contortus* (n=6) or with *H. placei* (n=6); and a third group of lambs was single challenged with *H. placei* (n=6), *H. contortus* (n=6), or remained uninfected throughout the trial period (Group Control, n=6). Animals serially infected with *H. placei* and then challenged with the same species presented the most intense immune response with the highest levels of anti-parasitic immunoglobulin and number of inflammatory cells in the abomasal mucosa. As a result, this group presented the lowest rate of parasite establishment (2.68% of the 4000 L3). Interestingly, the same did not occur in animals single challenged with *H. placei*, in which the rate of establishment was relatively high (25.3%), confirming that protective immune response to *H. placei* develops only when animals are repeatedly infected with this species. However, when the animals were previously infected serially with *H. placei* and then challenged with *H. contortus*, there was no evidence of significant protection (establishment of 19.18%). In comparison with *H. contortus*, strong immune response to *H. placei* occurs in lambs after serial infections, showing its poor adaptation to sheep.

Keywords: epidemiology, infection, inflammatory cells, nematode, resistance, ruminant.

1. Introduction

Haemonchus species are among the most important parasites of domestic ruminants from Tropical and Sub-Tropical areas worldwide. They are blood-sucking parasites that cause decrease in animal performance and, in extreme cases, death, particularly in young animals and periparturient females, which are more susceptible to infections.

Species of *Haemonchus* have complex histories with respect to host and geographic associations. According to Hoberg et al. (2004), biogeography and host distribution seem to be compatible with an African origin for *Haemonchus*, basal diversification driven by colonization among grazing and browsing antelopes with limited cospeciation and, subsequently, a complex history of host-switching to the Caprinae, Bovinae, Camelidae, and Giraffidae, and among other pecorans involving both core and satellite associations. The influence of domestic ruminants on the now cosmopolitan distributions for some species of *Haemonchus* is also likely. Only the species associated with domesticated Caprini and Bovini have distributions in the Nearctic and Neotropics and occur in satellite association among cervids and camelids.

In Brazil, there is strong evidence that *Haemonchus placei* and *Haemonchus similis* are well adapted to parasitism in cattle, while *Haemonchus contortus* is well adapted to small ruminants. Even when cattle and sheep share pastures, cross infections are rarely observed in the field (Amarante et al., 1997; Rocha et al., 2008). Similarly, in the tropics of the Caribbean islands (French West Indies), *H. similis* is the major parasite in bovine and *H. contortus* is the most important in small ruminants (Giudici et al., 1999), and larvae of *H. contortus* obtained from goats did not successfully establish after experimental infection in heifers (d'Alexis et al., 2012). In Africa, in the North Côte d'Ivoire, *H. contortus* is the main species in sheep and goats. However, about 10% of the worms recovered from goats belong to *H. placei* species. In cattle, *H. contortus* was very rare, while *H. placei* was the dominant species in zebu cattle and

taurine cattle; nevertheless, the proportion of *H. similis* was higher in zebu cattle than in taurine cattle (Achi et al., 2003).

However, the same does not occur in some areas of the world where *Haemonchus* species present a more generalist behavior regarding host specificity. A recent report from the USA showed a population of *H. contortus* very well adapted to cattle occurring in mixed infection with *H. placei* (Gasbarre et al., 2009). Under field condition, in the Sahelian areas of Mauritania, West Africa, mixed congeneric infections were frequent and the most striking finding was the role played by small ruminants in the survival strategy of *H. placei* under adverse climatic conditions: from the total of *H. placei,* 56% were found in sheep; 34% in goats, and only 10% in zebu cattle (Jacquiet et al., 1998). Therefore, the importance of the cross-infection in the maintenance of the different species in a delimited geographical area appears to be variable and it is a consequence of the adaptation of the parasite to survive in adverse conditions. Depending on the environmental conditions and on the availability of different species of ruminants sharing pastures, *Haemonchus* species may exhibit more generalist or more specialist behavior regarding host specificity.

It has been shown that *H. contortus* exhibits considerable ecological and biological plasticity to overcome unfavorable conditions, both in the external and host environments. The parasites could have either become more cold tolerant to the development and survival of the free-living stages, which would explain the apparent increasing importance of this parasite in the temperate climate countries of Europe, and/or developed special survival mechanisms of the parasitic stages within the host, to ensure between-year survival (reviewed by Waller et al., 2004).

Gastrointestinal nematode (GIN) infections stimulate the establishment of protective Th2 immune response, which plays an important role on the protection against such parasites in sheep (Andronicos et al., 2010). However, for parasites, it is advantageous to trick the host

into developing ineffective immune response, to find a suitable niche for maturation and propagation, and to do so without killing or unduly harming the host. Conversely, the host has to ideally generate effective immune response to expel the parasite and minimize its harmful effects (Anthony et al., 2007). *H. contortus* undergo several developmental stages within their mammalian host, each presenting different antigenic challenges to the immune system. There are distinct time points of immune activation correlating with the different life cycle stages of the parasite within the host tissue, comprising (i) early activation (within 1–2 days) corresponding to infection with the L3 stage, (ii) a second peak of activation (day 5–7 post challenged (pc)) corresponding to the rapid growth of the L4 blood feeding stage, and (iii) a down-regulation of immune activation during the chronic adult infection stage (Robinson et al., 2011). The IL-4 and IL-13 mRNA levels in the abomasal lymph node were up-regulated 14-fold and 30-fold, respectively, seven days after infection of naïve Nellore calves with *H. placei*, in comparison to a non–infected control group, evidencing the important role of an early Th2 polarization (Ibelli et al., 2011). In sheep infected with *H. contortus*, an early and significant rise in IL-4 mRNA levels was observed by day 1 pc, peaking on day 2 pc. Th2 cytokines, such as IL-4 and IL-3, induce the production of anti-parasite immunoglobulin (IgG, IgM, IgA and IgE) and also mast cell hyperplasia and eosinophilia (Robinson et al., 2011).

Rapid rejection or immune exclusion of challenge larvae is a well-recognized phenomenon in sheep hypersensitized by repeated infection with *H. contortus*. Mast cells and globule leukocytes present in the gastrointestinal mucosa are typically associated with this rapid rejection response. Carbohydrate binding proteins or lectins (intelectins and galectins) also form part of the immune response to GIN infection. Galectins constitute a large proportion of eosinophil protein and are released abundantly at sites of helminth infections, suggesting that eosinophils and their products may play a hitherto unrecognized role in the rapid rejection response of *H. contortus* infective larvae (Kemp et al., 2009). Galectin-11 is

involved in the changing of the viscosity and adhesiveness of gastrointestinal mucus. Such changes in the physiochemical properties of mucus are associated with the trapping and expulsion of *H. contortus* (Robinson et al., 2011).

Thereby, the aim of this trial was to study the immune response elicited by *H. contortus* and *H. placei* in lambs in order to assess whether the serial infections with both species confer protection against homologous or heterologous challenge. We also investigated the role played by immune response on parasite-host specificity.

2. Materials and methods

The assay was carried out making use 42 lambs with initial mean body weight of 21.3 kg, obtained from crosses of purebred Santa Ines sires with crossbred Santa Ines ewes. All of the lambs were acquired just after weaning (at 2–3 months of age) from a commercial farm located in Bofete, State of Sao Paulo. The animals were placed in pens with concrete floor in the facilities for small ruminants of the University.

After their arrival, fecal examination showed that 32 of the 42 animals were shedding nematode eggs, with mean of 597 eggs per gram of feces (EPG). *Haemonchus* spp. and *Trichostrongylus* spp. third stage larvae were found in the fecal cultures, identified according to the descriptions by Ueno & Gonçalves (1998). Thence, the animals were treated with albendazole (15 mg/kg, Valbazen®, Pfizer) and levamisole (10 mg/kg, Ripercol®, Fort Dodge) orally for three consecutive days, followed by treatment with trichlorfon (100 mg/kg, Neguvon® - Bayer) on single dose. Then, a series of fecal examination was performed to confirm the elimination of infection by nematodes.

The experiment reported was approved and conducted in accordance with the experimental protocol approved by the local Ethical Committee (protocol number 274-CEEA).

2.1. Experimental groups

The summary of the experimental design and the infection protocol used are presented in Figure 1. The distribution of the lambs in the experimental groups was based on their body weight. The serial infections (Si) were administered over a period of four weeks. One week after the last infection, all animals were treated with levamisole (10 mg/kg, Ripercol®, Fort Dodge). Fecal examinations confirmed that the infections had been eliminated from all animals after the anthelmintic treatment. Three days after treatment, the animals were artificially challenged with single doses of 4000 L3 of *H. placei* or *H. contortus*, except those from the group Control (Fig. 1).

