Instituto de Biociências

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

PG-BGA

Epidemiologia das infestações por *Oestrus ovis* em ovinos criados em Botucatu e influência da raça ovina no parasitismo

Bruna Fernanda da Silva

Tese apresentada ao Instituto de Biociências, Câmpus de Botucatu, UNESP, para obtenção do título de Doutor 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
2012

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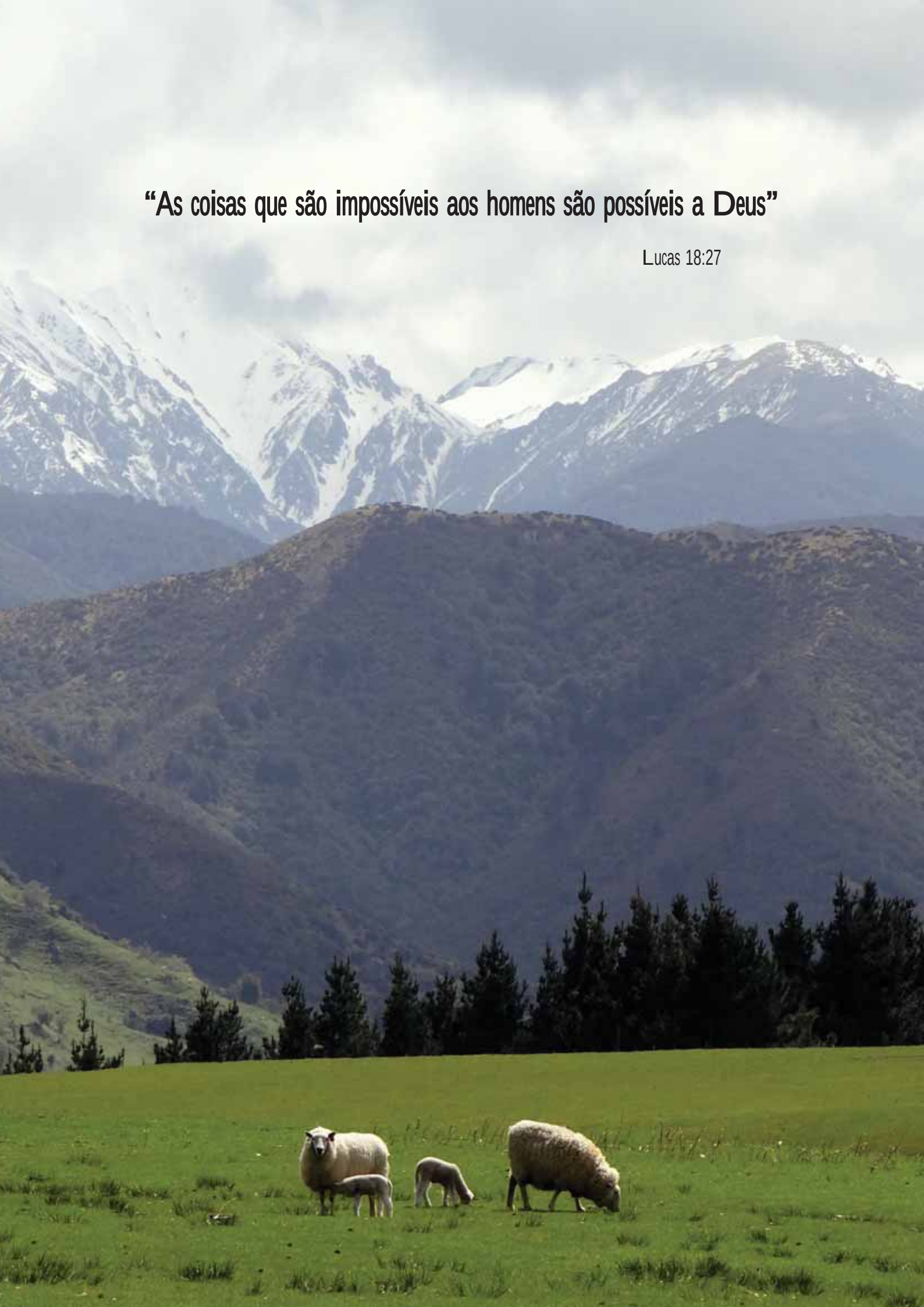

“As coisas que são impossíveis aos homens são possíveis a Deus”

Lucas 18:27

Dedico

Aos meus pais, José Horácio e Benedita e a minha irmã Beatriz

Meus exemplos de amor

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A Deus pelo dom da vida e por todas as maravilhas que tem feito em minha vida, por iluminar e abençoar meus caminhos e o meu trabalho.

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Muito obrigada!!!

"Escolhe um trabalho de que gostes, e não terás que trabalhar nem um dia na tua vida."

Confúcio

"Não conheço ninguém que conseguiu realizar seu sonho, sem sacrificar feriados e domingos pelo menos uma centena de vezes. O sucesso é construído à noite. Durante o dia você faz o que todos fazem. Mas, para obter um resultado diferente da maioria, você tem que ser especial. Se fizer igual a todo mundo, obterá os mesmos resultados. Não se compare à maioria, pois infelizmente ela não é modelo de sucesso. Se você quiser atingir uma meta especial, terá que estudar no horário em que os outros estão tomando cerveja com batata frita. Terá de planejar, enquanto os outros permanecem à frente da televisão. Terá de trabalhar enquanto os outros tomam sol à beira da piscina. A realização de um sonho depende de dedicação. Há muita gente que espera que o sonho se realize por mágica, mas toda mágica é ilusão e a ilusão não tira ninguém de onde está. Na verdade a ilusão é combustível dos perdedores, pois: 'Quem quer fazer alguma coisa, encontra um meio. Quem não quer fazer nada, encontra uma desculpa'."

(Desconheço a autoria)

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Resumo

A variação sazonal e a intensidade de infestação por larvas de *Oestrus ovis* em ovinos criados em Botucatu-SP foi avaliada de abril de 2008 a março de 2011. Dois cordeiros traçadores foram colocados, mensalmente, junto com um rebanho ovino, onde permaneceram por 28 dias. Após esse período, os traçadores foram sacrificados e as larvas de *O. ovis* recuperadas, identificadas e quantificadas de acordo com o estádio de desenvolvimento. Dos 72 cordeiros traçadores, 50% estavam infestados por larvas *O. ovis* com intensidade média de 16,8 larvas/cabeça com média de 7,8 larvas de primeiro estádio (L1), 5,3 de segundo (L2) e 3,7 de terceiro (L3). Sinais clínicos de oestrose foram mensalmente avaliados em todas as ovelhas do rebanho e a prevalência média de animais com sinais clínicos de oestrose foi de 13,4% (máxima de 31,4% em janeiro de 2009 e mínimo de 1% em setembro de 2010). Além disso, a prevalência do parasitismo por *O. ovis* e a intensidade de infestação foram avaliados em cabeças de ovinos obtidas de um abatedouro localizado em Itápolis – SP. Das 139 cabeças examinadas, 13,7% estavam parasitadas pelas larvas *O. ovis* e a intensidade de infestação média mensal variou de 1 até 10,2 larvas/cabeça com intensidade média geral de 4,5 larvas/cabeça. Do total de 85 larvas, 21,2% eram L1, 37,6% L2 e 41,2% L3. Os resultados demonstraram que as condições climáticas do Estado de São Paulo são favoráveis para a atividade da mosca e desenvolvimento dos estágios larvais praticamente durante todo o ano.

Em outro estudo, foi avaliada, comparativamente, a resistência de cordeiros de duas raças ovinas, Ile de France (IF) e Santa Inês (SI) contra infestações naturais por *O. ovis*, bem como a associação entre a ocorrência deste parasita com as infecções naturais por nematódeos gastrintestinais. Cordeiros machos SI (n = 12) e IF (n = 12) recém desmamados, foram mantidos juntos em um piquete de setembro a início de dezembro de 2009, quanto foram sacrificados. Todos os animais apresentaram infestação pelos

diferentes instares larvais de *O. ovis*, sem diferença entre as raças (P > 0,05). Os cordeiros da raça SI apresentaram em média 24,8 larvas, sendo que a intensidade de infestação variou entre 14 e 39 larvas, enquanto que os cordeiros da raça IF apresentaram em média 23,5 larvas, com infestação máxima e mínima, respectivamente de 11 e 36 larvas. Os cordeiros SI apresentaram a menor contagem de ovos de nematódeos por grama de fezes (OPG) e os menores números médios de *Haemonchus contortus*, *Trichostrongylus colubriformis* e *Strongyloides papillosus*, no entanto, não houve diferenças significativas entre as raças (P > 0,05). Foi observada relação inversa entre o número de larvas de *O. ovis* e nematódeos gastrintestinais em ambas as raças. Os cordeiros SI apresentaram aumento significativo no número de eosinófilos sanguíneos e nos níveis totais de IgE sérica e estas variáveis foram negativamente correlacionadas com as contagens de OPG e carga de *H. contortus* em ambas as raças. A resposta imune contra *O. ovis* e nematódeos gastrintestinais foram muito semelhantes nas duas raças (P > 0,05) e envolveu o recrutamento de células inflamatórias e produção de imunoglobulinas parasita-específicas no soro e muco. Os resultados indicaram que a presença dos anticorpos no soro e muco nasal não foram suficientes para proteger os animais contra a infestação pelas larvas de *O. ovis*, mas pareceram promover atraso no desenvolvimento larval, fato observado especialmente nos cordeiros SI. Em conclusão, não houve diferença no parasitismo por larvas de *O. ovis* entre as raças avaliadas, e os animais parasitados pelas larvas de *O. ovis* tendem a apresentar menor carga parasitária de nematódeos gastrintestinais.

Abstract

The seasonal factors which influence *Oestrus ovis* infestation in sheep in Botucatu-SP were determined from April 2008 until March 2011. Two tracer lambs were exposed monthly to natural infestation by *O. ovis* larvae for 28 consecutive days, by grazing with a sheep flock. Tracer animals were then euthanized and the larvae of *O. ovis* recovered from nasal and sinus cavities. Of the 72 tracer lambs, 50% were infested with *O. ovis* larvae and the mean intensity of infestation per head infested was 16.8 larvae with an average of 7.8 first instar (L1), 5.3 second instar (L2) and 3.7 third instar (L3). Clinic signs of oestrosis were evaluated in all sheep of the flock monthly and the average prevalence of animals with clinical signs of oestrosis was 13.4% (maximum of 31.4% in January 2009 and minimum of 1% in September 2010). Additionally, the *O. ovis* prevalence and infestation intensity were evaluated in heads from slaughtered sheep from Itápolis-SP. Of the 139 head examined 13.7% were parasitized by *O. ovis* larvae with monthly mean of intensity of infestation ranging from 1 until 10.2 larvae/infested head with general mean intensity of 4.5 larvae/infested head. Of the total of 85 larvae, 21.2% were L1, 37.6% L2 and 41.2% L3. The results suggest that the climatic conditions in São Paulo State are favorable to fly activity and larval development during the whole year.

In other study, were evaluated comparatively, the resistance in lambs of two sheep breeds, Ile de France (IF) and Santa Ines (SI) against *O. ovis* infestation, well as the association between the occurrence of this parasite with natural infections by gastrointestinal nematodes (GIN). SI (n=12) and IF (n=12) young male lambs weaned at two months of age were kept together in a paddock from September to early December 2009, when were sacrificed. All animals were infested by different larval instars of *O. ovis* without any statistical difference between breeds ($P > 0.05$). The SI lambs had an

average of 24.8 larvae, and the intensity of infection ranged between 14 and 39 larvae, while the IF lambs showed an average of 23.5 larvae with the minimum and maximum from 11 to 36 larvae, respectively. SI lambs presented the lowest nematode fecal egg counts (FEC) and the lowest mean numbers of *Haemonchus contortus*, *Trichostrongylus colubriformis* and *Strongyloides papillosus*, however, there was no significant differences between group means ($P > 0.05$). Inverse relationship between numbers of *O. ovis* larvae and gastrointestinal nematodes was observed in both breeds. SI sheep showed a significant increase in blood eosinophils and total IgE serum levels and these variables were negatively correlated with nematode FEC. The immune response against *O. ovis* and GIN were very similar in both breeds ($P > 0.05$) and involved the recruitment of inflammatory cells and the parasitic specific immunoglobulins production in serum and mucus. The results indicated that the presence of antibodies in serum or nasal mucus were not enough to protect them against *O. ovis* infestation, but can promote a delay in larval development, fact observed especially in SI lambs. In conclusion there was no breed difference regarding *O. ovis* infestation and in each breed, animals with more nasal bot fly larvae tended to display smaller worm burden.

Capítulo 1

Introdução

Oestrus ovis L. (Díptera: Oestridae) é um parasita cosmopolita causador de míiase cavitária e suas larvas são parasitas obrigatórios da cavidade nasal e seios paranasais de ovinos e caprinos (Zumpt, 1965).

A mosca de *O. ovis* é vivípara e deposita larvas de primeiro estádio (L1) diretamente no nariz dos ovinos e caprinos. Estas colonizam rapidamente as cavidades nasais, septo, turbinas e etmóide, e em seguida, mudam para larva de segundo estádio (L2) e migram para os seios frontais, onde irá completar seu desenvolvimento em larva de terceiro estádio (L3). As L3 maduras serão expelidas para o ambiente para o período de pupação que acontece no solo. A pupa dará origem a mosca, e após o acasalamento, as fêmeas grávidas atacam os ovinos para depositar as larvas, reiniciando o ciclo (Zumpt, 1965).

O desenvolvimento das larvas na cavidade nasal do hospedeiro bem como a atividade da mosca no meio ambiente é muito influenciado pelas condições climáticas do ambiente (Cobbett and Mitchell, 1941). O desenvolvimento larval pode variar entre 25-35 dias podendo se estender até nove meses dependendo da estação do ano e condições climáticas da região (Hall and Wall, 1995).

As larvas de *O. ovis* não são hematófagas e se alimentam de proteínas plasmáticas, de anticorpos que estão passando pela mucosa nasal durante o processo inflamatório, de mucina, albumina e colágeno da membrana basal (Frugère et al., 2000; Tabouret et al., 2003a). Embora seus ganchos e espinhos danifiquem as membranas nasais, a nutrição larval não é apenas mecânica, mas está relacionada principalmente a um processo bioquímico e grande liberação de oxido de nitrogênio (Angulo-Valadez et al., 2010).

O comprimento da larva e a coloração/tamanho dos espiraculos posteriores, por onde as larvas respiram, são um dos parâmetros utilizados para classificar as fases de desenvolvimento em L1, L2 ou L3 (Fig. 1) (Cepeda-Palacios et al., 1999).

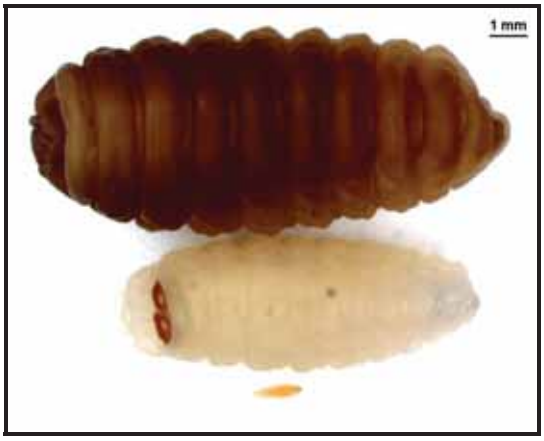

Figura 1. Larvas de *Oestrus ovis* em diferentes fases de desenvolvimento (vista dorsal). Foto: B. F. Silva, 2009.

A L1 é depositada na cavidade nasal dos ovinos com cerca de 1 mm de comprimento, possui ganchos orais relativamente pequenos e espiraculos posteriores não pigmentados (Fig. 2). A muda de L1 para L2 ocorre com cerca de 4 mm e nesta fase os espiraculos começam a ficar visíveis na cutícula da L1.

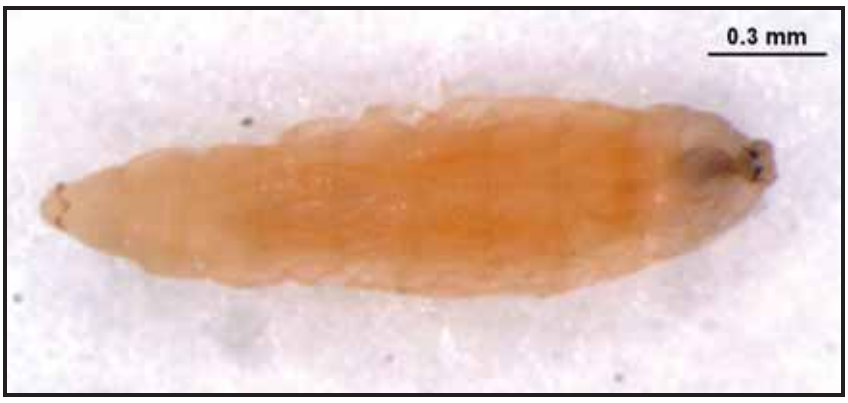

Figura 2. Vista ventral da larva de primeiro estádio (L1) de *Oestrus ovis*. Foto: B. F. Silva, 2009.

A L2 varia entre > 4 mm até 10 mm de comprimento e nesta fase os espiraculos posteriores são visíveis, inicialmente com coloração variando entre o amarelo-alaranjado até o marrom escuro (Fig. 3).

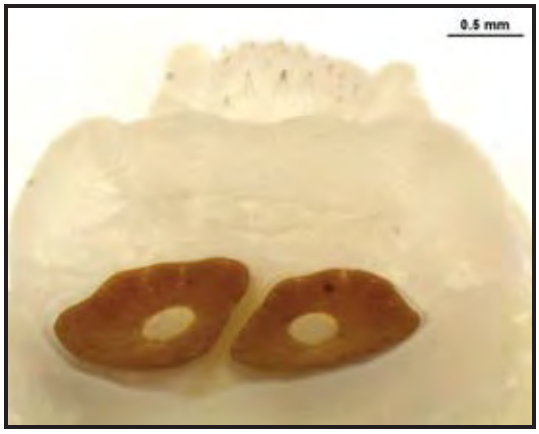

Figura 3. Detalhe da região posterior da larva de segundo estádio (L2) de *Oestrus ovis* onde estão localizados os espiraculos respiratórios. Foto: B. F. Silva, 2009.

A L3 possui comprimento entre >10 mm até 22 mm e os espiraculos apresentam coloração marrom escura. Conforme a maturação, a L3 começa apresentar listas dorsais pretas e mudam de cor, do branco para a cor creme e depois marrom claro até ficar com o corpo totalmente escuro, e então estará pronta para ser expelida pelo hospedeiro e pupar no solo (Fig. 4).

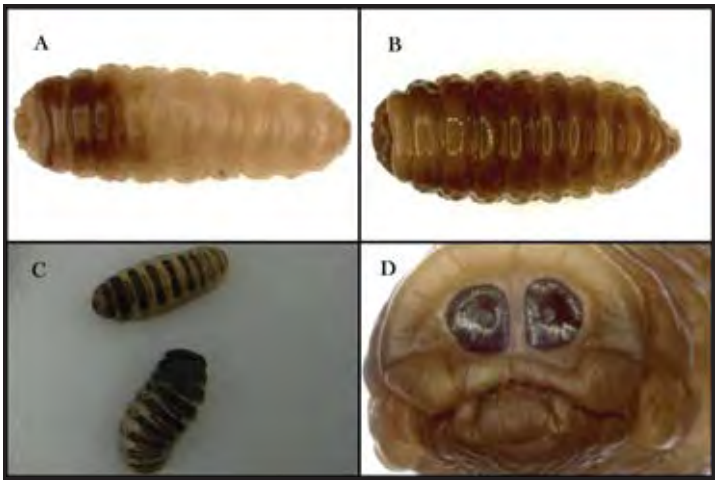

Figura 4. Larva de terceiro estádio (L3) de *Oestrus ovis* em diferentes fases de desenvolvimento (A, B e C); espiraculos respiratórios localizados na região posterior do corpo da larva (D). Foto: B. F. Silva, 2009.

A mosca adulta tem coloração acinzentada, possui pêlos e manchas escuras pelo corpo, o que facilita a camuflagem (Fig. 5). Além disso, voa rápido e possuem olhos grandes, o que facilita a localização de seus hospedeiros bem como, das fêmeas para a reprodução. A vida dos adultos é curta, pois não possuem peças bucais funcionais, e, portanto, não se alimentam no ambiente e dispõem apenas das reservas energéticas acumuladas durante a fase larval que acontece no aparelho nasal do hospedeiro (Angulo-Valadez et al., 2010). As fêmeas carregam consigo cerca de 500 larvas e são capazes de infestar vários ovinos / caprinos durante seu curto período de vida (Cobbett and Mitchell, 1941), além disso, machos e fêmeas emergem do pupario já sexualmente maduros, prontos para o acasalamento (Angulo-Valadez et al., 2010) adaptação que auxilia na economia de energia.

Figura 5. Vista dorsal da mosca adulta de *Oestrus ovis*. Foto: B. F. Silva, 2008.

A oestrose ou “bicho da cabeça”, como a enfermidade é comumente conhecida, afeta o bem estar e o desempenho produtivo dos animais parasitados, resultando em significativas perdas econômicas (Alcaide et al., 2003), como por exemplo, redução no ganho de peso (Horak and Snijders, 1974) e diminuição da produção de leite em até 9% (Dorchies et al., 2003).

Os distúrbios causados por este parasita começam no momento da postura das larvas, pois a mosca irrita os animais, que deixam de se alimentar para tentar se proteger dos seus ataques, escondendo o focinho no solo ou entre a lã de outros carneiros, balançando a cabeça e espirrando (Zumpt, 1965). Por sua vez, as larvas, pelos seus ganchos e espinhos, irritam e causam danos a mucosa nasal, provocando inflamação acompanhada de produção de exsudato mucoso (Fig. 6), que além de dificultar a respiração pode induzir infecções secundárias (Dorchies et al., 1998). O parasitismo pelas larvas de *O. ovis* causa rinite e sinusite nos animais, e o acumulo de muco nasal e os espirros frequentes são os principais sinais clínicos da infestação pelas larvas deste parasita em ovinos e caprinos (Angulo-Valadez et al., 2011).

Figura 6. Ovelhas da raça Bergamacia com corrimento nasal, sinal clínico característico do parasitismo por larvas de *Oestrus ovis*. Foto: B. F. Silva, 2008.

Epidemiologia

Oestrus ovis é um parasita distribuído mundialmente, mas a atividade da mosca no ambiente, o desenvolvimento larval no aparelho nasal do hospedeiro, bem como, o período de pupa que ocorre no solo é muito influenciado pelas condições climáticas do ambiente.

Os principais fatores climáticos que influenciam a atividade dos oestrideos são a temperatura, luminosidade e velocidade do vento, mas no caso do *O. ovis*, a temperatura é fator determinante para a larviposição (Cepeda-Palacios and Scholl, 2000). Em Baja California Sur, México, onde a temperatura diurna durante a primavera varia entre 9 – 35 °C foi observado que a atividade da mosca teve início quando a temperatura ultrapassou os 20 °C, mas a ‘temperatura ótima’ para a larviposição variou entre 26 - 28 °C. Porém, *O. ovis*, por um processo adaptativo, pode se ajustar as características climáticas da região onde habita e essa ‘temperatura ótima’ pode ser diferente

dependendo da região estudada (Cepeda-Palacios and Scholl, 2000), mas no geral, os ataques da mosca acontecem principalmente durante o período mais quente do dia.

Cobbett e Mitchell (1941) foram pioneiros em descrever a influencia do clima na epidemiologia do *O. ovis*. Dentre muitas descobertas, os pesquisadores observaram que durante o inverno o desenvolvimento larval na cavidade nasal dos animais é lento e que em locais onde o inverno é muito rigoroso, característico de regiões com clima temperado, a L1 cessa o desenvolvimento e entra em estado de hipobiose, ou seja, não há desenvolvimento larval neste período até que as condições climáticas voltem a ser favoráveis para o desenvolvimento. Da mesma forma, em países com clima muito quente, o desenvolvimento larval também é interrompido durante o período seco (Dorchies et al., 1998). Essa é uma das estratégias que pode assegurar a perpetuação do *O. ovis* em regiões em que as condições climáticas são extremas durante algum período do ano.

Devido a essa grande influencia do clima no desenvolvimento das larvas de *O. ovis*, a prevalência e intensidade de infestação é variável de acordo com o país ou região estudada, como pode ser observado na tabela abaixo (Tabela 1), bem como a prevalência dos diferentes instares larvais recuperados da cavidade nasal dos animais parasitados.

Em locais com clima temperado, como exemplo, na região sul da França (Dorchies et al., 2000) e região nordeste da Espanha (Gracia et al., 2010), as L1 foram predominantes durante o ano de estudo, constituindo respectivamente 85,1 e 78,4% da carga parasitária total. O oposto foi observado na Sicilia, Itália (Caracappa et al., 2000) e na região sudeste da Espanha (Alcaide et al., 2003) onde todos os diferentes instares larvais foram simultaneamente recuperados durante o ano de estudo e em proporções similares. Na Sicilia, por exemplo, a proporção de L1, L2 e L3 foi respectivamente,

41,4%, 29,1% e 29,6% o que indica que as condições climáticas da região estudada foram favoráveis para o desenvolvimento larval. Porém, em ambos os estudos, durante os meses frios, foi observado um período de desenvolvimento lento onde a proporção de L1 predominou sob os demais estádios larvais.

Tabela 1. Prevalência e intensidade de infestação por larvas de *Oestrus ovis* em ovinos.

Local	N*	Prevalência	Intensidade	Referência
Canadá	698	50,0%	2,5	(Fallis, 1940)
Estados Unidos	720	91,5%	25,6	(Meleney et al., 1962)
Nova Zelândia	1083	65.8%	3,1	(Kettle, 1973)
África do Sul	542	73,4%	15,2	(Horak, 1977)
Zimbábue	507	21,9%	1,1	(Pandey, 1989)
França (sudeste)	555	65,0%	24,8	(Yilma and Dorchies, 1991)
Itália (Sicilia)	841	55,8%	9,4	(Caracappa et al., 2000)
França (sul)	631	43,4%	10,8	(Dorchies et al., 2000)
Etiópia	248	77,4%	12,7	(Yilma and Genet, 2000)
Itália (Sardenha)	566	91,0%	19,0	(Scala et al., 2001)
Espanha (sudeste)	477	71,1%	18,5	(Alcaide et al., 2003)
Nigéria (norte)	116	62,1%	9,2	(Oniye et al., 2006)
Turquia (Konya)	624	59,0%	23,9	(Uslu and Dik, 2006)
Turquia (Kars)	387	40,3%	4,5	(Arslan et al., 2009)
Irã (Shiraz)	2002	49,7%	6,3	(Shoorijeh et al., 2009)
Espanha (nordeste)	120	84,2%	37,9	(Gracia et al., 2010)

*N = número de cabeças de ovinos examinadas, provenientes de abatedouros.

No Brasil, apesar da crescente observação de animais com sinais clínicos de oestrose, há poucos estudos epidemiológicos sobre esta enfermidade, e estes estão restritos a região Sul, onde as condições climáticas foram favoráveis em praticamente todos os meses do ano para a atividade da mosca de *O. ovis* e desenvolvimento larval na cavidade nasal dos ovinos (Ribeiro et al., 1990; Ramos et al., 2006).

Em Bagé – RS, das 144 cabeças de ovinos examinadas durante o período de um ano, 85,4% estavam parasitadas e 1639 larvas foram recuperadas, sendo que destas 68,6% eram L1, 12,3% L2 e 18,9% L3 (Ribeiro et al., 1990). Em Encruzilhada do Sul, a prevalência foi de 100% em animais abatidos com sinais clínicos de oestrose, com intensidade média de infestação de 23,8 larvas/animal e a prevalência de L1, L2 e L3 foi respectivamente de 78,9%, 14,5% e 6,6% (Oliveira et al., 1999). Já em estudo realizado em Santa Catarina, quando as temperaturas médias foram inferiores a 9,8 ºC, não foram constatadas larvas de *O. ovis* nos animais (Ramos et al., 2006).

Soroprevalência

No geral, o diagnóstico de oestrose é baseado em sinais clínicos e na detecção das larvas *post mortem*, porém, estudos demonstram que o teste ELISA (enzyme-linked immunosorbent assay) é sensível para detecção de anticorpos específicos anti *O. ovis*, utilizando antígenos totais ou produtos excretórios e secretórios das larvas (Papadopoulos et al., 2001; Alcaide et al., 2005a; Angulo-Valadez et al., 2008).

Alguns estudos, além de avaliar a prevalência por sorologia, também avaliaram fatores de risco associados com a oestrose. Em Yucatan – México, 30% dos ovinos avaliados estavam soropositivos para *O. ovis* e o tamanho do rebanho (> 25 animais) e a cor do nariz do ovino (escuro) foram associados como fatores de risco para a ocorrência da enfermidade (Murguia et al., 2000). Já na região sudeste da Alemanha a prevalência

de anticorpos foi de 50% nos ovinos examinados e o tamanho do rebanho (> 50 animais) foi o único fator de risco associado com a oestrose (Bauer et al., 2002).

Na região sudeste da Espanha, das 551 fazendas estudadas, apenas 18 fazendas estavam livres de animais soropositivos e em 115, todos os animais foram soropositivos para *O. ovis*. A prevalência média de animais soropositivos foi de 69,3% e foi observado que além do tamanho do rebanho (> 250 ovinos) e densidade da população ovina (> 100 ovinos por km^2) foram importantes fatores de risco para a ocorrência desse parasita na região estudada (Alcaide et al., 2005b).

Ovino x Caprino

Tanto ovinos como caprinos são parasitados por *O. ovis*, mas a prevalência do parasitismo é menor nos caprinos do que em ovinos. Em Pézenas, região sul da França, a prevalência, os sinais clínicos da infestação e a carga parasitária de *O. ovis* foi menor em caprinos do que em ovinos (Dorchies et al., 2000). Da mesma forma, na Grécia, a prevalência de animais com anticorpos específicos anti *O. ovis* foi menor em caprinos comparado aos ovinos (Papadopoulos et al., 2001; Papadopoulos et al., 2006).

Há algumas hipóteses para essa diferença no parasitismo por *O. ovis* entre caprinos e ovinos. Supõe-se que os caprinos aparentam ser mais sensíveis aos ataques da mosca e conseguem evitar o contato com a mesma de forma mais eficaz comparado aos ovinos (Dorchies et al., 1998; Angulo-Valadez et al., 2010). Acredita-se também que os caprinos co-evoluíram com o *O. ovis* por um período mais longo do que com os ovinos e talvez por isso sejam mais bem adaptados ao parasitismo (Angulo-Valadez et al., 2010).

Resposta imunológica

As infecções parasitárias caracterizam-se por estimular inúmeros mecanismos

imunológicos de defesa, sejam eles mediados por anticorpos ou por células e a eficiência da resposta imunológica depende do parasita em questão e do estágio da infecção.

A presença das larvas de *O. ovis* na cavidade nasal dos ovinos induz resposta imunológica celular, com recrutamento de leucócitos (linfócitos T e B, macrófagos) e granulócitos (eosinófilos, mastócitos e leucócitos globulares) na mucosa do trato nasal, e resposta imune humoral local e sistêmica com produção de imunoglobulina G (IgG) e imunoglobulina A (IgA) anti *O. ovis,* as quais são encontradas no soro e muco nasal dos ovinos parasitados (Tabouret et al., 2003b), o que sugere uma resposta imunológica tipo Th2 (Angulo-Valadez et al., 2011), similar ao que é observado na resposta imune contra o parasitismo por nematódeos gastrintestinais (Anthony et al., 2007; Rowe et al., 2008). Além disso, estudos demonstram que os produtos excretórios e secretórios das larvas de *O. ovis* causam reação de hipersensibilidade imediata em seus hospedeiros (Dorchies et al., 1998; Jacquiet et al., 2005).

A resposta inflamatória causada pelas larvas de *O. ovis* parece estar relacionada com a regulação da carga parasitária, pois promove redução no crescimento larval, mas por outro lado, não protege os hospedeiros contra o estabelecimento das larvas (Frugère et al., 2000; Jacquiet et al., 2005). Além disso, foi observado que os linfócitos de ovinos previamente infestados não responderam a estimulação com antígenos específicos (produtos excretórios e secretórios de L2 e L3) após a terceira infestação, ou seja, a capacidade de resposta dos linfócitos diminui de acordo com o número de exposições, sugerindo atividade imunossupressora pelo parasita (Jacquiet et al., 2005).