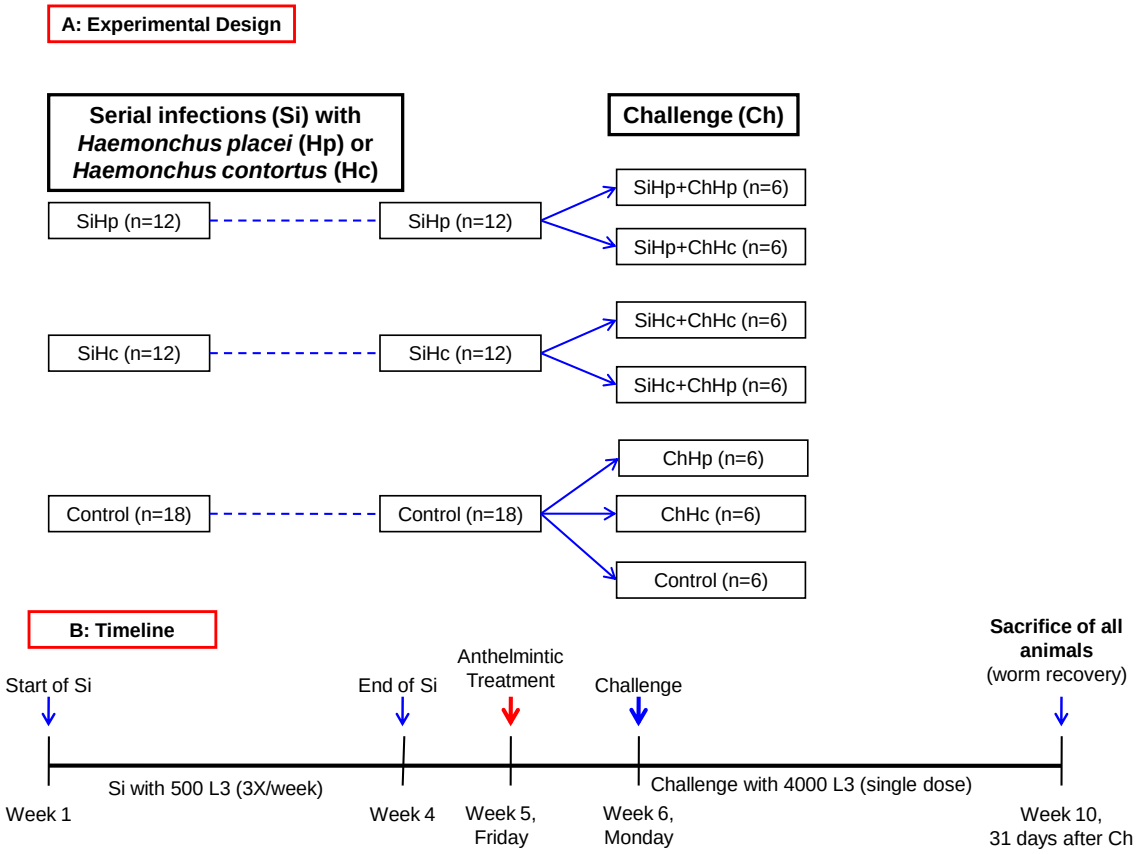

Figure 1. Experimental design: One group of lambs was serially infected (Si) 12 times (three times per week; on Monday, Wednesday and Friday), for four weeks, with 500 L3 of *H. placei* (Hp) and then challenged (Ch) with Hp (Group SiHp+ChHp) or with *H. contortus* (Group SiHp+ChHc); lambs of a second group were serially infected 12 times with 500 L3 of *H. contortus* (Hc) and then challenged with Hc (Group SiHc+ChHc) or with Hp (Group SiHc+ChHp); and the last group was single challenged with *H. placei* (Group ChHp), *H. contortus* (Group ChHc), or remained uninfected throughout the trial (Group Control). n = number of animals per group.

2.2. Production of infective larvae of Haemonchus placei and Haemonchus contortus

The infective larvae (L3) of *H. placei* and *H. contortus* that were used to infect donor animals had been preserved in liquid nitrogen and were both levamisole-susceptible. Both *Haemonchus* species had been previously identified based on the morphology of spicules of adult males (Achi et al., 2003).

Two male lambs, approximately six months old, were infected with *H. contortus* and used as donors for the production of L3 of this species, whereas two male calves, eight months old, were the donors of *H. placei*. The donor animals were kept indoors and before being infected, they received albendazole (15 mg/kg, Valbazen®, Pfizer) and levamisole (10 mg/kg, Ripercol®, Fort Dodge) orally for three consecutive days in order to eliminate any natural nematode infection. Their worm-free status was confirmed before the infection by a series of fecal examinations. They were kept in separate pens to avoid cross-contamination and, during the trial, they had free access to tap water and hay grass (*Cynodon dactylon* cv. Tifton 85), purchased from a farm with no ruminants, avoiding risks of food contamination by nematode infective larvae.

Each donor lamb was artificially infected, orally, with 10,000 L3 of *H. contortus* in a single dose, while each donor calf received 20,000 L3 of *H. placei*.

Fecal cultures from the feces of these donor animals (calves and sheep) were performed separately for the production of L3. All infective larvae were stored in distilled water and were <30 days old when used to infect the trial lambs.

2.3. Diet and health management

The experimental animals remained indoors and the concrete floor was washed every other day to minimize the risk of parasitic infection. The animals received decoquinate (Deccox® - Alpharma) according to the manufacturer's recommendations throughout the trial

period to prevent coccidiosis. They were vaccinated against clostridiosis (Sintoxan T Polivalente®, Merial S.A.) at the beginning of the experiment.

Lambs were fed with ground hay, Tifton 85 (*Cynodon* sp. cv.), and a commercial concentrate (Suplementa Ovino Campo® - Presence) in the proportion of 60 : 40 (concentrate : hay). The total diet, corresponding to 4.5% of their body weight, was calculated to provide average weight gain of 200 g/day (NRC, 2007); it contained 12.8% crude protein (CP), dry matter (DM) basis. The lambs had free access to tap water and mineral salt (Presensefós® - Presence).

2.4. Laboratory analysis

Fecal and blood samples were collected weekly. Additional samples were collected at the end of the serial infections (day 25), on day of anthelmintic treatment (day 32), and at the end of the study (day 65). All animals were euthanized 31 days after challenge for recovery and enumeration of parasites.

2.4.1. Fecal examination

Fecal samples were collected directly from the rectum of animals for nematode fecal egg count (FEC) determination. The FEC values were determined using a modified technique in which each nematode egg counted represented 100 EPG (Ueno & Gonçalves, 1998). Every week, fecal cultures for the production of L3 of gastrointestinal nematodes were prepared separately for each group. The larvae obtained were killed, stained with Lugol's iodine, and identified according to the descriptions by Ueno & Gonçalves (1998).

2.4.2. Hematology

Blood samples (5 mL) were collected by jugular vein puncture into Vacutainer tubes containing anti-coagulant (EDTA). Packed cell volume (PCV) was determined by microhematocrit centrifugation and total plasma protein levels (TPP) (g/dL) were estimated using a refractometer (Refractometer SPR-N, Atago). Plasma samples were stored at −20 °C until immunoglobulin measurement (ELISA).

Eosinophil counts in peripheral blood were made in a Neubauer's chamber after staining with Carpentier's solution (Dawkins et al., 1989); counts were expressed as the number of cells per μL of blood.

2.4.3. Enzyme-linked immunosorbent assay (ELISA)

The plasma levels of IgG and IgA antibodies against total antigen of third stage larvae (L3) and adult of *H. contortus* were estimated by ELISA Test. In addition, IgA levels in abomasal mucus were measured. The production of antigens of L3 and adults of *H. contortus* was previously described by Amarante et al. (2009).

The protocol used to measure the parasite-specific IgA levels in abomasal mucus and the parasite-specific plasma IgG and IgA levels was described by Silva et al. (2012). The only differences were in the dilutions used in the IgA analysis: plasma samples were diluted in PBS-GT (1:2) and rabbit anti-sheep IgA peroxidase conjugated was diluted at 1:5000 in the present trial. The protein concentration in the mucus samples was adjusted to 408.33 μg of protein/mL, diluted in PBS-GT (1:2).

2.4.4. Worm counts

The animals fasted for 12 hours before being sacrificed. The abomasum was opened along the curvature and its material was placed in a graduated beaker, washed with saline

solution so that the parasites adhered to the mucosa could be detached from the organ. The volume of 1 L was completed with saline solution. It was homogenized and divided in two containers of 500 ml each. One of the containers was immediately frozen for preservation of parasites and 5% formalin was added to the other container.

Worm identification and counting procedures were performed in the frozen material (50% of the content) as described by Ueno and Gonçalves (1998). The sample preserved with 5% formalin was also examined for the animals whose frozen material presented no worms.

2.4.5. Histology

Tissue samples taken from the fundic region of the abomasum were fixed in 4% buffered formaldehyde for 48 h. After that, the samples were dehydrated with alcohol and embedded in paraffin wax. Sections, 3 μm thick, were stained with giemsa or haematoxylin and eosin (H&E).

Mast cells were counted in the sections stained with giemsa and eosinophils, and globule leukocytes were counted in the sections stained with H&E. Cells were enumerated under a 10x eyepiece containing a calibrated graticule and 100x objective lens viewing an area of 0.01 mm^2. Inflammatory cells were enumerated in 30 fields of the abomasal mucosa, randomly selected, per animal. The counts were expressed as number of cells per mm^2 of mucosa.

2.5. Statistical Analysis

The responses were analyzed by one-way analysis of variance for the variables measured just once and by analysis of variance for repeated measures for the variables measured at several time points (IgG and IgA in plasma, EPG, blood eosinophils, PCV and TPP) using a Statistical Analysis System, version 9.2 (SAS Institute, Inc., Cary, NC, USA).

IgG, IgA, EPG, blood eosinophils and worm burden data were transformed to log 10(x + 1) prior to analysis. Values of $p < 0.05$ were considered statistically significant. Means were compared by Student's t-test at 5% significance level. Means are presented in results as arithmetic means (± standard error) of untransformed data.

3. Results

3.1. Fecal examination and worm counts

Most of the animals serially infected with *H. contortus* (9 out of 12) showed patency of the infection, i.e., eggs shed in feaces at 21 days after the beginning of the serial infections, while lambs serially infected with *H. placei* showed patency later, on day 32 after the beginning of the infections, when only three of the 12 animals shed eggs. The animals from the Control group, which did not receive serial or challenge infection, did not shed eggs in feaces throughout the trial.

The highest EPG means were displayed by lambs infected with *H. contortus*, regardless of the type of infection, either serial or challenge (Fig. 2). In contrast, infections with *H. placei* resulted in low EPG values. During the serial infection period (Fig. 2-A), the group infected with *H. contortus* presented significantly higher EPG means from day 21 up to day 32, differing from group serially infected with *H. placei* ($P<0.05$).