Foi observado que em ovinos imunossuprimidos, que receberam tratamento com corticóide, o desenvolvimento larval foi mais rápido comparado com o controle que não recebeu tratamento, onde as L2 foram recuperadas em maior proporção. Comparando o

grupo imunossuprimido com o grupo de ovinos que haviam sido previamente parasitados pelas larvas, o estabelecimento larval foi similar, porém as L2 do grupo imunossuprimido tiveram peso mais elevado (Jacquiet et al., 2005). Vale ressaltar que *O. ovis* só se alimenta durante a fase de vida parasitária, enquanto larva, e, portanto, a redução do peso da larva madura pode comprometer a viabilidade da mosca (Cepeda-Palacios et al., 2000).

Portanto, estes estudos demonstram que apesar dos ovinos sofrerem sucessivas infestações pelas larvas de *O. ovis*, a aquisição de resistência é muito difícil, oposto do que é observado no parasitismo por nematódeos gastrintestinais. Mas a resposta imunológica pode ao menos manter o parasitismo sob controle, regulando a carga parasitária ou afetando o crescimento das larvas, e, por consequência, a viabilidade das moscas.

Interação entre a infestação por* O. ovis *e a infecção por nematódeos gastrintestinais

É muito comum um animal ser parasitado por vários organismos simultaneamente, a exemplo dos ovinos, que comumente são parasitados por nematódeos gastrintestinais e por larvas de *O. ovis*. Vários trabalhos foram realizados a fim de avaliar a interação entre a infestação por larvas de *O. ovis* e as infecções com nematódeos gastrintestinais parasitas de ovinos, como por exemplo a interação entre *O. ovis* e o parasita do intestino delgado *Trichostrongylus colubriformis* (Yacob et al., 2002; 2004; 2006) ou pelo parasita do abomaso *Haemonchus contortus* (Dorchies et al., 1997b; Terefe et al., 2005).

Nestes trabalhos os ovinos foram divididos em quatro grupos de animais: infestados apenas com larvas de *O. ovis*; infectados apenas com nematódeo gastrointestinal (*T. colubriformis* ou *H. contortus*); ovinos infectados com ambos parasitas; e grupo controle que permaneceram livres de infestações/infecções pelos

referidos parasitas. Os pesquisadores observaram que a infecção no trato digestivo por nematódeos não modificou a biologia da população de *Oestrus* na cavidade nasal. Já a presença do *O. ovis* foi relacionada com significativa redução na eliminação de ovos pelos helmintos, redução no tamanho e fecundidade das fêmeas e na carga parasitária. Estas mudanças foram associadas com eosinofilia e significativas modificações na população tecidual de mastócitos, leucócitos e eosinófilos nos tratos respiratório e digestivo. Com base nesses resultados foi observado que a infecção parasitária em uma determinada região anatômica provoca "à distância" reações inflamatórias em todo o sistema de mucosa, pois foram observadas mudanças na população celular tecidual em região anatômica que não estava parasitada.

Porém, apesar de existir correlação negativa entre o parasitismo por *O. ovis* e nematódeos gastrintestinais, a regulação é de natureza transitória e desaparece quando as larvas de *O. ovis* são expelidas pelo hospedeiro ou após tratamento com antiparasitário. O mecanismo envolvido na regulação não é específico e está relacionado com forte ativação dos eosinófilos sanguíneos que age de forma inespecífica sobre os vermes (Yacob et al., 2006).

Controle

O controle do parasitismo por *O. ovis* é feito exclusivamente com o uso de antiparasitários, tais como os organofosforados, triclorfon e closantel, e as lactonas macrocíclicas, como a ivermectina (Dorchies et al., 1997a; Lucientes et al., 1998), moxidectina (Dorchies et al., 1996), doramectina (Oliveira et al., 2000; Dorchies et al., 2001) e eprinomectina (Hoste et al., 2004; Habela et al., 2006).

Considerações finais

Oestrus ovis é um parasita morfológica e biologicamente muito bem adaptado aos ovinos. Tais adaptações permitem sua sobrevivência sob condições climáticas adversas e extremas, bem como, a resposta imune do hospedeiro.

Apesar da elevada prevalência e intensidade de infestação observada mundialmente, a oestrose pode ser considerada uma enfermidade negligenciada no Brasil. Praticamente não há estudos epidemiológicos sobre este parasita, e apenas com tal conhecimento é possível predizer quais os fatores climáticos que favorecem a infestação, bem como, recomendar a melhor época de tratamento.

O Capítulo 2 da Tese intitulado “Epidemiology of *Oestrus ovis* in sheep in the southwest of Brazil” e o Capítulo 3 intitulado “Prevalence and intensity of *Oestrus ovis* infestation in sheep in central region of São Paulo State, Brazil” tiveram por objetivos avaliar a variação sazonal e a intensidade de infestação por larvas de *O. ovis* em ovinos criados em Botucatu-SP e em Itápolis-SP, respectivamente.

O Capítulo 4 intitulado “Parasitism by *Oestrus ovis*: Influence of sheep breed and nematode infections” e o Capítulo 5 intitulado “Cellular and humoral immune responses in sheep naturally infested with *Oestrus ovis* (Diptera: Oestridae) and with nematode infections” tiveram por objetivos avaliar comparativamente, a resistência de cordeiros de duas raças ovinas, Ile de France e Santa Inês, contra infestações naturais por *O. ovis*, bem como a associação entre a ocorrência deste parasita com as infecções naturais por nematódeos gastrintestinais e a resposta imunológica envolvida na proteção contra estes parasitas.

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Capítulo 2

Epidemiology of *Oestrus ovis* in sheep in the southwest of Brazil[a]

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Abstract

The seasonal factors which influence *Oestrus ovis* infestation in sheep in São Paulo State in the southwest of Brazil were determined from April 2008 until March 2011. Two tracer lambs were exposed monthly to natural infestation by *O. ovis* larvae for 28 consecutive days, by grazing with a sheep flock. Tracer animals were then euthanized and the larvae of *O. ovis* recovered from nasal and sinus cavities. Of the 72 tracer lambs, 50% were infested with *O. ovis* larvae and the mean intensity of infestation per head infested was 16.8 larvae with an average of 7.8 L1, 5.3 L2 and 3.7 L3. In addition, clinic signs of oestrosis were evaluated in all sheep of the flock monthly and the average prevalence of animals with clinical signs of oestrosis was 13.4% (maximum of 31.4% in January 2009 and minimum of 1% in September 2010). The results suggest that evolution and development of larval instars practically occurs

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throughout the entire year, but *O. ovis* larval infestation was especially frequent during spring and summer months.

Key words: *Oestrus ovis*; epidemiology; sheep; control.

Introduction

Oestrosis is a worldwide myiasis caused by larvae of the fly *Oestrus ovis* (Linné 1761, Diptera: Oestridae), which are obligatory parasites of nasal and sinus cavities of sheep and goats. The female fly is viviparous and deposits larvae in or around the nostrils of its host. These early first instars attach to the mucous membranes in the nasal cavities, change to second instars and move up to the sinuses where it completes development in mature third instars, which are expelled for pupation under the soil (Zumpt, 1965). The length of this parasitic portion of the life cycle is quite variable from a few weeks to several months depending on the season and climatic conditions (Hall and Wall, 1995). Clinical respiratory signs such as seromucous or purulent nasal discharge, frequent sneezing and dyspnea, may severely impair the health of infested animals. Those pathological affects causes serious economical losses in small ruminant livestock (Alcaide et al., 2003), i.e., reduction in live weight gain (Horak and Snijders, 1974) and decrease in milk production of almost 9% (Dorchies et al., 2003).

Numerous studies regarding the epidemiology of *O. ovis* have been carried out in many countries but results are likely to be influenced by varying geographical and epidemiological conditions. In southwest France, for example, the infestation was present in 65% of the heads with mean intensity of 24.8 larvae/infested head consisting mainly of first larval instar (Yilma and Dorchies, 1991). In Sicily, Italy, the prevalence of infestation was 55.8% and all different larval stages were simultaneously recovered in similar proportions (Caracappa et al., 2000). In Brazil there are only few studies

about this parasite and these are restricted to States in South region, where favorable climatic conditions to *O. ovis* parasitism are observed throughout the year (Ribeiro et al., 1990; Oliveira et al., 1999; Ramos et al., 2006), excepting periods with temperature less than 9 °C when no larvae were recovered from tracer sheep (Ramos et al., 2006).

Oestrosis can be considered a neglected disease in Brazil, despite its increasing occurrence according to farmers and veterinary clinician's perception. Therefore, it is imperative more research about the epidemiology of the disease, since only with such knowledge it will be possible to predict environmental factors favoring infestation as well as to recommend the best strategies for oestrosis prophylaxis. This work was conducted to determine the seasonal variation of *O. ovis* infestation in sheep in the southwest of Brazil over a period of three years.

Materials and methods

Study location

The study was carried out in Botucatu, São Paulo state, Brazil, at an altitude of 786 m a.s.l.. Climate data referring to averages of temperatures, relative air humidity and rainfall were obtained by the Department of Environmental Science, Agronomical Science College, UNESP, located 8 km from the experimental site (Fig. 1 A and B).

For each month, from April 2008 until March 2011, two tracer lambs were exposed to natural infestation with *O. ovis* larvae for 28 consecutive days, while grazing together always with the same sheep flock. Immediately thereafter the tracer lambs were euthanized. Heads were removed and cut open along their longitudinal and sagittal axis. All larvae present in nasal cavity (nasal passage, septum, middle meatus and conchae) and frontal sinus were collected and counted. Larvae were preserved into 70% alcohol

and identified according to their stage of development based on description of the Zumpt (1965) and Capelle (1966).

Nasal discharge score (clinic signs of oestrosis) were recorded also in animals of the sheep flock on the first day of each month and in tracer sheep before euthanasia.

According to clinical signs, the scores of nasal discharge (ND) were the following: no ND = 0; dyspnea without nasal mucous ND = 1; dyspnea with sero-mucous ND = 2; and dyspnea with muco-purulent ND = 3. Results are presented as prevalence of sheep with clinical signs of oestrosis (%) and mean of ND score.

Management of tracer lambs

In total, over the three years of observations, 72 Ile de France weathered male lambs, were purchased from farms located in São Paulo State. Twelve lambs were purchased every six months, with initial age between two and five months.

Immediately after arrival at the University facilities, animals were housed, vaccinated against clostridial infections (Sintoxan Polivalente®, Merial, Brazil) and orally drenched, once daily, for three consecutive days with levamisole phosphate (10 mg/kg, Ripercol® L 150 F, Fort Dodge) and albendazole (10 mg/kg, Valbazen® 10 Cobalto, Pfizer). One week later, the same protocol was carried with triclorfon (100 mg/kg, Neguvon®, Bayer S.A.) to remove any existing infestation of *O. ovis* and infection by nematodes. Tracer lambs that stayed housed for more than one month, received an additional treatment with triclorfon (100 mg/kg; Neguvon® - Bayer S.A.) one week before being taken to the farm to remove any infestation by *O. ovis*.

While housed, the animals were fed on concentrate (Tech Ovin Unique, Socil®, with 18% of crude protein) in an amount corresponding to 1% of their mean live weight and had free access to Tifton hay and tap water. Decoquinate (Deccox®, Alpharma) was added to the commercial feed to prevent coccidiosis.

Sheep flock management

The sheep farm where the tracer lambs were placed belongs to University and consisted in156 Bergamacia sheep at the beginning of the study and 202 animals at the end. The sheep were kept in rotational grazing on *Panicum maximum* cv Tanzania grass and, every year, from June until early November, due to low amount of forage at this time, the animals received additional diet with corn silage once daily.

To avoid mortality of animals due to parasitism by gastrointestinal nematodes, the sheep flock received anthelmintic treatments with levamisole phosphate (10 mg/kg, Ripercol® L 150 F, Fort Dodge) or moxidectin (0.2 mg/kg, Cydectin® Fort Dodge). Details about these treatments are presented in Table 1.

Statistical analyses

Descriptive statistical analyses were performed in agreement with Bush et al. (1997). The following terms were used:

Prevalence: the number of hosts infested with *O. ovis* larvae divided by the number of hosts examined;

Intensity of infestation: the number of *O. ovis* larvae in a single infested host;

Mean intensity of infestation: the total number of *O. ovis* larvae found divided by the number of hosts infected with that parasite.

Results

Clinical signs of oestrosis in the sheep flock

The average prevalence of animals with clinical signs of oestrosis was 13.4% with maximum of 31.4% in January 2009 and minimum of 1% in September 2010. The overall ND average score during all years was 1.4 with minimum of 1 and of 1.8 (Fig. 2).

Prevalence and intensity of infestation in tracer sheep

O. ovis larvae were recovered from 50% of the tracer sheep during the three years of experiment. The mean intensity of infestation was 16.8 larvae per infested head with an average of 7.8 L1, 5.3 L2 and 3.7 L3 (Fig. 1 C).

The highest number of larvae recovered from a single sheep was 66 in September 2008 and of these, 64 were first stage (L1) and two second stage (L2). The highest mean larval burden was found at the beginning of spring in September and October 2008 (40 and 41 larvae/infested head, respectively).

O. ovis larvae were especially present during the spring and summer months (starting in September until March), except in 2009 when many larvae were also recovered during autumn (April to June 2009). This high rate of larvae recovery coincided with mean temperatures between 20 ºC and 25 ºC and the relative air humidity of around 70% (Fig. 1 A and B). No larvae were recovered in July when the minimum mean temperatures were < 14 ºC.

Of the total of 606 larvae, 46.4% was L1, while L2 and L3 represented 31.3% and 22.3% of the total larval burden, respectively. The majority of L1 were located in the nasal cavity (99.6%). In contrast L3 were found especially in the frontal sinus (97.8%). L2 were recovered from both sites: 40% from nasal cavity and 60% from frontal sinus.

The highest intensity of infestation occurred mainly during the first two years of the experiment. Considering the total number of larvae, 46.9 % was recovered in the first and 32.5 % in the second year. The lowest intensity of infestation was observed in third year with 20.6% of the total larvae recovered.

Most of the tracer lambs that were parasitized with *O. ovis* (30 of 36) did not show any clinical signs of oestrosis during the experiment. Only six animals showed

signs, i.e., dyspnea and sero-mucous discharge with average score of 1.7. The larval burden of these animals were variable, ranging from 12 to 66 larvae, constituted especially by L1 and L2.

Discussion

The prevalence of *O. ovis* in tracer lambs was 50% which is less than that observed in Bagé, Rio Grande do Sul State, Brazil, where 85.4% of sheep were infested (Ribeiro et al., 1990). However in Bagé, the animals were exposed to *O. ovis* infestation for a longer time period (from one month until one year) while in the present experiment the sheep were exposed for just 28 days. Moreover, others factors may have influenced the *O. ovis* epidemiology, like the climatic conditions of each region, size of sheep flock and management, which can result in variable prevalence demonstrated by many studies conducted in slaughterhouses in different countries (Yilma and Dorchies, 1991; Abo-Shehada et al., 2000; Scala et al., 2002; Alcaide et al., 2003; Arslan et al., 2009; Shoorijeh et al., 2009).

The mean intensity of infestation reported in this study (16.8) was similar than those previously reported for Sardinia, Italy (Scala et al., 2001) and southwest of Spain (Alcaide et al., 2003) with mean intensity of 19 and 18.5 larvae by infested head, respectively, but lower than that found in southwest of France with 24.8 larvae (Yilma and Dorchies, 1991) and in northeast of Spain with 37.9 (Gracia et al., 2010). It is of interest to note that a high percentage of first stage larvae were found by those authors, especially during the colder months. This was not observed in the present study where the proportion of different larval instars did not show variation throughout the experimental months.

The respective percentages of L1, L2 and L3 were 46.4%, 31.3%, 22.3%. The presence of different instars indicates that endogenous development and re-infestation with L1 occurred simultaneously. Otherwise, in southwest region of France, under temperate weather, L1 predominated with 90.5% of the total burden, while L2 (6.0%) and L3 (3.5%) displayed a low proportion (Yilma and Dorchies, 1991). A greater burden of L1 indicates the occurrence of a period of hypobiosis during the year, when *O. ovis* cease the development, or at least suggests a decrease in the rate of larval maturation. This was not observed in the present experiment, where it was observed synchronous evolution of all larval stages without any evidence of hypobiosis.