After challenge (Fig. 2-B), the groups infected with *H. contortus* (SiHp+ChHc and ChHc) presented EPG means significantly different from those of the groups SiHp+ChHp and SiHc+ChHp on day 63. At that moment, the group challenged only with *H. contortus* (ChHc) presented mean significantly higher ($P<0.05$) than the group previously infected with the same species (SiHc+ChHc).

The group SiHp+ChHp presented the lowest worm burden mean, which differed ($P<0.05$) from the groups ChHp, ChHc, and SiHp+ChHc (Table 1).

One animal from the group SiHp+ChHp and one from group SiHc+ChHp did not harbor any parasites, while one lamb from the group SiHc+ChHc had just one female specimen of *Haemonchus.* The lamb from the group SiHp+ChHp did not shed eggs in any occasion, while the other two animals (one from SiHc+ChHc and one from SiHc+ChHp) passed eggs during the serial infections, but not after challenge.

The parasite establishment rates were higher in the groups that received only the challenge infection (4000 L3), i.e., groups not infected serially. The mean values were 25.28% in the group ChHp and 22.85% in ChHc. The groups SiHp+ChHc and SiHc+ChHp that received the heterologous infection showed rates of 19.18% and 15.75%, respectively. The lower establishment rate was presented by the groups that received homologous challenge after being previously serially infected: 2.68% in SiHp+ChHp and 10.23% in SiHc+ChHc.

3.2. Hematology

Lower values of PCV were observed in the groups infected with *Haemonchus* in comparison with the uninfected control group. However, no clinical signs of haemonchosis, such as anemia or hypoproteinemia were observed. There was significant interaction between Time*Group ($P<0.0001$) during the serial infections with regards to PCV results (Fig. 3-A). In general, there was a progressive reduction in the PCV values of animals infected with either *H. contortus* or *H. placei*, while the uninfected lambs (Control group) presented mean PCV values between 33.7% and 37.4% during the serial infections. The lambs that received serial infections with *H. placei* showed lower values than the *H. contortus* group throughout the serial infection period, with significant differences between groups on days 0, 14 and 21 ($P<0.05$). The *H. placei* group presented means significantly lower than the control group on

days 21, 25 and 32. Significant difference between the control group and the *H. contortus* infected animals occurred only on day 14.

After challenge (Fig. 3-B), there was significant interaction between Time*Group ($P<0.05$). The uninfected control group remained with the highest PCV means (35.5-37.2%), while the group SiHp+ChHc presented the lowest values with the minimum PCV mean on day 63 (29.2%). However, this group showed means significantly lower than the control group only on the last two sampling days (63 and 65), and the same occurred with the group ChHp. In addition, the groups SiHp+ChHp and ChHc presented means significantly lower than the group Control on day 65.

The levels of TPP remained within the normal range in all groups during the serial infections (Fig. 4-A). During this period, the group *H. placei* presented values significantly ($P<0.05$) higher than the group Control on days 25 and 28. After challenge (Fig. 4-B), there were no significant differences between group means ($P>0.05$).

In relation to blood eosinophils, significant increase in the averages occurred in the groups infected serially with *H. placei* or *H. contortus*, which showed values significantly higher ($P<0.05$) compared to the group Control on days 14, 21, 25, 28 and 32 (Fig. 5-A). After challenge, there was an increase in the averages of circulating eosinophils on the challenged groups one week after infection, followed by a tendency of reduction until the end of the trial (Fig. 5-B). Seven days after challenge (day 42), all groups presented mean eosinophil numbers statistically different from the group Control ($P<0.05$), except the group ChHp.

3.3. Immunology

3.3.1. Plasma IgG against antigens of Haemonchus contortus

The serial infections with *H. placei* induced high production of IgG against L3 antigens, while the same did not occur in animals serially infected with *H. contortus* (Fig. 6-A). The increase of IgG levels started 21 days after the beginning of the serial infections with *H. placei* and persisted until day 32 (when all animals received anthelmintic). During this period (21 to 32), the means of the group infected with *H. placei* were statistically different from those of the group Control. After challenge, IgG levels remained relatively high in the groups SiHp+ChHp and SiHp+ChHc until the end of the trial compared to the other groups (Fig. 6-B). The group SiHp+ChHp presented IgG levels significantly higher ($P<0.05$) than the control group on days 42, 49, 56, 63 and 65. The same occurred with the group SiHp+ChHc, excepting day 42. No other group showed levels significantly higher than the control group, except the group challenged with *H. placei* (ChHp) on day 63. Significant interaction between Time*Group occurred during the serial infections period ($P<0.0001$) and after the challenge period ($P<0.05$).

The clear difference in the production of IgG against L3 observed between groups infected serially with *H. placei* and *H. contortus* did not occur in ELISAs performed with antigens obtained from *H. contortus* adult specimens. During the serial infection period, statistical difference between infected and control groups were the following: the group *H. placei* with higher IgG levels than the group Control on days 14, 21, 25, 28 and 32; and the group *H. contortus* higher than the group Control on days 25, 28 and 32 (Fig. 7-A). After challenge, the groups SiHp+ChHp and SiHp+ChHc presented higher IgG means ($P<0.05$) than the group Control on days 49 and 56 post infection (Fig. 7-B).

3.3.2. Plasma IgA against antigens of Haemonchus contortus

The IgA levels against L3 antigens presented the same trend observed with the IgG levels in the group serially infected with *H. placei.* There was an increase in values starting 21 days after the beginning of the serial infections, which persisted until day 32 (Fig. 8-A). A significant interaction was recorded between Time*Group ($P<0.0001$) and the *H. placei* group means were significantly higher than *H. contortus* and control group means on days 25, 28 and 32 ($P<0.05$). After challenge (Fig. 8-B), on days 42, 49 and 56, the groups SiHp+ChHp, SiHp+ChHc, SiHc+ChHc, and SiHc+ChHp showed significant difference in comparison to group Control ($P<0.05$), except the group SiHc+ChHp on day 42, SiHc+ChHc on day 49, and SiHp+ChHc on day 56. The single challenge groups (ChHp and ChHc) showed increased levels of IgA, which differed from the group Control, only on day 56. A significant interaction was observed between Time*Group after challenge ($P<0.0001$).

The levels of IgA against adult antigens during of the serial infections were higher for the *H. placei* group compared to the *H. contortus* and Control groups (Fig. 9-A). On day 25, the *H. placei* mean was significantly higher than those of the other two groups ($P <0.05$), and on the same day, the *H. contortus* mean was higher than the Control mean. Significant interaction was recorded between Time*Group ($P<0.002$). After challenge, the groups that received previous serial infections (with *H. contortus* or *H. placei*) showed IgA means statistically different from the group Control on day 42 (Fig. 9-B). Significant interaction was observed between Time*Group ($P<0.002$).

3.3.3. IgA against antigens of Haemonchus contortus in the abomasal mucus

The lowest levels of IgA anti-L3 (Fig. 10-A) and anti-Adult (Fig. 10-B) antigens in the abomasal mucus were recorded in the group Control (0.04 ± 0.05 and 0.03 ± 0.01, respectively), while the highest levels were observed in the group SiHp+ChHc (0.50 ± 0.29

and 0.43 ± 0.17, respectively). However, there was no statistical difference between group means.

3.4. Histology

Regarding mucosal mast cells, eosinophils, and globule leukocytes, the group SiHp+ChHp presented the highest numbers of cells per mm^2 of abomasal mucosa (Fig. 11). Means of the infected groups were statistically higher than those of the group Control ($P<0.05$), except for the globule leukocytes group means, which were relatively low in all groups with the highest mean in the SiHp+ChHp group (11 ± 3).

4. Discussion

Animals serially infected with *H. placei* and then challenged with the same species showed the most intense immune response with the highest levels of anti-parasitic immunoglobulin and inflammatory cell numbers in the abomasal mucosa. Consequently, this group presented the lowest rate of parasite establishment. Inflammatory cell numbers in abomasal mucosa and parasite specific IgA were inversely associated with *H. contortus* worm burden and FEC in sheep (Amarante et al., 2005). Increased numbers of eosinophils, both in blood and tissues, are frequently associated with the expression of resistance to nematodes (Dawkins et al., 1989; Balic et al., 2006) and the killing potential of *H. contortus* L3 by eosinophils has been demonstrated *in vitro* (Terefe et al., 2007).

Interestingly, the same did not occur in animals challenged once with *H. placei* (group ChHp), in which the rate of establishment was relatively high (25.3% of the 4000 L3), confirming that protective immune response to *H. placei* only develops when animals are repeatedly infected with this species. Lower rates of establishment of *H. placei* in sheep were

reported by Riggs (2001) after single infection with 4000 L3 of *H. placei* from cattle or sheep origins, of 3.7% and 6.7% respectively.

It is important to mention that in the Elisa tests, we used antigens from *H. contortus* and the high levels of IgG and IgA in plasma in animals infected once with *H. placei* demonstrated that both *Haemonchus* species share common or similar antigens. This fact was demonstrated by vaccination of calves with intestinal membrane glycoproteins from *H. contortus* that conferred substantial protection against *H. placei*, both in terms of reduced egg output and adult worm numbers (Bassetto et al., 2011). However, when animals were previously infected serially with *H. placei* and then challenged with the other species, *H. contortus*, there was no evidence of significant protection. Therefore, this lack of cross-protection was unexpected.