Among the climatic factors influencing oestrid fly activity, temperature, light intensity, and wind are recognized as the most important, but in the case of *O. ovis*, it has been reported that temperature is the principal factor determining fly activity (Cepeda-Palacios and Scholl, 2000). During all years of the study, averages of maximum temperatures were between 20.8 ºC and 29.8 ºC. It has been demonstrated that 20 ºC was the minimum temperature for fly activity while optimum temperature appear to range between 26 and 28 ºC (Cepeda-Palacios and Scholl, 2000). Therefore, the temperature conditions of the present study were optimal for fly activity during all months of the experimental years, excepted in two winter months (June and July), when regardless of mean maximum temperature of around 25 ºC, the mean of minimum were always less than 14 ºC. In these climatic conditions the fly activity was possibly reduced, especially in July, which coincided with reduction in number of sheep at the farm with clinical signs of oestrosis.

Nasal discharges and sneezing were evident in sheep from the farm during all study, but only six of the 72 tracer animals had clinical symptoms of oestrosis. The delay between the first infestation and the appearance of clinical signs suggests the

possible involvement of a hypersensitivity phenomenon in the modulation of the infection and these first infestations are possibly not associated with overt oestrosis clinical signs (Dorchies et al., 1998). Probably, due the short period between the infestation and euthanasia, most of the tracer sheep did not have time to develop and display immune response with sneezing and nasal discharge, which are characteristic clinical signs of parasitism by *O. ovis*, and the opposite occurred with the sheep from the farm, continuously exposed to infestation.

Among sheep from farm, the mean prevalence of animals with some clinical signs of oestrosis was relatively low (13.4%) with average score of 1.4. Studies of *O. ovis* distribution analysis demonstrated that the numbers of larvae recovered in the sheep population followed a negative-binomial distribution (Bart and Minar, 1992; Abo-Shehada et al., 2000) and similar results were observed with gastrointestinal nematode infections in sheep: a relatively small part of the flock constituted by susceptible animals was responsible for the excretion of the majority of gastrointestinal nematode eggs to environment (Sréter et al., 1994; Stear et al., 1998). Other possible explanation may be related with the immune response; in this case, oestrosis symptoms could be due to hypersensitivity reactions displayed only by a minor percentage of the flock.

A variation in *O. ovis* larval number was observed between the experimental years: the two first years concentrated 79.4% of total larval number recovered while just 20.6% of larval number was recovered during the last experimental year. These differences between the burdens in experimental years were probably a consequence of administration of broad spectrum anthelmintic to control gastrointestinal nematodes infection in sheep flock where the tracer lambs were kept. Initially levamisole phosphate was used and in the second and third year also moxidectin, a macrocycle

lactone that acts against *O. ovis* larvae (Dorchies et al., 1996). These treatments with moxidectin were necessary due to anthelmintic resistance to other anthelmintics and probably caused reduction in *O. ovis* population in the farm.

In conclusion, it is possible to affirm that several annual generations of adult flies may occur in the region and probably in other areas with similar climatic conditions. New studies will be necessary to evaluate the impact of oestrosis in sheep production, as well the best strategies of prophylaxis.

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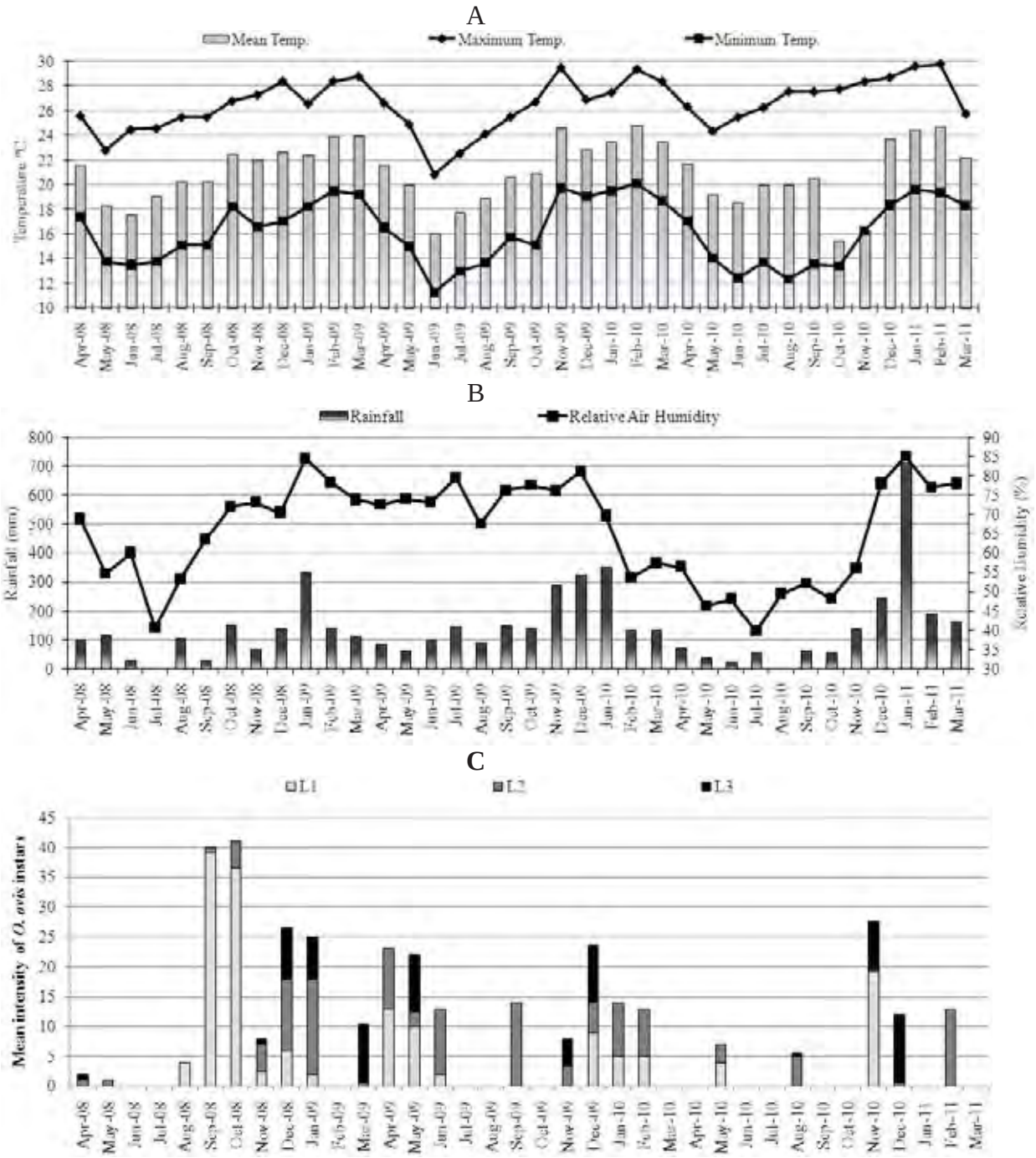

Figure 1. Average of maximum, minimum and mean monthly temperatures (A); monthly rainfall and relative air humidity (B); and monthly mean intensity of first larval instar (L1), second larval instar (L2) and third larval instar (L3) of *Oestrus ovis* infestation in tracer sheep (C) from April 2008 until March 2011.

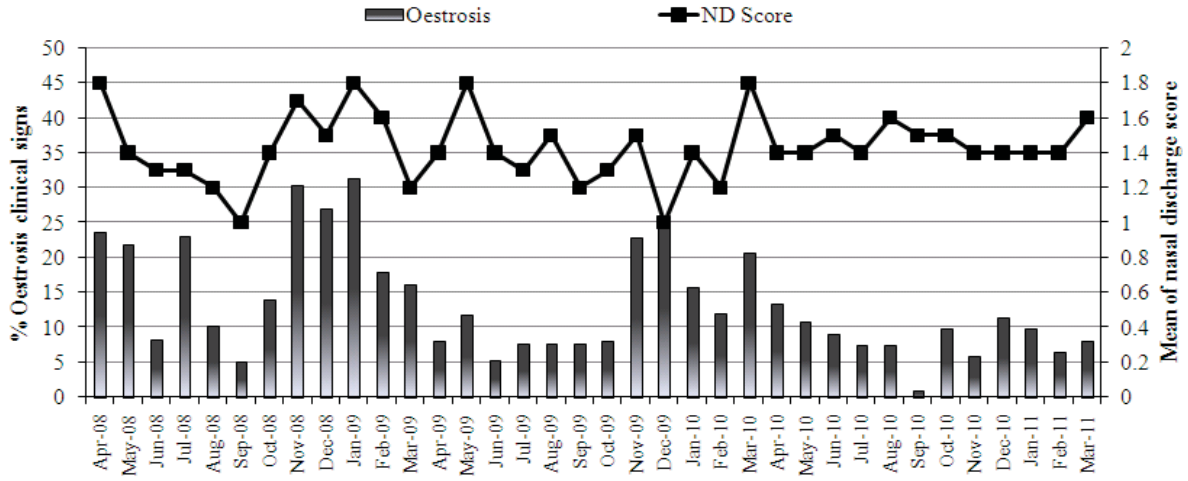

Figure 2. Prevalence of clinical signs of oestrosis (%) in the sheep of the flock and mean nasal discharge score (ND) in those animals displaying clinical signs. Dyspnea without nasal mucous: ND = 1; dyspnea with sero-mucous: ND = 2; and dyspnea with muco-purulent: ND = 3.

Table 1. Percentage of animals of the sheep flock that received anthelmintic treatment with levamisole phosphate or moxidectin from April 2008 to March 2011.

Year		Jan	Feb	Mar	Apr	Mai	Jun	Jul	Aug	Sep	Oct	Nov	Dec
2008	Levamisole				9.2	13.8	0	100	36.8	7.2	100	0	100
	Moxidectin				0	0	0	0	0	0	0	0	0
2009	Levamisole	0	100	0	100	0	0	23.3	12.9	0	4.5	5.9	20.3
	Moxidectin	0	0	0	62.9	0	0	0	0	0	0	0	0
2010	Levamisole	9.9	5.4	0	21.3	1.5	100	10.9	0	0	0	0	30.7
	Moxidectin	0	0	0	0	0	0	37.1	21.8	0	25.7	9.4	6.4
2011	Levamisole	0	5.0	0									
	Moxidectin	24.3	26.7	9.9									

Capítulo 3

Prevalence and intensity of *Oestrus ovis* infestation in sheep in central region of São Paulo State, Brazil[b]

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Abstract

Heads of 139 sheep were examined with the aim of determining the *Oestrus ovis* prevalence and infestation intensity in central region of São Paulo State, Brazil. Heads from slaughtered sheep were examined and the different *O. ovis* larval instars (L1, L2 and L3) were recovered from the nasal and sinus cavities. *O. ovis* larvae were detected in 13.7% of sheep. The monthly mean intensity of infestation ranged from 1 until 10.2 larvae/infested head with general mean intensity of 4.5 larvae/infested head. Of the total of 85 larvae, 21.2% were L1, 37.6% L2 and 41.2% L3. The present results demonstrate

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that oestrosis is an important parasitic disease in sheep of the central region of São Paulo State, which must be considered in programs of prophylaxis of sheep diseases.

Key-words: *Oestrus ovis*; sheep; prevalence; infestation.

Introduction

Oestrus ovis (Diptera: Oestridae), the sheep nasal bot fly, is a cosmopolitan parasite whose larvae develop in the head sinuses and nasal passages of sheep and goats in all sheep-farming areas of the world. The female fly is viviparous and deposit larvae in or around the nostrils of its host and these early first instars attach to the mucous membranes in the nasal cavities, change to second instars and move up to the sinuses where it completes development in mature third instars, which are expelled for pupation under the soil (Zumpt, 1965). The length of this parasitic portion of the life cycle is quite variable from a few weeks to several months depending on the season and climatic conditions (Cobbett and Mitchell, 1941). Clinical respiratory signs such as seromucous or purulent nasal discharge, frequent sneezing and dyspnea, may severely impair the health of affected animals (Dorchies et al., 1998).

O. ovis can thrive in different environments and has adapted to the climate prevailing wherever sheep are kept (Horak, 1977) and the ability to adapt to different environments allows to the natural persistence of infestation and the difficulties for its control (Alcaide et al., 2005). Many surveys have demonstrated the high prevalence of ovine oestrosis in numerous areas all over the world, e.g. Zimbabwe (Pandey, 1989), France (Yilma and Dorchies, 1991), Italy (Caracappa et al., 2000), Spain (Alcaide et al., 2003) and Greece (Papadopoulos et al., 2010). Oestrosis is commonly seen in sheep in Brazil but there are few studies about the epidemiology of this parasite and most of

them are restricted to States in south region where favorable climatic conditions are observed throughout the year.

In Rio Grande do Sul State, the prevalence of oestrosis in sheep was of 85.4% in Bagé (Ribeiro et al., 1990) and in Encruzilhada do Sul, the mean intensity of infestation was 23.8 larvae (Oliveira et al., 1999). In Santa Catarina State the *O. ovis* infestation intensity were greater during the spring and summer months and no larvae were recovered from tracer sheep when the temperature was less than 9 °C (Ramos et al., 2006).

The first study about *O. ovis* epidemiology in São Paulo State was conducted recently in southwest region where prevalence was 50% with mean intensity of 16.8 larvae per animal with the highest infestation rate in spring and summer time (Silva et al., 2011). São Paulo State has different climatic conditions in each region and small sheep farms could be found in the most part of this State. Therefore detailed research about the epidemiology of parasitic infestation is required to recommend the best strategies of oestrosis prophylaxis in small ruminants. This study aimed to determine the *O. ovis* prevalence and infestation intensity in slaughtered sheep from central region of São Paulo State, Brazil.

Materials and methods

A total of 139 sheep heads were examined from July 2009 to February 2010. The animals were originated from Itápolis, Ibitinga, Borborema, Guarantã and Tapinas, counties from the central region of São Paulo State. The monthly numbers of sheep slaughtered in an abattoir in Itápolis city was variable and heads from all animals were examined. The maximum and minimum numbers of heads examined per month were 26 in July and August 2009 and nine in October 2009, respectively (Table 2).

Most of the sheep were ≤ 1 year old (89.2%), Santa Ines (80.6%) breed and its crosses with Dorper breed. Information on prior antiparasitic treatment and sheep management were not available. Climate data were obtained by the Center of Meteorological and Climate Research Applied to Agriculture (CEPAGRI / UNICAMP). The annual rainfall average for the last 10 years was 1416.5 mm and the annual mean temperature average for this period was 22.9 °C (Table 1).

Heads of slaughtered sheep were separated from the carcasses, put into an individual plastic bag to avoid the transfer of larvae from one head to another and to collect those that came out of nasal cavities. They were transported to Laboratory inside a cool box with ice. Heads were cut open along their longitudinal and sagittal axis. All larvae present in nasal cavity (nasal passage, septum, middle meatus and conchae) and frontal sinus were collected and counted. Larvae were preserved in 70% alcohol and identified according to their stage of development based on description of the Zumpt (1965) and Capelle (1966).

Statistical analyses

Descriptive statistical analyses were performed in agreement with Bush et al. (1997). The following terms were used:

Prevalence: the number of hosts infested with *O. ovis* larvae divided by the number of hosts examined;

Intensity of infestation: the number of *O. ovis* larvae in a single infested host;

Mean intensity of infestation: the total number of *O. ovis* larvae found divided by the number of hosts infected with that parasite.

Results

Of the 139 heads examined, 19 (13.7 %) were infested with *O. ovis* larvae with minimum and maximum prevalence of the 7.7 % in August 2009 and 20.0 % in February 2010, respectively (Table 2). The monthly mean intensity of infestation ranged from 1 to 10.2 larvae/infested head with general mean of intensity of 4.5 larvae/infested head.

In October 2009 no *O. ovis* larvae was recovered, coinciding with the lowest number of heads examined. Of the total of 85 larvae, 21.2% was L1, while L2 and L3 represented 37.6% and 41.2%. The sheep heads collected were derived mainly from young animals (≤ 1 year old) but the prevalence of *O. ovis* larvae were similar between the ages groups (Table 3).

Discussion

The prevalence of *O. ovis* infestation (13.7 %) in this study was lower than those reported in similar studies carried out in other countries such as France, where 65% of sheep was infested with *O. ovis* larvae (Yilma and Dorchies, 1991) or in Sicily with 55.8% of prevalence (Caracappa et al., 2000) and Turkey with 40.6% (Arslan et al., 2009). Higher prevalence was also observed in other Brazilian studies: 85.4% in southern Brazil in sheep exposed to natural infestation during one year (Ribeiro et al., 1990) and 50% of prevalence in tracer sheep in Botucatu, São Paulo State, Brazil (Silva et al., 2011).

Despite the lower prevalence observed in the present study, *O. ovis* larvae were present in all experimental months, except in October, coinciding with the lowest number of sheep heads examined. Probably if more heads had been monthly examined we would have more chances to find infested sheep, like is observed in studies done in

other countries where the sheep slaughter routine is high and frequent. In Sicily, as example, 70 heads were examined per month (Caracappa et al., 2000) whereas in the present study in the maximum were examined 26 sheep heads per month. In São Paulo State, Brazil, large slaughterhouses are common which are specialized in slaughtering cattle, poultry and pigs. Slaughter of sheep, in general, is restricted to small abattoirs and with low slaughter routine, making it difficult to collect a larger number of heads to examine.

Many factors can influence the *O. ovis* epidemiology such as sheep management practices and the particular climatic conditions. The low prevalence of oestrosis in these animals may have been influenced by previous treatment with drugs, such as macrocyclic lactones, which are usually used for prophylaxis of infections with gastrointestinal nematodes and have action against *O. ovis* larvae instars (Dorchies et al., 1996; 1997; Lucientes et al., 1998). Influence of anthelmintic treatment on *O. ovis* epidemiology was also observed in Botucatu, where a reduction in infestation of tracer sheep coincided with the treatment of the flock with macrocyclic lactones (Silva et al., 2011).

Among the climatic factor influencing oestrid fly activity, temperature, light intensity and wind is considered the most important, but in case of *O. ovis*, the temperature is the principal factor affecting the larvipositional activity of gravid females (Cepeda-Palacios and Scholl, 2000). Although the sheep heads were examined during the eight months, the climatic conditions of the region of the sheep origin should be favorable to *O. ovis* activity during all months of the year, where the mean of temperature is around 22 ºC and maximum and minimum averages around 16.3 and 29.6 ºC. Previous studies demonstrated that *O. ovis* fly activity start when the temperature is around 20 ºC, but the major peak of strikes was seen between 25 and 28

ºC (Horak, 1977; Cepeda-Palacios and Scholl, 2000) that coincide with the average of maximum temperature in this place.

It is interesting to note that mainly L2 and L3 *O. ovis* larval instars were recovered in this study. Probably the climatic conditions were suitable to endogenous larval development and L1 were able to change to L2 in a short period of time. Study showed that larvae in sheep head can have a rapid development and reach the maturity in three weeks (Cobbett and Mitchell, 1941). In Santa Catarina State, Brazil, greater L3 numbers also were recovered from tracer sheep (Ramos et al., 2006), but probably because animals were exposed to infestation for 28 days and then were housed for more 20 days before slaughter, larvae had enough time to complete the development.

In conclusion, despite of the lack of information on prior antiparasitic treatment and sheep management, which may influence oestrosis prevalence, all different *O. ovis* larvae instars were recovered from slaughtered animals. This suggests the existence of favorable climatic conditions for the fly activity and larval development of *O. ovis* in sheep from central region of São Paulo State.

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The authors declare that they have no conflict of interested.

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Table 1. Average of maximum, minimum and mean monthly temperature and rainfall in the last 10 years in Itápolis city, São Paulo State.

Month	Temperature (ºC)			Rainfall (mm)
	Minimum	Maximum	Mean	
January	19.5	30.9	25.2	235.9
February	19.7	31.1	25.4	204.5
March	19.0	30.8	24.9	158.2
April	16.4	29.5	22.9	78.9
May	13.8	27.6	20.7	65.0
June	12.4	26.6	19.5	43.5
July	11.9	26.9	19.4	22.9
August	13.4	29.3	21.4	25.7
September	15.5	30.3	22.9	70.6
October	17.1	30.6	23.9	121.2
November	17.9	30.8	24.3	147.7
December	19.0	30.5	24.7	242.4
Annual	16.3	29.6	22.9	1416.5
Min	11.9	26.6	19.4	22.9
Max	19.7	31.1	25.4	242.4

Source: CEPAGRI / UNICAMP

Table 2. Numbers of heads infested with *Oestrus ovis* per month, mean larval burden per head, number of first, second and third larval stage (L1-L3) and total number of larvae found per month in sheep from central region of São Paulo State, Brazil.

Month	Number of heads examined	Number of heads infested	Prevalence (%)	Mean larval burden	L1	L2	L3
July 2009	26	5	19.2	10.2	17	19	15
August 2009	26	2	7.7	2.0	0	0	4
September 2009	13	2	15.4	1.0	0	0	2
October 2009	9	0	0	0	0	0	0
November 2009	12	1	8.4	4	0	0	4
December 2009	16	3	18.8	4.7	1	9	4
January 2010	17	2	11.8	1.0	0	0	2
February 2010	20	4	20.0	2.0	0	4	4
Total	139	19			18	32	35
Mean			13.7	4.5			

Table 3. Prevalence of infestation by *Oestrus ovis* according to sheep age.

Age	Heads examined/heads infested	Prevalence (%)
1 ≤	124/16	12.9
2	8/2	25.0
≥ 3	7/1	14.3
Total	139/19	13.7

Capítulo 4

Parasitism by *Oestrus ovis*: Influence of sheep breed and nematode infections[c]

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Abstract

Previous studies showed that Santa Ines (SI) hair sheep were more resistant to gastrointestinal nematode infections (GIN) than Ile de France (IF) sheep. The present experiment aimed to evaluate if that reported resistance difference against GIN also occurred against *Oestrus ovis* infestation and also to evaluate the influence of *O. ovis* infestation on the gastrointestinal nematodes (GIN) infections. SI (n=12) and IF (n=12) young male lambs were weaned at two months of age and moved to a paddock (0.3 ha) with *Brachiaria decumbens* grass, where they also received concentrate ration. The

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animals were kept together during the experimental period (September to early December 2009). Fecal and blood samples were taken from all animals every two weeks and body weight and nasal discharge score (oestrosis clinic signs) were recorded on the same occasion. In early December 2009, all lambs were sacrificed and *O. ovis* larvae and GIN were recovered, counted and identified according to the larval stage. All animals were infested by different larval instars of *O. ovis* without any statistical difference between breeds ($P > 0.05$). The SI lambs had an average of 24.8 larvae, and the intensity of infection ranged between 14 and 39 larvae, while the IF lambs showed an average of 23.5 larvae with the minimum and maximum from 11 to 36 larvae, respectively. SI lambs presented the lowest nematode fecal egg counts (FEC) and the lowest mean numbers of *Haemonchus contortus*, *Trichostrongylus colubriformis* and *Strongyloides papillosus*, however, there was no significant differences between group means ($P > 0.05$). Inverse relationship between numbers of *O. ovis* larvae and gastrointestinal nematodes was observed in both breeds. SI sheep showed a significant increase in blood eosinophils and total IgE serum levels and these variables were negatively correlated with nematode FEC. A negative correlation was observed between total IgE serum level and *H. contortus* burden in both breeds. In conclusion, there was no breed difference regarding *O. ovis* infestation and in each breed, animals with more nasal bot fly larvae tended to display smaller worm burden.

Key words: *Oestrus ovis*, nematodes, genetic resistance, breed-effect, sheep, total IgE

Introduction

Infections caused by gastrointestinal nematodes are the major constraint in small ruminant production, however concurrent infections of sheep with nasal bot fly *Oestrus ovis* and nematodes of the digestive tract are common (Yacob et al., 2002). *O. ovis*

causes cavitary myiasis in sheep and goats with clinical respiratory signs such as seromucous or purulent nasal discharge, frequent sneezing and dyspnea, which may severely impair their health. Those pathogenic effects cause serious economical losses in the small ruminant livestock (Alcaide et al., 2003).

Haemonchus contortus and *Trichostrongylus colubriformis* are the major gastrointestinal nematodes infecting sheep in Brazil (Amarante et al., 2004; Ramos et al., 2004; Louvandini et al., 2006). The increasing development of resistances to anthelmintics in these nematode populations (Almeida et al., 2010) imposes the need to find alternative approaches less dependent on anthelmintic treatments for parasitic control. Such strategies include, for example, pasture decontamination using different herbivore species (Fernandes et al., 2004; Rocha et al., 2008) and the breeding of genetically resistant animals (Amarante et al., 2004; Good et al., 2006; Alba-Hurtado et al., 2010).

Epidemiological observations from field studies indicated that sheep selected for resistance to gastrointestinal nematodes were more infected with the nasal bot fly *O. ovis* than susceptible animals (Yacob et al., 2001). Many studies have shown negative interactions between parasitism by *O. ovis* larvae and gastrointestinal nematodes (Dorchies et al., 1997; Yacob et al., 2002; 2004; 2006; 2008; Terefe et al., 2005). The presence of *O. ovis* larvae is correlated with significant reductions in nematode egg excretion, worm fecundity and worm burdens. It was clear that *O. ovis* larvae had an antagonistic effect on the worm populations but in contrast, the presence of nematodes was not related with any change in the biology and larval burden of *O. ovis* (Yacob et al., 2002; Terefe et al., 2005).

Sheep of the Santa Ines breed originated from the northeast of Brazil but has been widely distributed throughout the southeast region of Brazil in the last few years. It

shows greater resistance to *H. contortus* infection than sheep of European breeds, common in the same region (Amarante et al., 2004; Rocha et al., 2004; Bricarello et al., 2005; Amarante et al., 2009). However, there is no information available about the comparative resistance of Santa Ines and Ile de France sheep breeds to natural infestation by *O. ovis*. Therefore, the purpose of this study was to examine the comparative resistance to a natural infestation by *O. ovis* larvae in young lambs of two sheep breeds (Santa Ines and Ile de France) and determine the interaction of this insect infestation with naturally acquired gastrointestinal nematode infections.

Material and Methods

Study location

The study was carried out in Botucatu, São Paulo state, Brazil, at an altitude of 786 m a.s.l.. Climate data relating to averages in temperatures, relative air humidity and rainfall were obtained by the Department of Environmental Science, Agronomical Science College, UNESP, located at 8 km from experimental site. The average monthly relative humidity was always higher than 76% throughout the experimental period that occurred from September to December 2009. As usual for the region, rainfall was abundant (150.5 mm in September, 141.8 mm in October, 289.0 mm in November and 327.1 mm in December) with a total 908.3 mm. Maximum and minimum mean temperature ranged from 25.5 °C to 15.8 °C in September and from 29.5 °C to 19.8 °C in November. The time of the year chosen to conduct the experiment was based on an epidemiological study carried out recently in the same area that showed a high incidence of oestrosis in sheep from September to December (unpublished data).

Animals and experimental design

Twelve young intact male Santa Ines (SI) and Ile de France (IF) lambs were purchased from farms located in São Paulo state. Four lambs were acquired from each farm to assure a minimum of genetic variability in each breed. All lambs were born in June 2009, except four Ile de France lambs, which were born in May. Lambs were weaned at two months of age and moved in late August to University facilities. The animals were kept exclusively in pasture during the experimental period in a paddock (0.3 ha) with *Brachiaria decumbens* grass, where they had free access to tap water. In order to prevent massive infection by GIN, the animals were also supplemented, daily, with commercial feed (Tech Ovin Unique, Socil®, with 18% of crude protein) in an amount corresponding to 3% of their mean live weight. The amount of the commercial diet was adjusted every 14 days according to lambs body weight gain. Decoquinate (Deccox®, Alpharma) was added to the commercial feed to prevent coccidiosis. Early in the trial, lambs received specific vaccine against clostridial infections (Sintoxan Polivalente®, Merial, Brazil)

Fecal and blood samples were taken, body weight and nasal discharge score (oestrosis clinic signs) were recorded from all animals every two weeks.

Fecal examination revealed that animals were infected with gastrointestinal nematodes at their arrival to University facilities. In order to start the trial with animals in the same conditions, they were drenched orally, once daily, for three consecutive days with levamisole phosphate (10 mg/kg, Ripercol® L 150 F, Fort Dodge) and albendazole (10 mg/kg, Valbazen® 10 Cobalto, Pfizer). The results obtained in the first sampling were not included in the analysis. Two SI lambs died early in the trial of unknown causes and the data for these animals were excluded from analyses.

At six months of age, in early December 2009, the animals were euthanized for *O. ovis* larvae identification and counting and worm burden determination. The fresh carcass weight was also recorded in an electronic balance (Modelo BCW 15, Welmy®).

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 McMaster technique, in which each nematode egg counted represented 100 eggs per gram (EPG) of feces. Composite fecal cultures for the production of infective larvae of gastrointestinal nematodes were prepared separately for each breed, and the larvae obtained were killed, stained with Lugol's iodine and identified, according to the descriptions of Keith (1953).

Pasture infectivity

To determine the number of third stage larvae (L3) per kilogram of dry matter (L3/kg DM), grass samples were collected manually from paddock, close to the soil, approximately every 3.5 m. The collector followed a W-track on the paddock (Taylor 1939). Samples were processed in the laboratory, according to Niezen et al. (1998), and the larvae obtained were killed, stained with Lugol's iodine and identified, according to the descriptions of Keith (1953).

Hematology

Blood samples were collected by jugular vein puncture into vacutainer tubes with and without anti-coagulant (EDTA). Packed cell volume (PCV) was determined by micro-haematocrit centrifugation and total plasma protein levels (g/dL) were estimated using a refractometer (Refractometer SPR-N, Atago). The 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. Serum samples were stored at -20 °C until immunoglobulin measurements and serum albumin was determined using a kit (Protal método colorimétrico® – Laborlab) and absorbance was read at 625 nm. The albumin/globulin ratio was estimated by the formula: albumin/globulin = concentration of albumin / (total protein – albumin concentration).

Serology – sandwich enzyme immunoassay (EIA) for IgE antibody

Total IgE levels in the animal's sera were measured using sandwich enzyme immunoassay (EIA) as previously described (Shaw et al., 1997).

Oestrus ovis: clinic signs and larvae count post mortem

Signs of infestation and their clinical severity were measured by the score of nasal discharge (ND). The score was the following: no ND = 0; only dyspnea without secretion = 1; sero-mucous ND = 2; and muco-purulent ND = 3.

After euthanasia, heads were removed and cut open along their longitudinal and sagittal axis. All larvae present in nasal cavity (nasal passage, septum, middle meatus and conchae) and frontal sinus were collected and counted. Larvae were preserved into 70% alcohol and identified according to their stage of development based on description of the Zumpt (1965) and Capelle (1966).

Worm counts

The abomasum was opened along its greater curvature and the contents placed in a container. A 10% aliquot of the abomasal contents was preserved in 5% formalin. The mucosal layers of all abomasums were soaked in saline solution at 38 °C for 6 h. All content of the digested material was collected and preserved in 5% formalin. A similar procedure was used to process the small intestine, but a 10% aliquot of the material

digested was collected. The large intestine was opened and a 10% aliquot of the contents was collected and preserved. Worm identification and counting procedures were performed in the preserved material as describe by Ueno and Gonçalves (1998).

Statistical analyses

Significant differences between groups for all variables were assessed by one-way analysis of variance using Minitab 11.21 statistical software (Minitab Inc., USA). Group means were considered statistically different when $P < 0.05$. EPG, worm burden, *Oestrus* larval numbers, eosinophils/μL and nasal discharge score data were transformed using $\log_{10}$ (x+1) prior to analysis. The results in figures and tables are expressed as arithmetic means (± standard error of the mean). Spearman's correlation coefficient between variables was assessed using the same software. To test if there was difference between breeds regarding proportions of larval instars of *O. ovis*, the Chi-square test for trend was performed using SAS (release 9.2).

Results

Oestrus ovis: clinic signs and larvae count post mortem

The animals, from both breeds, showed no oestrosis clinics signs in the first month of trial (September), but, from October until the end of experiment, most of the lambs showed oestrosis clinic signs with average of scores ranging from 0.5 to 1.7. On 18th November the IF lambs showed average score (1.7) significantly higher ($P < 0.05$) than SI lambs (0.8).