In comparison with *H. placei*, the serial infection with *H. contortus* conferred reduced protection against the homologous challenge infection. The group serially infected with *H. contortus* and then challenged with the same species (SiHc+ChHc) presented 409 worms in average, while the group that received only the single infection with the same species (ChHc) presented 914 worms, corresponding to a protection of 44.8% conferred by the previous serial infection. Using different protocols of serial infections, Emery et al. (2000) also observed similar protection (43% or 56%) in lambs after challenge with *H. contortus*. Possibly, significant protection against *H. contortus* only develops when animals ingest L3s for a longer period of time. Bricarello et al. (2005) observed that progressive decline in FEC of Santa Ines lambs, indicating development of immune response, started only nine weeks after the beginning of the serial infections with 300 *H. contortus* L3 three times weekly and, that the degree of resistance was greatly influenced by the protein content of the diet. Similarly, sheep infected three times weekly for 15 weeks at four rates, ranging from 600 to 4800 L3 per week, presented a peak of *H. contortus* burdens between 6 and 9 weeks of infection. Establishment

of incoming larvae was not influenced by infection rate: it declined from 45% in the first 4 weeks of infection to insignificant levels during the final 6 weeks (Barger et al., 1985).

The animals used in the present trial had been exposed to nematode infection in the farm of origin. This previous contact with the gastrointestinal nematode might have conferred some protection against the experimental infections during the trial. However, the relatively high establishment rate displayed by the groups that received only the single infection demonstrated that this protection did not persist in the absence of continuous income of infective larvae.

The present results indicate the important role of immune response in preventing a successful establishment of *H. placei* in sheep. Le Jambre (1983) suggested that *H. contortus* use a host mediated response in sheep to limit their competitor *H. placei* and that the resulting exclusion and dislodgement of *H. placei* act as a major pre-mating barrier to species hybridization. He observed that established *H. contortus* excluded the establishment of *H. placei*. However, established infections of adult *H. placei* could not exclude *H. contortus*. Furthermore, the exclusion or dislodgement of *H. placei* was abrogated by injecting the host with dexamethasone (Le Jambre, 1983).

Our results demonstrate that, at least in part, the immune response may play an important role in the host-parasite specificity. In comparison with *H. contortus, H. placei* is unable to evade the immune mechanisms of lambs, which are able to recognize the invasion by the “strange” species and, due to constant exposition can activate an effective immune response, thus, the serial infections contributes on efficiency of acquired immunity. It is known that helminth infections and the corresponding host immune response are products of a prolonged dynamic co-evolution between the host and its parasite. Regarding the success of the host-parasite relationship, Dinnen (1963) postulated that the selective pressure provided by the contemporary immune response is likely to be more precise and may favor the survival

of only those variants of the metazoan parasite presenting a sufficiently reduced antigenic disparity with the host. The degree of antigenic disparity may be dependent on the evolutionary selection of genetic components of both the host and the parasite. In addition, a minimum or threshold level of antigenic information would be necessary for the stimulation of immunological response. The role of the immunological response in the "adapted" host-parasite relationship is to control the parasitic burden, rather than to cause complete elimination of the infection.

In Brazil, natural mixed infections may occur when sheep and cattle share pastures, but cross infections between *Haemonchus* species are usually insignificant (Santiago et al., 1975; Amarante et al., 1997, Rocha et al., 2008; Brasil et al., 2012). This aspect can be explained in part by the results obtained in the present study, which demonstrated that sheep repeatedly infected with *H. placei* acquire strong resistance to this parasite. This also explains the extinction of *H. placei* from farms grazed only by sheep in our environment.

Differences in the host specificity of *Haemonchus* species, apparently influenced by the environment, have been reported. In wet tropical areas, where the environmental conditions favor high degrees of infection due to favorable conditions for the development and survival of the free living stages on pastures, there is a high degree of host-parasite specificity, as observed in Brazil (Santiago et al., 1975; Amarante et al., 1997, Rocha et al., 2008; Brasil et al., 2012), Australia (Le Jambre, 1983), French West Indies (Giudici et al., 1999; d'Alexis et al., 2012), and the North Côte d'Ivoire (Achi et al., 2003). Possibly, in these environments, there is a high selective pressure to produce antigenic convergence between the parasite and the host, while in semi-arid environments with low rate of infection, like Mauritania (Jacquiet et al., 1998), a threshold level of antigenic stimulation may not be reached and, in part, would explain hosts harboring mixed infections.

In conclusion, the host-parasite specificity evolution may be influenced by the interaction between the host immune response and the number of incoming larvae, which is greatly influenced by the environment. In wet tropical areas with high level of pasture contamination with infective larvae, parasites apparently tend to be more specialized, while in more adverse conditions they tend to be more generalist, i.e., they are able to establish successfully in different host species. Thus, in our environment, we can predict that, with time, *Haemonchus* specialization tends to increase, i.e., *H. contortus* will be even more adapted to small ruminants and, in turn, *H. placei* will be even more adapted to parasitism in cattle. This biological aspect of the host-parasite specificity can be exploited as an option for the prophylaxis of gastrointestinal nematode infections though the use of grazing strategies employing different species of ruminants (Fernandes et al., 2004).

In comparison with *H. contortus*, strong immune response to *H. placei* occurs in lambs after serial infections, showing its poor adaptation to sheep, which may explains its extinction in areas grazed only by small ruminants in Brazil. However, field studies will be necessary to better evaluate the interaction between infections by *H. placei* and *H. contortus* in sheep.

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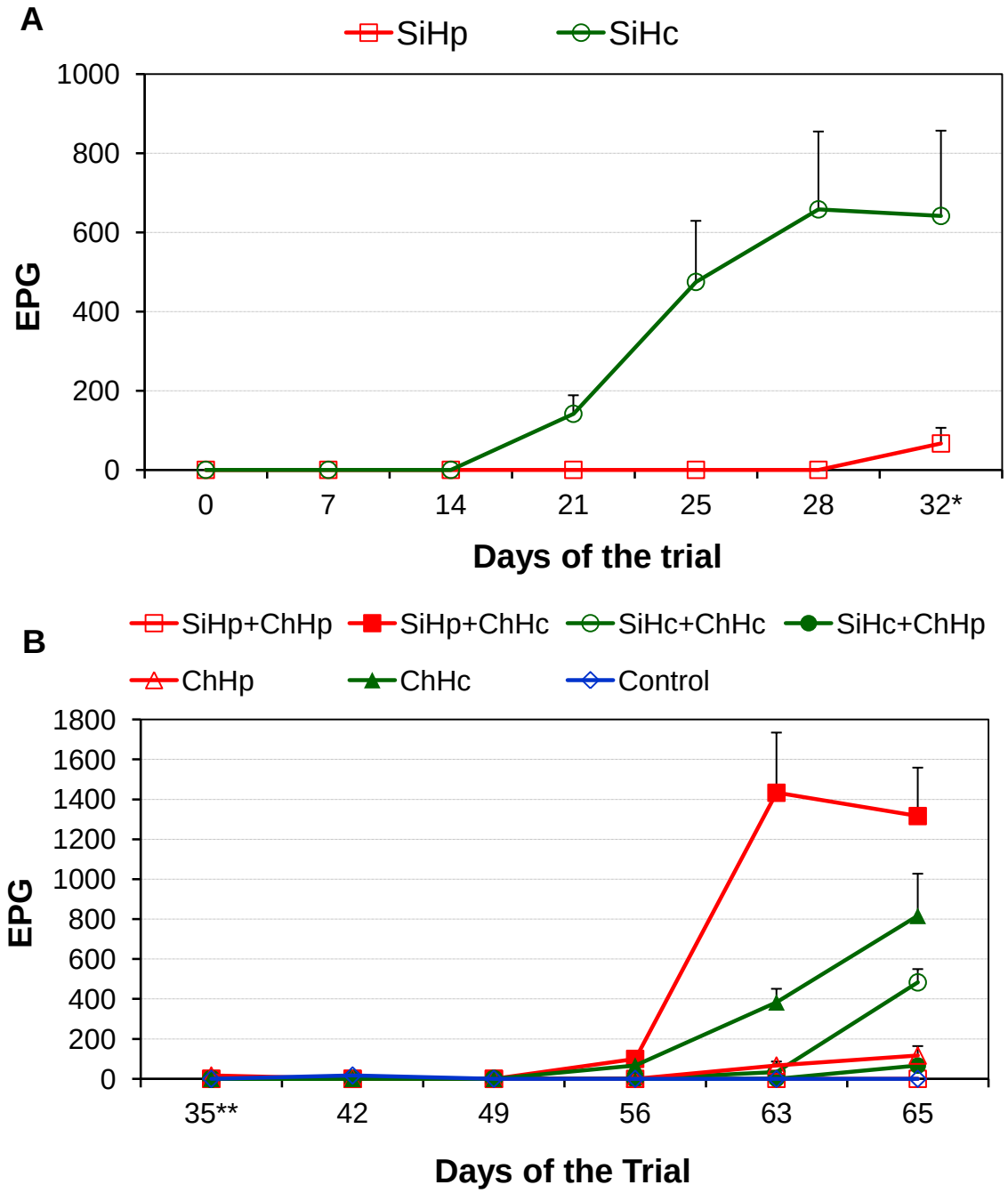

Figure 2. Mean number of eggs per gram of feces (EPG) of the lambs serially infected (Si), with 500 L3 (three times per week), of either *Haemonchus contortus* (Hc) or *Haemonchus placei* (Hp) from day 0 to day 25. On day 32* all animals received anthelmintic treatment (A). On day 35** animals were challenged (Ch) with 4000 L3 (B). The group Control remained uninfected during the trial. Bars: standard error.

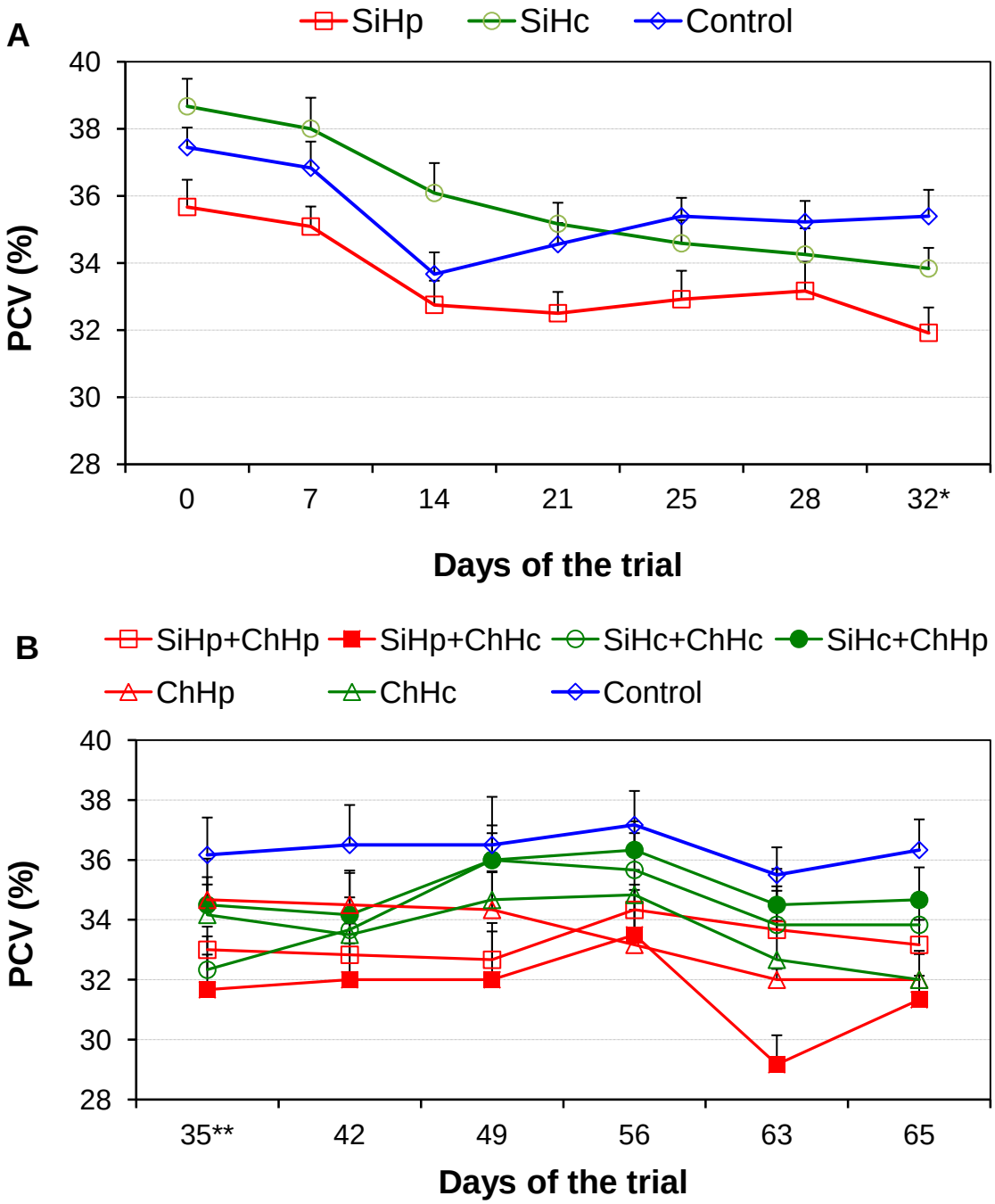

Figure 3. Mean packed cell volume (PCV) of the lambs serially infected (Si) with 500 L3 (three times per week), of either *Haemonchus contortus* (Hc) or *Haemonchus placei* (Hp) from day 0 to day 25. On day 32* all animals received anthelmintic treatment (A). On day 35** animals were challenged (Ch) with 4000 L3 (B). The group Control remained uninfected during the trial. Bars: standard error.

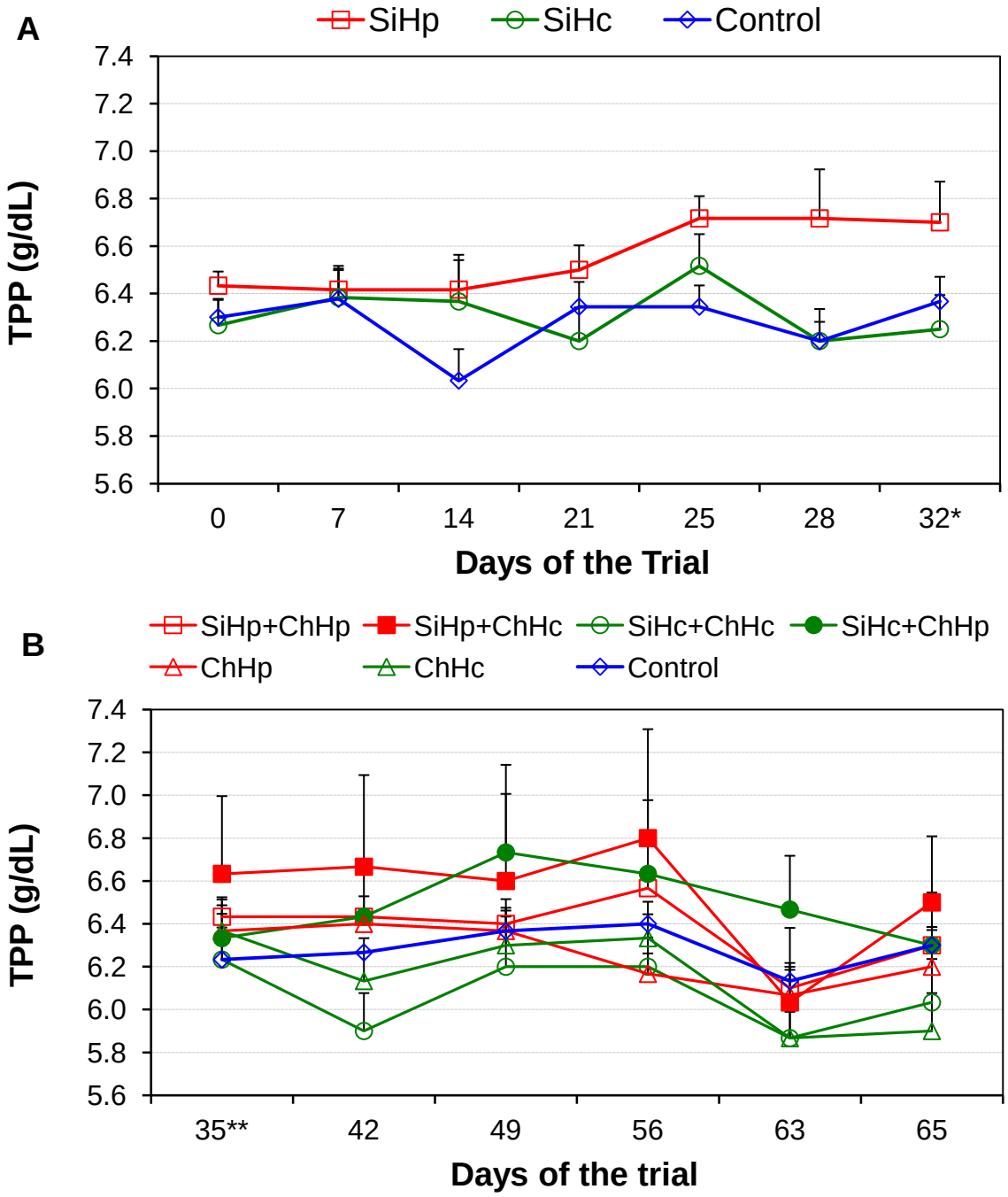

Figure 4. Average of total plasma protein (TPP) of the lambs serially infected (Si) with 500 L3 (three times per week), of either *Haemonchus contortus* (Hc) or *Haemonchus placei* (Hp) from day 0 to day 25. On day 32* all animals received anthelmintic treatment (A). On day 35** animals were challenged (Ch) with 4000 L3 (B). The group Control remained uninfected during the trial. Bars: standard error.