When euthanized, all three *O. ovis* larval instars were observed with prevalence of 100% in both breeds (Table 1). The mean intensity of infestation was 24.8 larvae / animal in SI lambs, with the minimum of 14 and maximum of 39 larvae per animal. In IF lambs the mean intensity of infestation was 23.5 larvae / animal and the number of

larvae ranged from 11 to 36 larvae. There was no significant difference between breeds ($P > 0.05$). The first stage larvae (L1) were predominant in both breeds (Table 1). However, there was significant difference ($P < 0.05$) between breeds regarding the proportion of larval instars of *O. ovis*: The total larval burden in SI lambs was composed by 75.8% L1, 11.7% L2 and 12.5% L3, while IF lambs had 57.5% L1, 18.4% L2 and 24.1% L3.

Fecal examination

At arrival of the experimental site, the mean of Strongyle and *Strongyloides papillosus* FEC were, respectively, of 2275 and 900 EPG in IF lambs and of 3660 and 30 EPG in SI lambs. *Haemonchus* spp. was the predominant genus in fecal cultures (93% in IF and 83% in SI) followed by *Trichostrongylus* spp. (7% in IF and 17% SI). Fifteen days after the anthelmintic treatment, mean FEC dropped to 60 and 20 EPG (Strongyle and *S. papillosus*, respectively) in SI lambs and 158 and 75 EPG (Strongyle and *S. papillosus*, respectively) in IF lambs.

During the sampling period, the Strongyle FEC in IF lambs increased gradually until 4th November and thereafter there was a slight decrease in the mean FEC. The FEC in SI lambs also increased during the first collections with the maximum mean FEC of 781.8 (± 194.4) on 21st October, but then the mean Strongyle FEC decreased and was near zero in the last sampling (80.0 ± 32.7). The *S. papillosus* FEC also increased gradually throughout the experiment in both breeds, with IF lambs displaying a higher mean than SI lambs (725.0 ± 263.7 and 250.0 ± 80.6, respectively) in the last collection. Despite the IF lambs showing the highest Strongyle and *S. papillosus* FEC (Fig. 1), no significant difference was observed between breeds during the experiment ($P > 0.05$). The animals were also observed to be infected with *Moniezia* spp.

It was noted that there was variability in susceptibility to GIN parasitism within each breed. Within SI animals, there were three resistant lambs, whose FEC remained low from the beginning of the experiment (< 500 EPG), while others showed relatively high FEC with a maximum of 19,600 EPG in one animal, but over the course of the trial these lambs developed protective immunity that resulted in a sharp FEC reduction that were close to zero in all animals at the end of the experiment. Among the IF lambs, there were resistant lambs; susceptible animals that acquired resistance throughout the experiment; and four susceptible lambs, which remained with high FEC, with counts up to 6600 EPG. Despite the high FEC observed in these IF lambs, salvage anthelmintic treatments were not necessary during the experiment.

Haemonchus spp. was the predominant nematode in fecal cultures ranging between 67% and 98% in IF and between 86% and 98% in SI, followed by *Trichostrongylus* spp. ranging from 2% and 33% in IF and from 2% and 14% in SI lambs. *S. papillosus* larvae were also found in fecal cultures in both breeds in all sampling.

Nematode larvae on pasture

The *Haemonchus* spp. infective larvae were the major species recovered from herbage. In the first collection no infective larvae was recovered, but from the 23rd September significant numbers of *Haemonchus* spp. infective larvae per kilogram dry matter (L3/kg DM) were recovered with a peak of 1713.7 L3/kg DM on 4th November. Infective larvae of *Trichostrongylus* spp. were recovered during the experiment but only in two samplings and in low numbers (41.1 L3/kg DM on 18th November and 59.5 L3/kg DM on 2nd December).

Hematology

The lambs of both breeds had mean packed cell volume (PCV), total plasma protein (TPP), albumin and globulin within the normal range for sheep. Initially the mean of PCV was 33% (± 1.0%) and decreased gradually throughout the experiment until reaching the lowest mean values of 27.6% (± 0.7%) in IF lambs and 27.7% (± 0.8%) in SI lambs on 4th November (Fig. 2 A). The TPP in IF lambs ranged between 5.4 and 6.0 g/dL and in SI lambs from 5.1 to 6.0 g/dL throughout the experiment ($P > 0.05$) (Fig. 2B).

Albumin values ranged between 3.0 and 3.9 g/dL in IF lambs and between 3.1 and 3.8 g/dL in the SI ($P > 0.05$) (Fig. 2C). The ratio of albumin/globulin during the experiment ranged from 2.1 to 2.7 g/dL in IF lambs and between 2.1 to 2.5 g/dL in SI ($P > 0.05$).

In all experimental weeks, the SI lambs had significantly higher mean number of blood eosinophils than IF lambs ($P < 0.05$), except on 7th October and 2nd December, when there was no significant difference between the breeds. IF lambs had mean (± SE) values between 189.6 (±49.7) and 814.6 (±181.9) eosinophils/µL while the average of the SI ranged from 460.0 (±145.6) to 2131.8 (±222.6) eosinophils/µL of blood throughout the experiment (Fig. 3).

Serology – sandwich enzyme immunoassay (EIA) for IgE antibody

In all experimental weeks, the SI lambs had higher total serum IgE levels compared with IF lambs (Fig. 4), which were significantly higher on 23rd September, 21st October, 4th November and 2nd December ($P < 0.05$).

Weight gain

IF lambs showed mean daily weight gain (200 g/day) significantly ($P < 0.01$) higher than SI lambs (154 g/day). The animals were sacrificed at 6-7 months of age, when IF lambs showed a mean of 40.6 kg (± 1.87) and the SI 27.9 kg (± 1.50). Likewise, the average hot carcass weight was significantly higher in IF lambs (16.6 kg ± 0.94) compared with SI (11.4 ± 0.66 kg) ($P < 0.05$).

Worm counts

The following species were found in gastrointestinal contents of the animals: *H. contortus*, *T. colubriformis* and *S. papillosus* (Table 1). The mean worm counts were always higher in IF lambs than SI, but without significant differences between breeds ($P > 0.05$). *Moniezia* spp. specimens were also recovered from most of the lambs of both breeds in small numbers.

Correlation coefficients

Although the experimental groups were composed of a limited number of animals, correlation coefficients were calculated between the number of *H. contortus*, *T. colubriformis*, *S. papillosus* and *O. ovis* separately for each breed. Negative correlation coefficients were observed between *O. ovis* x *H. contortus* and *O. ovis* x *T. colubriformis* worm burdens in both breeds, however, significant correlation occurred only between *O. ovis* larvae x *S. papillosus* ($r = -0.614$; $P < 0.05$) just in SI lambs (Table 2).

Significant negative correlations were observed in SI lambs between total IgE x *S. papillosus* worm burden ($r = -0.648$; $P < 0.05$) and in IF lambs between total IgE x *H. contortus* worm burden ($r = -0.594$; $P < 0.05$). In SI animals correlation between total IgE x *H. contortus* was also negative ($r = -0.576$, $P = 0.08$) (Table 3).

In some sampling dates, significant negative correlations were observed just in SI lambs: on 21st October and 4th November between total IgE x Strongyle's FEC (r = -0.631 and r = -0.699, respectively; $P < 0.05$); on 18th November and 2nd December between total IgE x FEC of *S. papillosus* (r = -0.968; r = -0.752, respectively; $P < 0.05$); and between blood eosinophils x FEC of Strongyle (r = -0.764 e r = -0.745, respectively; $P < 0.05$).

Discussion

Sheep of the SI breed are known to exhibit greater resistance to gastrointestinal infections than sheep of European breeds (Amarante et al., 2004; 2009; Rocha et al., 2004; Bricarello et al., 2005). Such difference between breeds was not observed regarding *O. ovis* infestation, which were similar in both breeds. However, epidemiological observations from field studies indicated that sheep selected for resistance to gastrointestinal nematodes were more infected with the nasal bot fly, *O. ovis* than susceptible ones (Yacob et al., 2001).

All different larval instars were recovered from animals of both breeds, but the proportions of different instars were different between the breeds with higher proportion of L1 in SI lambs. Possibly, there was delay in larval development in SI lambs caused by a more intense immune response in comparison with IF. In other studies it was demonstrated that immune response against *O. ovis* can impair larval development (Frugeré et al., 2000; Angulo-Valadez et al., 2007).

In the present study young animals (less than six months old) were evaluated. If the trial had had a longer duration, perhaps statistical difference between the breeds could have been evidenced. However, some studies showed that despite sheep of all ages being affected by oestrosis, the largest infestations were found in sheep more than

4-years-old (Abo-Shehada et al., 2000; Uslu and Dik, 2006; Arslan et al., 2009; Shoorijeh et al., 2009). These results indicate that the ability of animals to become oestrosis' resistant is very limited, in contrast to what occurs with GIN infections.

Concurrent infections of sheep with *O. ovis* and nematodes of the digestive tract are common in the field as demonstrated in the present experiment, in which *H. contortus* was the predominant nematode species in lambs of both breeds, followed by *T. colubriformis* and *S. papillosus*, in agreement with previous studies in the same region (Amarante et al., 2004). However, the number of parasites recovered from the lambs in the present experiment was much lower than it was observed previously by Amarante et al. (2004), who reported, on average, *H. contortus* worm burden almost two times higher in SI sheep and approximately six times higher in IF animals than in the present trial. Similarly for *T. colubrifomis* worm burden, almost nine times higher in SI animals and almost 17 times higher in IF sheep.

The relatively low parasite worm burden observed in animals of the present experiment may have been due to the influence of two factors. First, parasitism by *O. ovis* may have induced nonspecific immune response that indirectly protected against gastrointestinal nematodes. Several studies have shown that there is negative interaction between the parasitism by larvae of *O. ovis* and gastrointestinal nematodes (Dorchies et al., 1997; Yacob et al., 2002; 2004; 2006; 2008; Terefe et al., 2005). It was observed that the FEC were significantly higher in lambs infected only with *H. contortus* compared to lambs infected with both *H. contortus* and *O. ovis*. In this case the production of blood eosinophils and high inflammatory cellular activity in tissue stimulated by the presence of *O. ovis* larvae conferred protection against *H. contortus* (Dorchies et al. 1997; Terefe et al., 2005). The same was also observed in experimental infections with *T. colubriformis* and *O. ovis* (Yacob et al., 2002; 2004; 2006). Similarly,

negative correlation coefficients were observed between *O. ovis* and GIN worm burden in the present trial. In addition, animals infected with *O. ovis* seem to be more tolerant to the pathogenic effects of haemoncose because parasitism by *O. ovis* reduced the length of *H. contortus*, and thus minimized the consumption of blood by the parasite (Terefe et al., 2005). This may be an additional reason why no lamb showed clinical signs of parasitic gastroenteritis, such as anemia accompanied by bottle jaw or diarrhea.

Secondly, the experimental animals received a high quality diet consisting of commercial feed with 18% crude protein, the amount of 3% of body weight per day in addition to grazing high quality braquiaria grass (*B. decumbens*). The high quality food may also have favored the immune response of animals against GIN. Bricarello et al. (2005) observed in SI and IF sheep breeds, artificially infected with *H. contortus*, that high level of protein in the diet resulted in increased resistance of the SI compared to IF sheep. Increased resistance and resilience were also observed in grazing SI lambs naturally infected with gastrointestinal nematodes that received a diet supplemented with high protein content (Louvandini et al., 2006). Therefore, the low GIN parasite burdens displayed by sheep in the present experiment may have been due to the interaction of several factors, specially the high nutritional standards associated with the indirect effects of *O. ovis* infestation.

Eosinophilia and higher level of total IgE in the SI lambs indicate that the immune responses of these animals against parasites were more pronounced than that of IF. Significant negative correlation between total IgE and Strongyle and *S. papillosus* FEC were observed on two occasions in the SI which was not observed in IF lambs. Similar results were observed in sheep selected for resistance, artificially infected with *T. colubriformis*, which presented higher levels of total IgE in intestinal lymph and lower FEC when compared to susceptible sheep (Pernthaner et al., 2005). Furthermore,

the peripheral eosinophilia and increased serum IgE were associated with the reduced infection in Gulf Coast Native lambs naturally infected by *H. contortus* (Shakya et al., 2009). Moreover, despite IF lambs having serum total IgE levels lower than SI lambs, a significant negative correlation was observed in IF lambs between total IgE and *H. contortus* worm burden. These results demonstrate that the intra-breed differences observed in the level of infection by GIN may be explained by the efficiency of the immune response. In Nelore cattle infected naturally by *Haemonchus placei* and *Cooperia punctata,* a resistant group also had significantly higher total serum IgE levels and higher mean of blood eosinophil counts than a susceptible group (Bricarello et al., 2007). Therefore, it has been suggested that total IgE levels might be considered useful as a marker to identify resistant and susceptible cattle and sheep to nematode infections (Bricarello et al., 2007; Shakya et al., 2009).

The mean number of blood eosinophils was significantly higher in SI than in IF in almost all samplings and a significant negative correlation was observed between the number of eosinophils/μL and FEC in SI lambs on two occasions. These results confirm that eosinophilia can be more pronounced in more resistant animals. In Mexico, eosinophilia was also associated with resistance to *H. contortus* infection in native Criollo lambs that presented more eosinophils and a smaller worm burden than Suffolk lambs (Alba-Hurtado et al., 2010). The presence of *O. ovis* larvae in the nasal cavity of sheep is also related to a strong activation of eosinophils (Jacquiet et al., 2005; Yacob et al., 2006). The experimental animals showed variation in eosinophil values throughout the study that could be related to variations in the degree of exposition to infective stages of parasites that might have occurred in the experimental site.

The results obtained also reinforced the importance of identifying animals in a flock that are highly susceptible to parasites, which was the case of four IF lambs.

These animals are responsible for a large majority of pasture contamination and, once identified, they should be removed from the flock (Sréter et al., 1994; Amarante et al., 1998; Stear et al., 1998; Bassetto et al., 2009), a procedure that could minimize the economic losses due to parasitism by GIN, reducing pasture contamination by infective larvae and, consequently, reducing the need of anthelmintic prophylactic treatments. Moreover, the resistance is a heritable characteristic (Sréter et al., 1994; Gruner et al., 2004).

The IF lambs showed significantly higher weight gain compared to the SI, averaging 200 g/day versus 154 g/day, respectively. Animals of the same breeds and age, kept indoors, free of parasitic infections, and receiving a similar diet, presented body weight gain of 256 g/day (IF lambs) and 223 g/day (SI lambs) (Bricarello et al., 2005). At least in part, the lower body weight gain of the animals of the present experiment in comparison with those studied by Bricarello et al. (2005) could be due to oestrosis and GIN infections. The negative influence of GIN on body weight gain has been well demonstrated. In ewes, *O. ovis* induced a decrease of almost 9% in milk production (Dorchies et al., 2003). However, further studies will be necessary to assess the influence of oestrosis in sheep productivity in our environmental conditions.

In conclusion, there was no breed difference regarding *O. ovis* infestation in SI and IF young sheep and animals with more nasal bot fly larvae tended to display smaller worm burden.