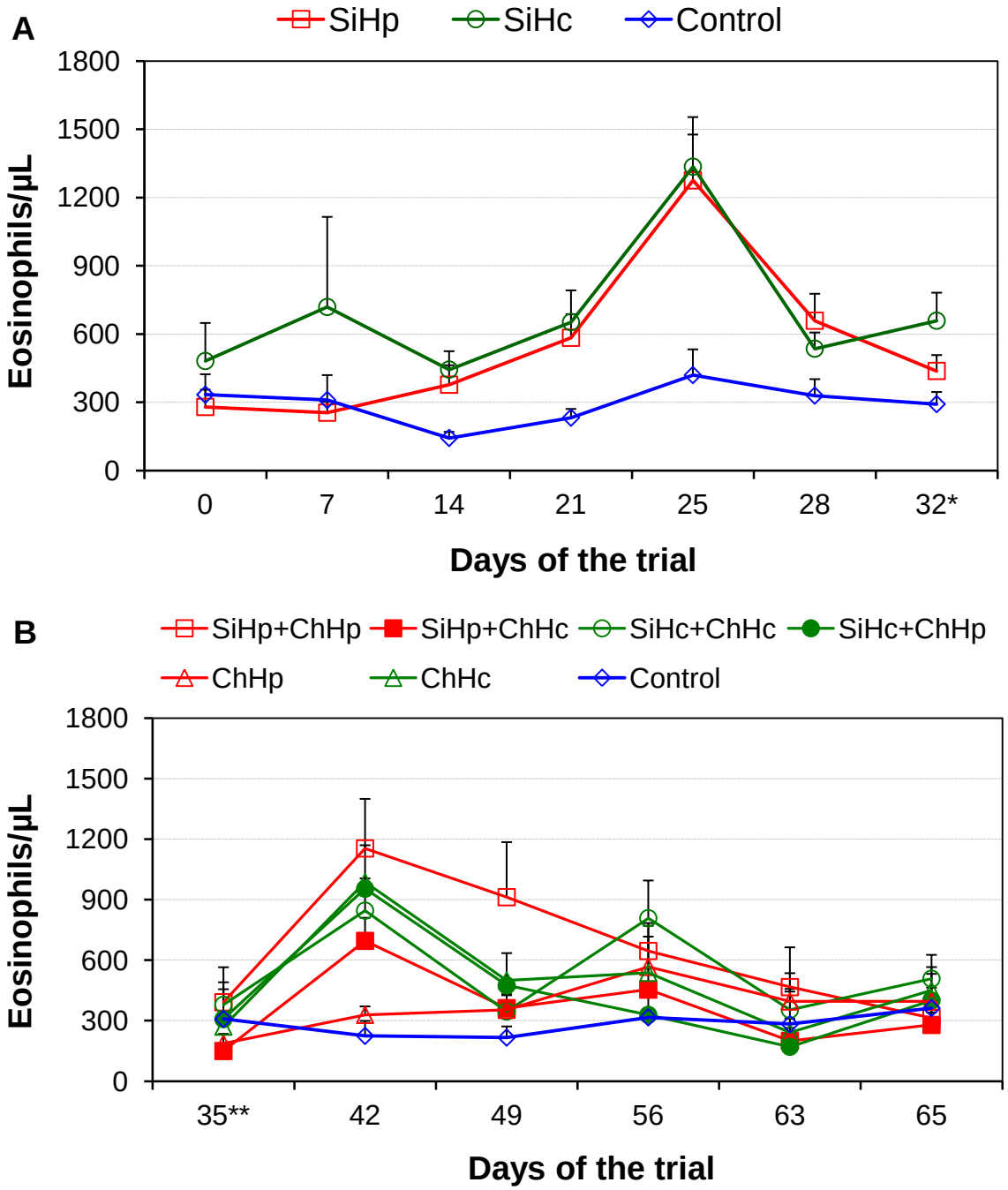

Figure 5. Mean of blood eosinophils per µL of blood of the lambs serially infected (Si) with 500 L3 (three times per week), of either *Haemonchus contortus* (Hc) or *Haemonchus placei* (Hp) from day 0 to day 25. On day 32* all animals received anthelmintic treatment (A). On day 35** animals were challenged (Ch) with 4000 L3 (B). The group Control remained uninfected during the trial. Bars: standard error.

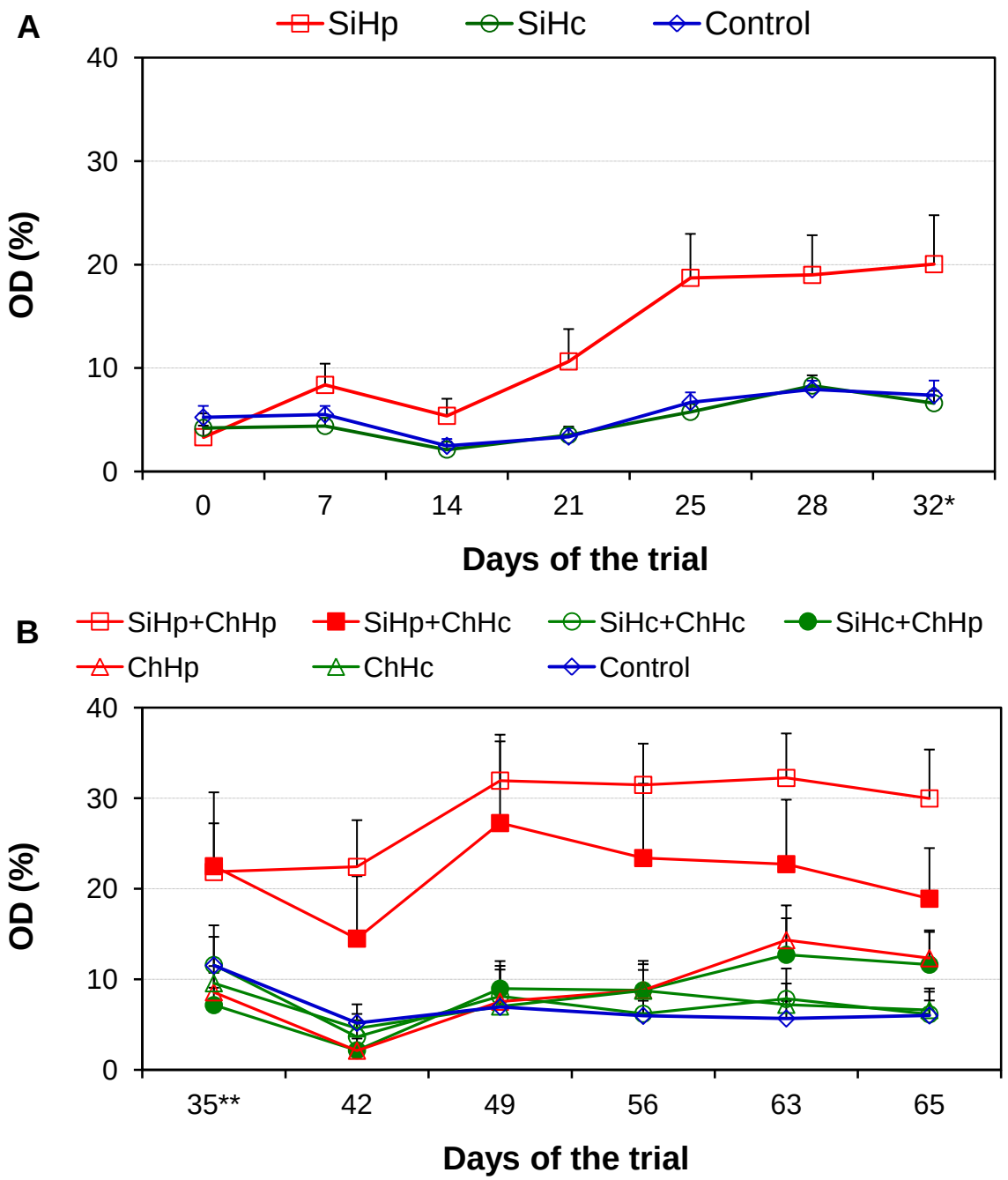

Figure 6. Mean levels of plasma IgG anti-L3 of *Haemonchus contortus* in lambs serially infected (Si), with 500 L3 (three times per week), of either *H. contortus* (Hc) or *Haemonchus placei* (Hp) from day 0 to day 25. On day 32* all animals received anthelmintic treatment (A). On day 35** animals were challenged (Ch) with 4000 L3 (B). The group Control remained uninfected during the trial. Bars: standard error.

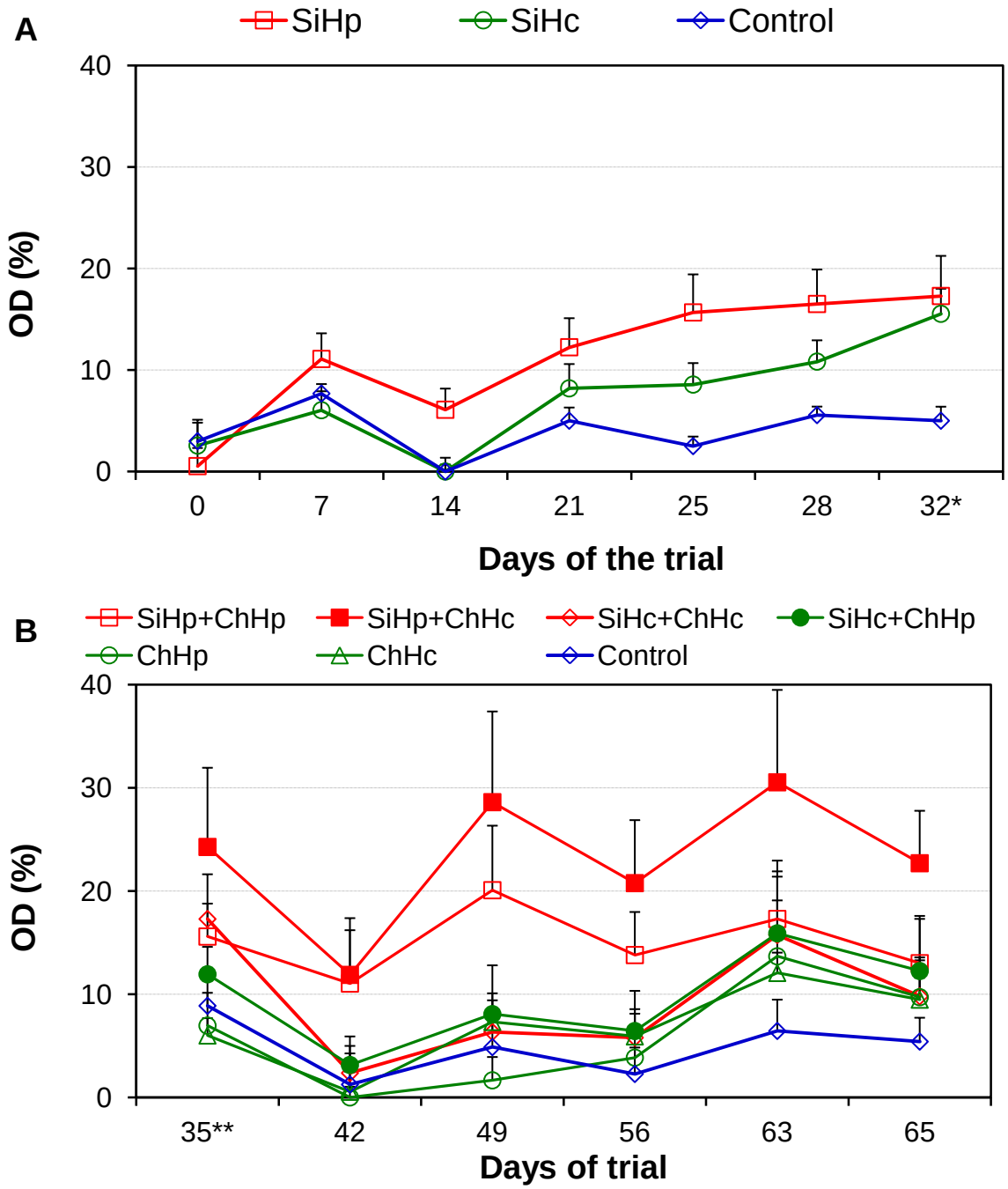

Figure 7. Mean levels of plasma IgG anti-Adult of *Haemonchus contortus* in lambs serially infected (Si), with 500 L3 (three times per week), of either *H. contortus* (Hc) or *Haemonchus placei* (Hp) from day 0 to day 25. On day 32* all animals received anthelmintic treatment (A). On day 35** animals were challenged (Ch) with 4000 L3 (B). The group Control remained uninfected during the trial. Bars: standard error.

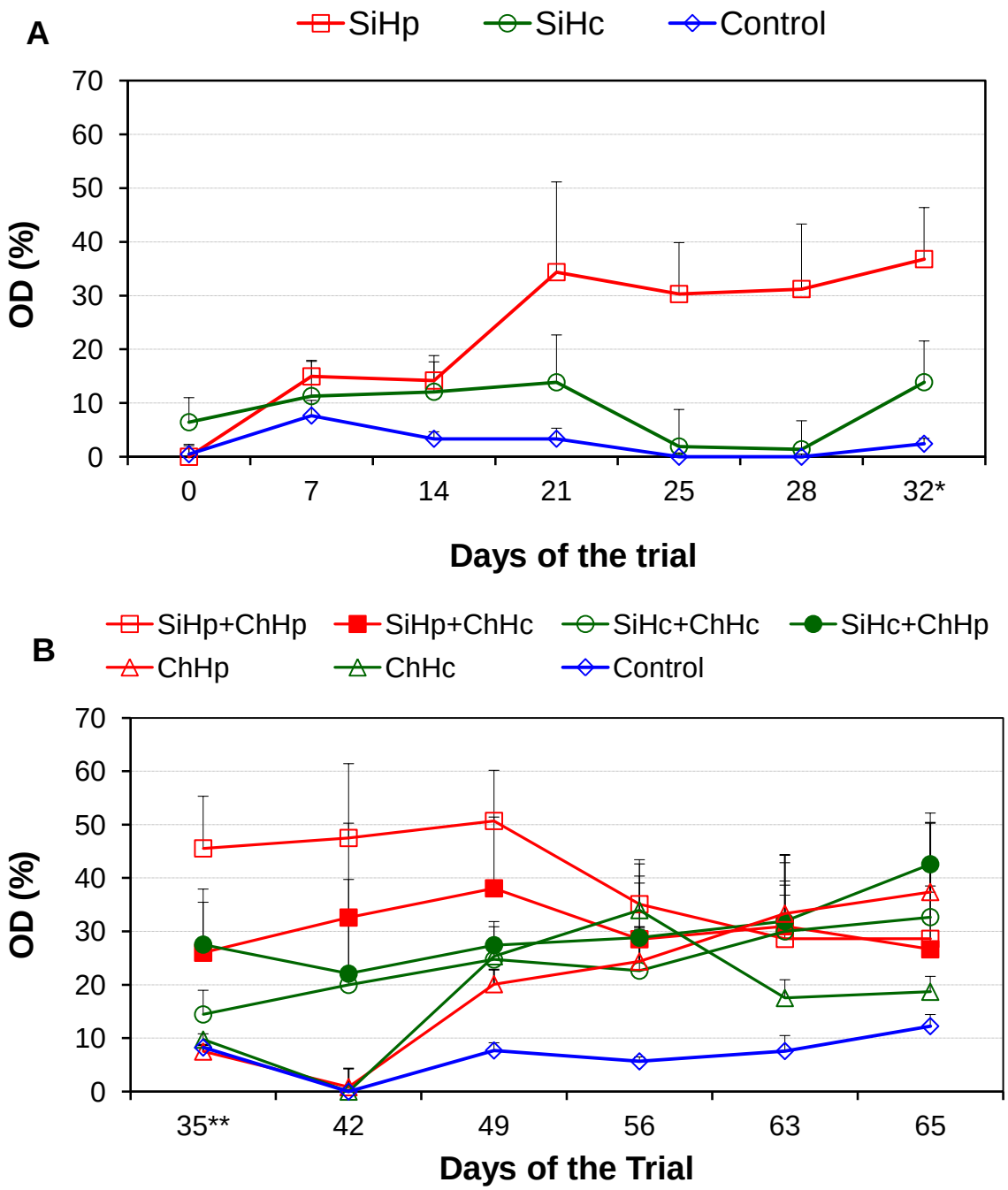

Figure 8. Mean levels of plasma IgA anti-L3 of *Haemonchus contortus* in lambs serially infected (Si), with 500 L3 (three times per week), of either *H. contortus* (Hc) or *Haemonchus placei* (Hp) from day 0 to day 25. On day 32* all animals received anthelmintic treatment (A). On day 35** animals were challenged (Ch) with 4000 L3 (B). The group Control remained uninfected during the trial. Bars: standard error.

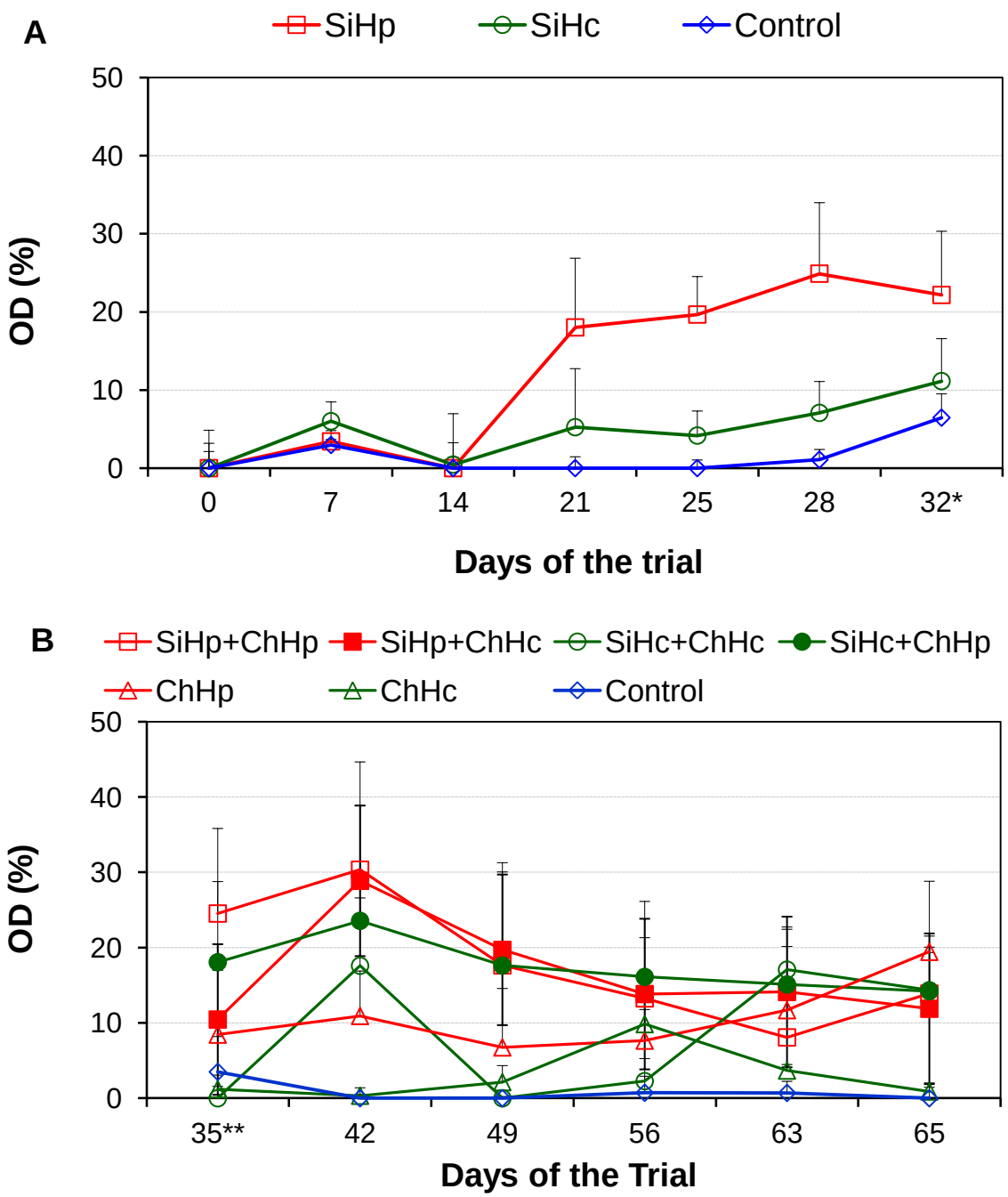

Figure 9. Mean levels of plasma IgA anti-Adult of *Haemonchus contortus* in lambs serially infected (Si), with 500 L3 (three times per week), of either *Haemonchus contortus* (Hc) or *Haemonchus placei* (Hp) from day 0 to day 25. On day 32*all animals received anthelmintic treatment (A). On day 35** animals were challenged (Ch) with 4000 L3 (B). The group Control remained uninfected during the trial. Bars: standard error.