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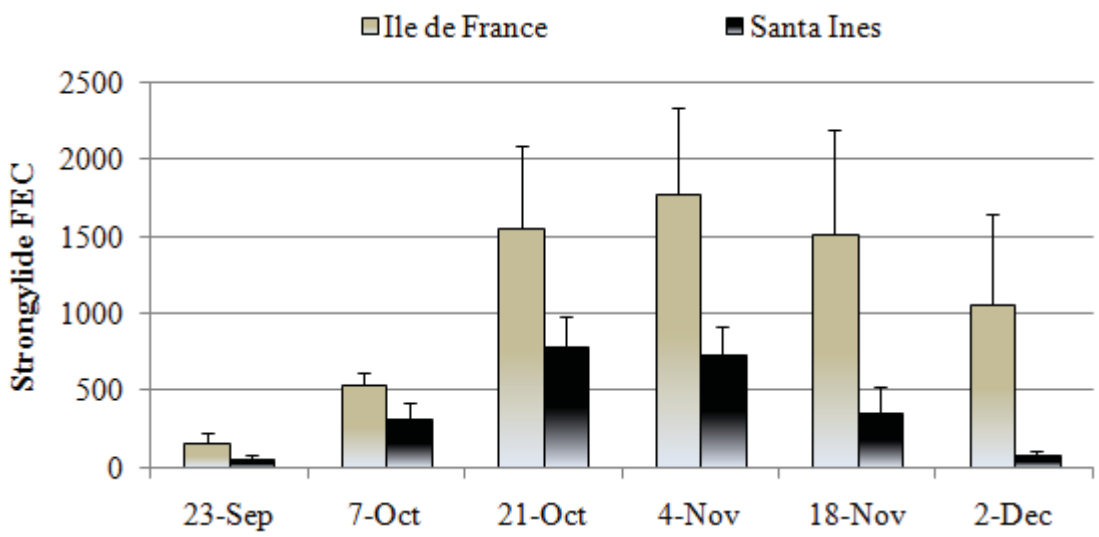
A



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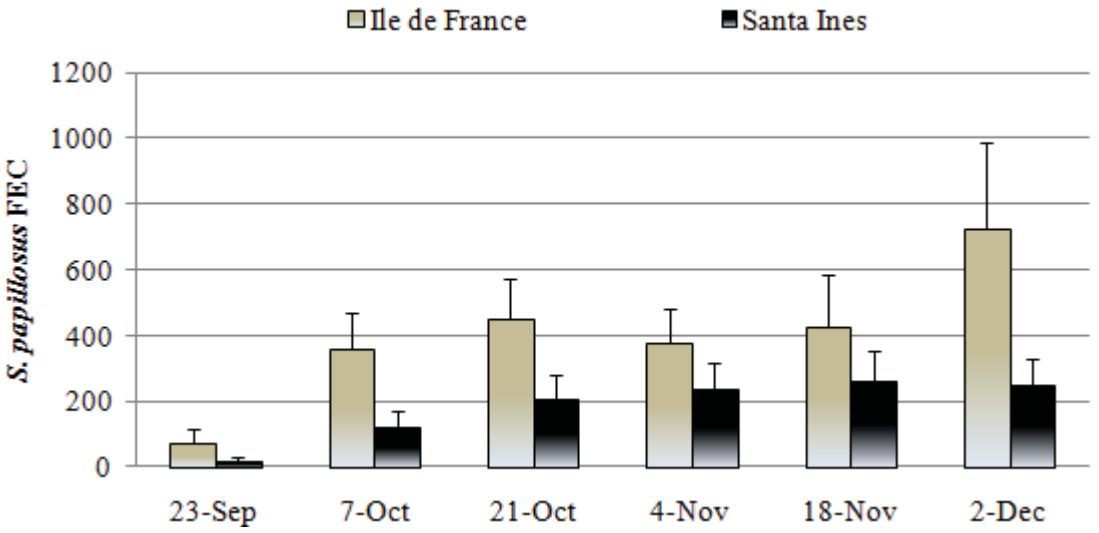

Figure 1. Mean of fecal egg counts (FEC) of the Strongyle (*H. contortus* and *T. colubriformis*) (A) and *Strongyloides papillosus* (B) of the Ile de France and Santa Ines male lambs naturally infested with *Oestrus ovis* and gastrointestinal nematodes. Bars are standard error. There was no significant difference between group means ($P > 0.05$).

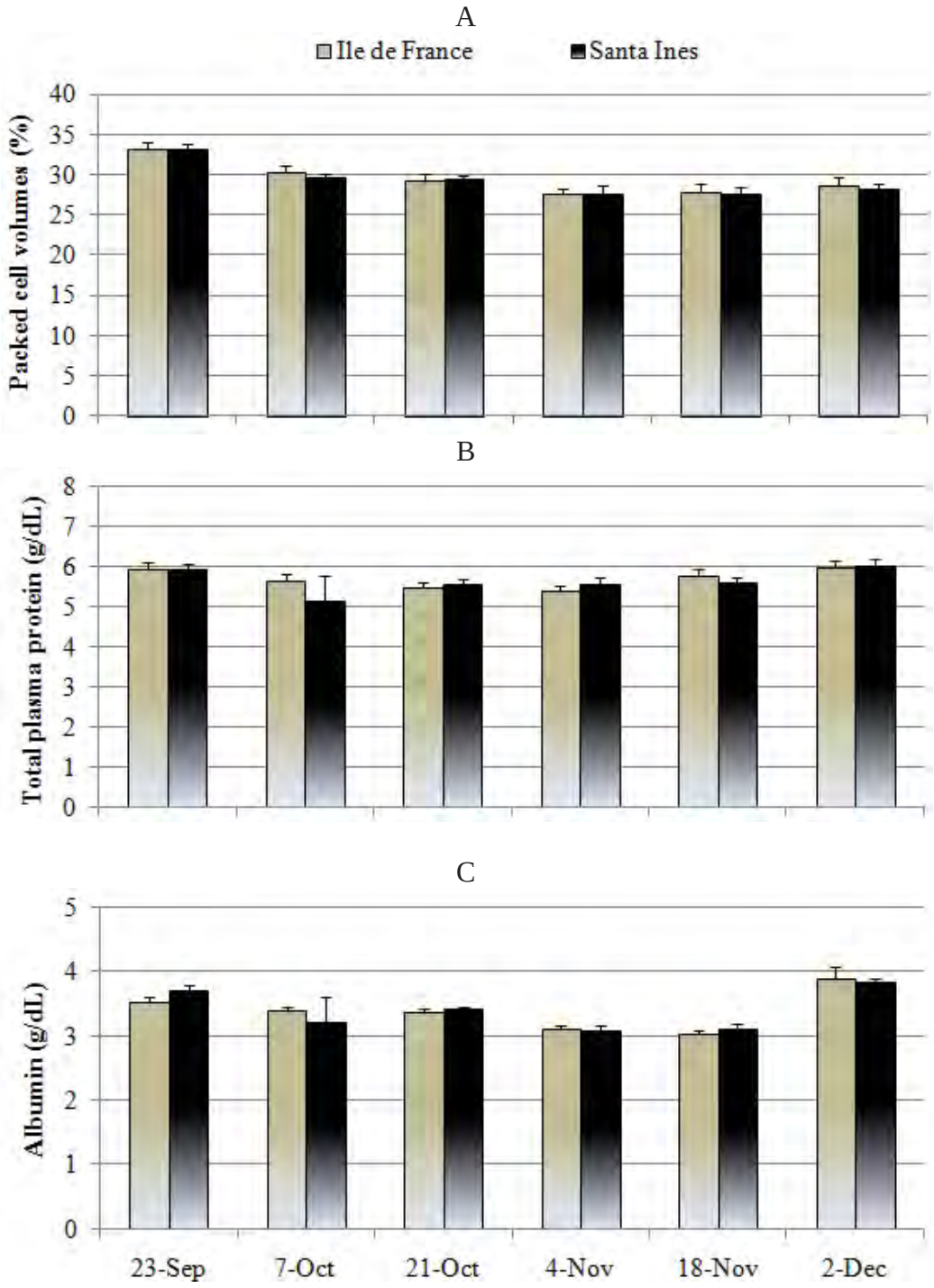

Figure 2. Mean packed cell volume (A), total plasma protein (B) and albumin (C) of the Ile de France and Santa Ines male lambs naturally infested by *Oestrus ovis* and gastrointestinal nematodes. Bars are standard error. There was no significant difference between group means ($P > 0.05$).

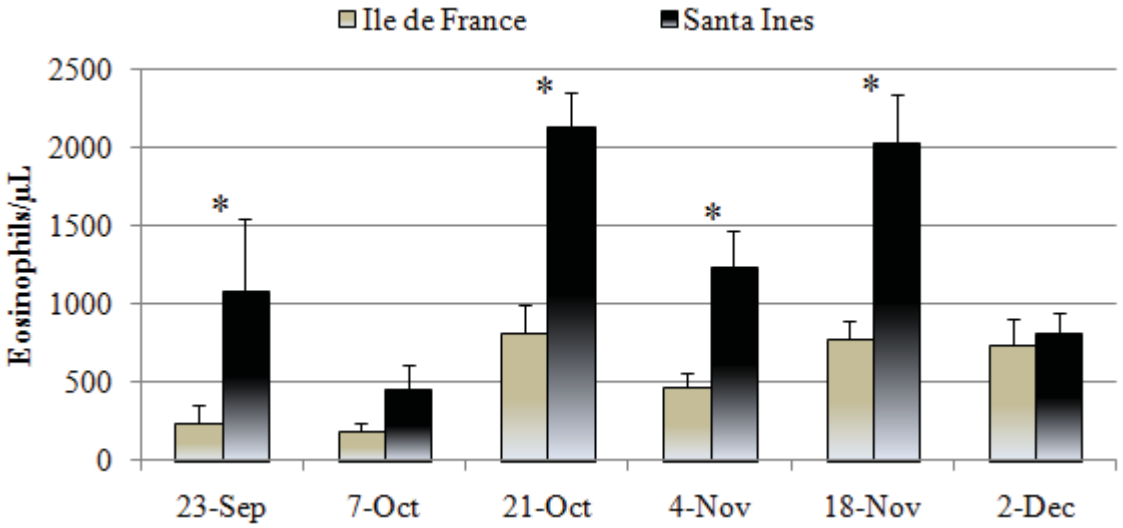

Figure 3. Means of blood eosinophils (eosinophils/µL) in Ile de France and Santa Ines male lambs naturally infested by *Oestrus ovis* and gastrointestinal nematodes. Bars are standard error. Data on which a significant ($P < 0.05$) difference was found between the groups are indicated with an asterisk (*).

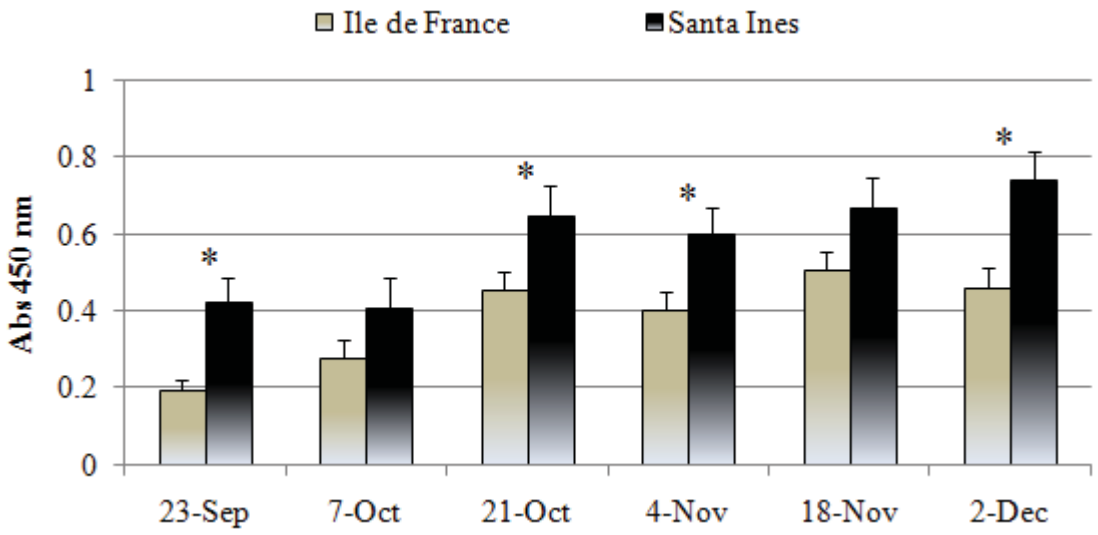

Figure 4. Means of total IgE level in sera in Ile de France and Santa Ines males lambs naturally infested by *Oestrus ovis* and gastrointestinal nematodes. Bars are standard error. Data on which a significant ($P < 0.05$) difference was found between the groups are indicated with an asterisk (*).

Table 1. Mean number of parasites in Ile de France and Santa Ines males lambs naturally infested by *Oestrus ovis* and gastrointestinal nematodes.

Species	Stage of development	Santa Ines	Ile de France
H. contortus	Early L4	503.0 (0-1640)	626.0 (0-1744)
	Late L4	264.0 (0-1020)	115.9 (0-520)
	L5 and adults	108.4 (0-734)	900.0 (0-4630)
	Total burden	876.0 (0-2704)	1642 (0-5520)
T. colubriformis	Total burden	565.0 (150-1350)	774.0 (140-1800)
S. papillosus	Total burden	472.0 (0-2140)	967.0 (50-3980)
O. ovis	L1	18.8 (7-34)	13.5 (1-29)
	L2	2.9 (0-7)	4.3 (0-13)
	L3	3.1 (0-10)	5.7 (0-13)
	Total burden	24.8 (14-39)	23.5 (11-36)

There was no significant difference between group means ($P > 0.05$). Minimum and maximum values are in parenthesis.

Table 2. Correlation coefficients between numbers of *H. contortus* (Hc), *T. colubriformis* (Tc), *S. papillosus* (Sp) and *O. ovis* (Oo) in Ile de France and Santa Ines male lambs.

Breed		Hc	Tc	Sp
Ile de France	Tc	-0.382		
	Sp	0.070	-0.032	
	Oo	-0.333	-0.323	-0.102
Santa Ines	Tc	-0.164		
	Sp	0.552	-0.224	
	Oo	-0.395	-0.249	-0.614*

* $P < 0.05$.

Table 3. Correlation coefficients between numbers of blood eosinophil/μL (last sampling), total IgE (last sampling), *H. contortus* (Hc), *T. colubriformis* (Tc), *S. papilosus* (Sp) and *O. ovis* (Oo) in Ile de France and Santa Ines male lambs.

Breed		Eosinophil	IgE
Ile de France	IgE	-0.343	
	Hc	0.175	-0.594**
	Tc	0.326	-0.095
	Sp	-0.448	-0.154
	Oo	0.133	0.375
Santa Ines	IgE	0.200	
	Hc	-0.139	-0.576*
	Tc	0.164	0.261
	Sp	0.006	-0.648 **
	Oo	-0.219	0.529

* P = 0.08; ** P < 0.05

Capítulo 5

Cellular and humoral immune responses in sheep naturally infested with *Oestrus ovis* (Diptera: Oestridae) and with nematode infections[d]

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Abstract

This study was carried out to evaluate the humoral and cellular immune response in Ile de France (IF) and Santa Ines (SI) young sheep naturally infested by *Oestrus ovis* and gastrointestinal nematodes (GIN). Mast cells, eosinophils and globule leucocytes were enumerated in the upper respiratory tract (septum, middle meatus and ventral nasal conchae) and in the mucosa of abomasum and small intestine. Immunoglobulin G (IgG) levels in serum samples and immunoglobulin A (IgA) levels in mucus from the nasal, abomasum and small intestine mucosas were determined against *O. ovis*, *Haemonchus contortus* and *Trichostrongylus colubriformis* antigens. The immune response against *O. ovis* and GIN are very similar in both breeds and involve the recruitment of inflammatory cells and immunoglobulins production in serum and mucus. Significant positive correlation was observed in both breeds between the number of *O. ovis* larvae x

[d] Artigo redigido de acordo com as normas do periódico científico Veterinary Parasitology.

IgG anti-*Oestrus* crude extract (IF: $r = 0.58$; SI: $r = 0.66$; $P < 0.05$), between *O. ovis* larvae x IgG anti-*Oestrus* excretory and secretory products (IF: $r = 0.59$; SI: $r = 0.63$; $P < 0.05$). But apparently, the presence of antibodies in serum or nasal mucus as well as inflammatory cells, were not efficient in the protection against *O. ovis* infestation. Regarding GIN, the levels of immunoglobulins and the inflammatory cell numbers in gastrointestinal mucosa presented significant inverse relationship only with *H. contortus* worm burden in Santa Ines animals. Probably this was one of the reasons why these animals showed the lowest FEC and worm burden compared to IF, since *H. contortus* was the more prevalent GIN during the experiment. In conclusion, the immune response against *O. ovis* and GIN are very similar and involve the recruitment of inflammatory cells and production of immunoglobulins against the parasites that can be detected in serum and mucus. However, *O. ovis* seems to be very well adapted to sheep and the immune response against their larvae promotes just the delay in larval development but not the expulsion of the parasite.

Key words: Immune response; Sheep; *Oestrus ovis*; *Haemonchus contortus*; *Trichostrongylus colubriformis*.

Introduction

Mixed infections by dipteran larvae and helminthes are quite common in ruminants, i.e. sheep, are normally parasitized by gastrointestinal nematodes and by *Oestrus ovis* larvae. These parasites infections stimulate many immune mechanisms of defense that can be mediate by antibody or cells, but the efficiency of this immune response depends of the parasite and the infection stage.