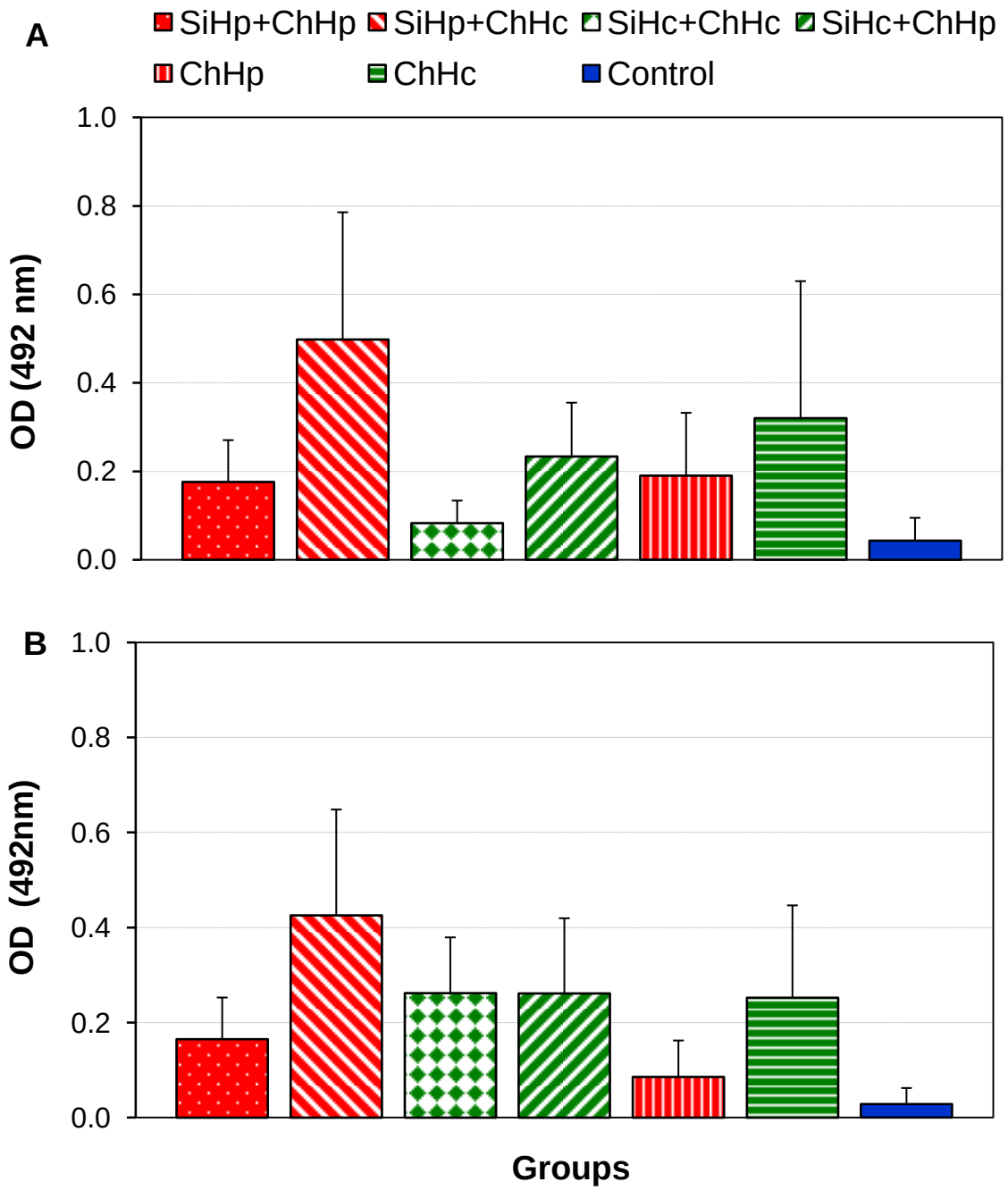

Figure 10. Mean levels of IgA in abomasal mucus anti-L3 (A) and anti-adult (B) of *Haemonchus contortus* in lambs serially infected (Si) with 500 L3 (three times per week) of either *H. contortus* (Hc) or *Haemonchus placei* (Hp) during four weeks. One week after the last Si, all animals received anthelmintic treatment and, three days later the animals were challenged (Ch) with either 4000 L3 Hc or Hp. The group Control remained uninfected during the trial. Bars: standard error. There was no significant difference between group means (P>0.05).

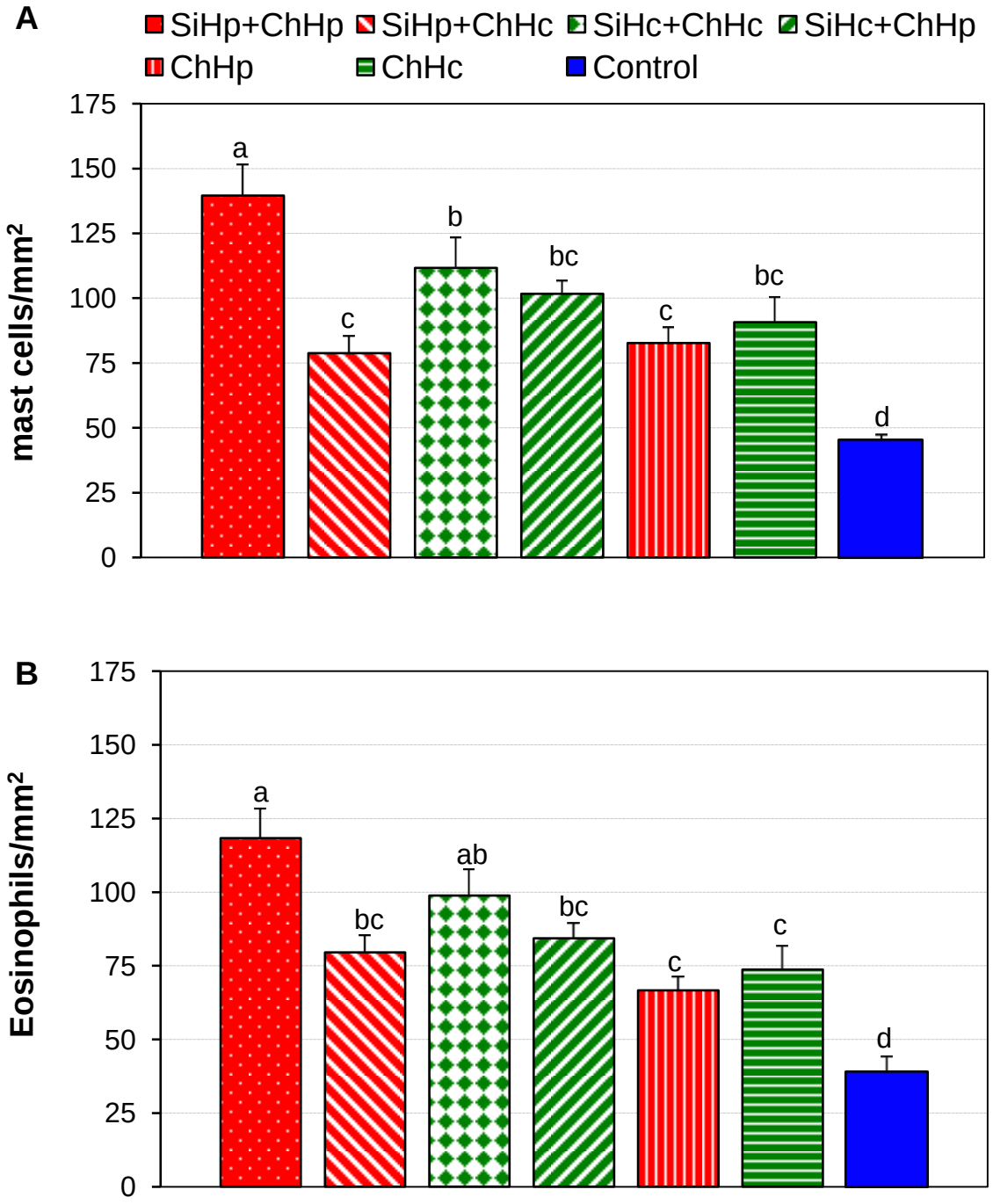

Figure 11. Mean number of mucosal mast cells (A) and eosinophils (B) per mm^2 from abomasal mucosa in lambs serially infected (Si) with 500 L3 (three times per week) of either *H. contortus* (Hc) or *Haemonchus placei* (Hp) during four weeks. One week after the last Si, all animals received antithelmintic treatment and, three days later the animals were challenged (Ch) with either 4000 L3 Hc or Hp. The group Control remained uninfected during the trial. Significant differences ($P < 0.05$) between the groups are indicated by different letters. Bars: standard error.

Table 1. Mean number (± standard error) of parasites from lambs serially infected (Si) with 500 L3 (three times per week) of *Haemonchus contortus* (Hc) or *Haemonchus placei* (Hp) for four weeks. One week after the last serial infection, all animals received anthelmintic treatment and, three days later the animals were challenge (Ch) with 4000 L3 Hc or Hp.

Groups	**Stage of development**			
	Juvenile L4	**Juvenile L5**	**Adult**	**Total Burden**
SiHp+ChHp	6 (± 6)	42 (± 41)	60 (± 51) a	107 (± 97) a
SiHc+ChHc	9 (± 7)	3 (± 1)	398 (± 350) ab	409 (± 350) ab
SiHp+ChHc	27 (± 25)	42 (± 34)	698 (± 387) b	767 (± 372) bc
SiHc+ChHp	0	153 (± 97)	477 (± 261) ab	630 (± 292) abc
ChHp	4 (± 3)	60 (± 34)	947 (± 265) b	1011 (± 281) c
ChHc	13 (± 8)	68 (± 33)	832 (± 349) b	914 (± 332) bc

Arithmetic means with different letters in the column are significantly different ($p<0.05$).