Proinflammatory immune reactions are characteristic of *O. ovis* infestation and involve the recruitments of cells (mast cells, eosinophils, macrophages, T and B

lymphocytes) and the secretion of immunoglobulins that suggest a type Th2 immune response (Angulo-Valadez et al., 2011) similar with the protective immune response against gastrointestinal parasitism by nematodes (Anthony et al., 2007; Rowe et al., 2008). Many studies about the relationship between *O. ovis* and helminth co-infections revealed that there are antagonist interaction between *O. ovis* larvae and the Strongylide nematodes *Trichostrongylus colubriformis* and *Haemonchus contortus*. The infection of the digestive tract with nematodes did not modify the biology of *Oestrus* populations, but in contrast, infections with *O. ovis* was related to significant reductions in nematode egg excretion and worm burdens. These changes were associated with significant modifications in populations of mast cells, globule leucocytes and eosinophils in the respiratory and digestive tracts. They also indicate that parasitic infection in one particular anatomical site induces "at distance" inflammatory reactions of the whole mucosal system (Dorchies et al., 1997; Yacob et al., 2002; Terefe et al., 2005).

This study was carried out to evaluate the humoral and cellular immune response in Ile de France and Santa Ines young sheep naturally infested by *O. ovis* and with nematode infections. We used samples from previously published study (Silva et al., 2011b) that demonstrated no breed difference regarding *O. ovis* infestation, but that revealed that animals with more nasal bot fly larvae tended to display smaller worm burden. In the present experiment, we investigated which are the inflammatory cell population and immunoglobulins involved in the protection against those parasites.

Material and Methods

Animals

The cellular and humoral response was evaluated in the upper respiratory tract (septum, middle meatus and ventral nasal conchae) and in the digestive tract

(abomasum - fundic region and small intestine - 1 m from the pylorus) of the Ile de France (IF) and Santa Ines (SI) young sheep naturally infested with *O. ovis* larvae and infected with gastrointestinal nematodes (GIN). The experimental design has been describe previously (Silva et al., 2011b). Briefly, 12 IF and 12 SI lambs were purchased from different farms located in Sao Paulo State. Four lambs were acquired from each farm to assure a minimum of genetic variability in each breed. All lambs were born in June 2009, except four IF lambs, which were born in May. Lambs, weaned at two months of age, were moved in late August to University facilities. The animals were kept exclusively in pasture during the experimental period in a paddock (0.3 ha) with *Brachiaria decumbens* grass, where they had free access to tap water.

At the beginning of the trial, in order to start the study with animals in the same conditions, all lambs were treated with anthelmintics (levamisole phosphate + albendazole). Two SI lambs died early in the trial of unknown cause and the data for these animals were excluded from analyses.

At six months of age, in early December 2009, the animals were euthanized. Blood serum, tissue and mucus samples were collected for immunological and histological analysis.

Hematology

Blood samples were collected by jugular vein puncture into vacutainer tubes without EDTA and serum samples were stored at -20 °C until use for immunoglobulin G (IgG) measurements.

Histology

Immediately after death, tissue samples were taken from three anatomical regions in the upper respiratory tract, i.e. septum, middle meatus and ventral nasal

conchae and from two sites of the digestive tract, i.e. abomasum (fundic region) and small intestine (1 m from the pylorus) for counting of mucosal mast cells, eosinophils and globule leucocytes. All tissue samples were fixed in 10% buffered formaldehyde for 48 h.. Afterwards, the samples were dehydrated with alcohol and embedded in paraffin wax. Sections, 2 μm thick, were stained with toluidine blue 1% or haematoxylin and eosin (H&E).

Mast cells were counted in sections stained with toluidine blue and eosinophils and globule leucocytes in sections stained with H&E. Cells were enumerated under a 10x eye piece containing a calibrated graticule and 100x objective lens viewing an area of 0.01 mm^2. Thirty fields, randomly selected, were observed per animal for each histological region and the mean numbers of cell/surface were calculated and compared between the groups. The counts were expressed as number of cells per mm^2 of mucosa.

Mucus

Mucus was taken from the nasal cavities, abomasum and small intestine mucosas to determine the levels of immunoglobulin A (IgA).

While the larvae of *O. ovis* were collected, mucus from nasal mucosa was extracted by lightly scraping the mucosal surface with a glass slide and mucus was stored in a falcon tube at -20 °C until processing. A 5 cm piece of abomasum and small intestine were sampled for the extraction of mucus and stored at -20 °C until processing. Tissues were thawed and mucus was scraped off with a glass slide. The scrapings were collected in a falcon tube on ice. Three milliliters of ice cold PBS supplemented with protease inhibitors (Complete®, Roche) was added to each sample. The samples were shaken for 1 h at 4 °C and centrifuged for 30 min at 4 °C and 3000 x g. The supernatant was collected and centrifuged again for 30 min at 4 °C and 15000 x g (Kanobana et al., 2002). Protein concentrations were determined using a kit (Protal método

colorimétrico® – Laborlab, Brazil) and the samples of abomasum mucus were adjusted to protein concentration of 0.4 g/dL; small intestine to 0.1 g/dL and nasal mucus to 0.7 g/dL using PBS supplemented with protease inhibitors.

Enzyme-linked immunosorbent assay (ELISA)

IgG levels in serum samples were determined against excretory and secretory products (ESP) and crude extract (CE) antigens from second instar (L2) *O. ovis* larvae; and against third stage larvae (L3) and adults (L5) of *H. contortus* and *T. colubriformis* antigens.

IgA levels in nasal mucus was tested against excretory and secretory products (ESP) and crude extract (CE) from L2 *O. ovis* larvae; abomasal mucus was tested against L3 and L5 of *H. contortus* and small intestine mucus against L3 and L5 of *T. colubriformis*.

Parasites used in antigen production

Second instar of *O. ovis* (L2) were collected from naturally infested sheep heads and they were washed several times in phosphate-buffered saline (PBS pH 7.2) with 240.000 UI of penicillin and 100 mg/ml of streptomycin and the viability of larvae were checked under a stereomicroscope.

The excretory and secretory products were obtained from five L2 maintained in a culture medium *in vitro*. The L2 were placed in a tube containing 10 ml RPMI-1640 (Sigma; 8758) with penicillin and streptomycin and were incubated in darkness for 24 h in a 5% CO_2 atmosphere at 37 °C. Supernatants were collected, centrifuged at 2000 x g for 20 min at 4 °C and stored at -80 °C until use.

To obtain the crude extract, 10 L2 were fragmented/homogenized, using homogenizer (T10 basic, IKA), in 5 ml of the PBS pH 7.2 supplemented with protease

inhibitor (Complete®, Roche). The extract was centrifuged at 15000 x g for 30 min at 4 °C and supernatants were collected and centrifuged newly. Protein concentrations of *O. ovis* antigens were determined using a kit (Bicinchoninic Acid Protein Assay Kit – Sigma) and absorbance was read at 562 nm. The antigen extracts were stored in aliquots at -80 °C until further use.

The production of antigens of infective third stage larvae (L3) and adults (L5) of *H. contortus* and *T. colubriformis* were previously described by Amarante et al. (2009) and Cardia et al. (2011), respectively.

Parasite-specific serum IgG

Polystyrene micro-titer plates (Nunc, USA) were coated with 100 µl of the different antigens (5µg/ml) diluted in carbonate-bicarbonate buffer (pH 9.6); plates were incubated overnight at 4 °C. All subsequent incubations were carried out for 1 h at 37 °C using, in each well, with a total of 100 µl of reagents. Between each step, plates were washed three times with ultra pure water (EASYpure II UV, Barnstead, USA) containing 0.05% Tween 20 (ProPure® - Amresco).

After coating, blocking was carried out with 0.1 % Gelatin (Amresco, USA) and 0.05 % Tween 20 (ProPure® - Amresco) in PBS 7.2 (PBS-GT). Serum samples were diluted in PBS-GT (1:500) and applied in duplicate. Plates were then incubated with rabbit-anti sheep IgG peroxidase conjugated diluted at 1:10000 (A130-101P, Bethyl Laboratories, Inc. USA). Finally, OPD substrate solution (1,2-phenylenediamine dihydrochloride, Dako, Denmark) was added to each well and the enzymatic reaction was allowed to proceed at room temperature, in the dark for 15 min and stopped with sulphuric acid solution at 5% and plates were immediately read using an automated ELISA reader (Biotrak II, Amersham-Biosciences, UK) at 492 nm.

The positive standard serum for *O. ovis* was obtained from a sheep evaluated by titration of the all serum samples tested from this experiment and as negative control, serum samples was obtained from young animals kept indoors that had no contact with adult bot flies. The standard positive serum for *H. contortus* and *T. colubriformis* were obtained from a sheep repeatedly infected with these nematodes. Results were expressed as the percentage of the optical density value (OD) of the positive standard serum and employing the following formula: % OD = [(OD mean of the tested serum – OD mean of blank) / (OD mean of the positive standard serum – OD mean of blank)] x 100 (Kanobana et al., 2001).

Parasite-specific mucus IgA

The ELISA reactions for parasite-specific mucus IgA were as previously described for serum analysis with 1:10 mucus dilution to abomasum and nasal mucus and with 1:2 mucus dilution to small intestine. Rabbit-anti sheep IgA peroxidase conjugated was diluted at 1:10000 (A130-108P, Bethyl Laboratories, Inc. USA). Finally, OPD substrate solution (1,2-phenylenediamine dihydrochloride, Dako, Denmark) was added to each well and the enzymatic reaction was allowed to proceed at room temperature, in the dark for 15 min and stopped with sulphuric acid solution at 5% and plates were immediately read using an automated ELISA reader (Biotrak II, Amersham-Biosciences, UK) at 492 nm. The results were expressed as the percentage of OD of sample minus OD of blank (Kanobana et al., 2001).

Statistical analyses

Significant differences between groups for all variables were assessed by oneway analysis of variance using statistical software, Minitab 11.21 (Minitab Inc., USA). Group means were compared using Tukey test, significant level at 5%. The data

relative to cell counts, IgG and IgA was transformed to $\log_{10}(x+1)$. Statistical significance was taken as $P < 0.05$. Spearman's correlation coefficient between variables was assessed using the same software. To test if there was effect of the time in serum IgG levels, the repeated measures analyses was performed using SAS (release 9.2). The figures and tables in the results are expressed as arithmetic means (± standard error of the mean).

Results

The data on FEC, nematode and *O. ovis* burdens of IF and SI lambs has been presented in detail by (Silva et al., 2011b). In brief, the IF lambs showed the highest Strongyle and *S. papillosus* FEC and worm burden, but no statistical significant difference was observed between breeds during the experiment. FEC were relatively low in most animals, but were high in a small number of sheep, especially IF.

All three *O. ovis* larval instars were observed with prevalence of 100% in both breeds. The mean intensity of infestation in IF and SI lambs was 23.5 and 24.8 larvae/animal, respectively. There was no difference between breeds.

No significant differences between groups were found in the number of inflammatory cells counted in nasal and digestive mucosa, except in globules leucocytes/mm^2 average in abomasum that was significantly higher in IF than in SI lambs ($P < 0.05$) (Fig. 1).

The serum IgG anti-*Oestrus* crude extract was higher than the mean values of IgG anti-*Oestrus* excretory and secretory products in both breeds. In the first month of experiment (September 2009) the IgG anti-*Oestrus* levels were close to zero (Fig. 2), but started to increase on 7th October 2009 at the same time that the animals of both breeds started to display clinical signs of oestrosis. The serum IgG anti-*Oestrus* levels

increased gradually throughout the experiment until reaching the highest mean value in the last collection day (2nd December 2009), being similar in both breeds ($P > 0.05$).

At the beginning of experiment, all lambs were parasitized by GIN, therefore, they already displayed relatively high levels of serum IgG anti-L5 and anti-L3 of *H. contortus* (Fig. 3) and *T. colubriformis* (Fig. 4). There was no difference between the breeds to antigens tested ($P > 0.05$) and there was low variation in serum IgG levels against GIN throughout of experiment, excepting the level of IgG anti-L5 of *T. colubriformis* and anti-L3 of *H. contortus* that significantly increased until the end of experiment in both breeds ($P < 0.05$).

The IgA in nasal mucus anti-*Oestrus* CE was higher than the mean values of IgA anti-*Oestrus* ESP (Fig. 5A). The IF lambs showed higher levels of IgA than SI lambs, but no significant difference was observed between the breeds ($P > 0.05$).

The IgA in gastrointestinal mucus anti-L5 and anti-L3 of *H. contortus* (Fig. 5B) and anti-L5 of *T. colubriformis* (Fig. 5C) were higher in SI lambs than in IF, but no significant difference was observed between breeds ($P > 0.05$).

Correlation coefficients between parasitological and immunological variables are presented in Tables 1, 2 and 3. Although the experimental groups were composed of a limited number of animals, significant ($P < 0.05$) positive correlation was observed in both breeds between the number of *O. ovis* larvae x IgG anti-*Oestrus* crude extract in IF ($r = 0.58$) and SI ($r = 0.66$), between *O. ovis* larvae x IgG anti-*Oestrus* excretory and secretory in IF ($r = 0.59$) and SI ($r = 0.63$).

IF lambs showed significant positive correlation between the number of *O. ovis* larvae x globule leucocytes from nasal meatus ($r = 0.71$; $P < 0.05$).

Regarding to GIN burden and immune response, significant correlations were observed just in SI lambs: abomasum mast cells x *H. contortus* burden ($r = -0.73$; $P <$

0.05); IgG anti-L3 Hc x *H. contortus* burden (r = -0.72; P < 0.05); IgA anti-L5 Hc x *H. contortus* burden (r = -0.61; P = 0.07); and mast cells from small intestine x *T. colubriformis* burden (r = 0.60; P = 0.07).

No significant correlation coefficients was observed between inflammatory cells from nasal tract x GIN tract (Table 4), excepting between globule leucocytes from nasal conchae x small intestine in IF lambs (r = 0.63; P < 0.05).

Discussion

The parasitism with GIN and *O. ovis* causes an increase in inflammatory cell numbers in mucosas of the upper respiratory and gastrointestinal tracts and also the production of anti-parasite specific immunoglobulins (Yacob et al., 2002; Bricarello et al., 2005; Terefe et al., 2005; Cardia et al., 2011), changes that were also observed in the present study. Such immune response was similar in animals of both breeds and resulted in no breed difference regarding *O. ovis* infestation or GIN worm burdens. However, SI lambs showed a higher proportion of L1of *O. ovis* compared to IF, indicating a more efficient immune response of the former breed (Silva et al., 2011b). The immune response is involved in the regulation of *O. ovis* populations (Jacquiet et al., 2005), and may have an inhibitory effect on *O. ovis* larval growth, delaying development (Frugère et al., 2000; Angulo-Valadez et al., 2007b).

At the beginning of this experiment the serum IgG levels anti-*O. ovis* was close to zero in SI and IF lambs, probably because the lambs never have had previous contact with this parasite, but after the first experimental month, most of lambs started to show clinical signs of oestrosis, i.e., dyspnea and nasal discharge, coinciding with the significantly gradual rise of serum IgG levels. In *O. ovis* infestation, humoral systemic response of IgG usually reaches seroconversion 2-4 weeks post-first infection and the

highest levels are observed during the development of L2 and L3 larvae (Alcaide et al., 2005; Angulo-Valadez et al., 2011).

In an epidemiological study with tracer lambs exposed to *O. ovis* infestation during 28 days, several animals presented considerable parasitic burden, but no clinical signs of oestrosis (Silva et al., 2011a). This indicates that the major symptoms of infestation, nasal discharge and frequent sneezing, are immunologically mediated, i.e., depends of the acquisition of immune response against the parasite. These symptoms are more intense in some animals indicating hypersensitivity. In animals with this clinical manifestations, larvae, especially L1s in the nasal cavities, are at high risk of becoming trapped in dense mucus, asphyxed and expulsed from the host (Angulo-Valadez et al., 2011).

A large percentage of the animals do not display intense symptoms of infestation. Probably, there is a strategy of immune tolerance on the part of the host in order to avoid self-damage or a strategy on the part of the parasite to avoid hyperimmunostimulation that may lead to a “self-curing” phenomenon, similar to that observed in gastrointestinal helminth parasites. Studies suggest that *O. ovis* uses immunosuppressive strategies such as the depletion of lymphocytes and the degradation of immunoglobulins to evade defensive attacks from the host (Tabouret et al., 2003; Jacquiet et al., 2005) and L1 have an important role in the regulation of inflammatory reactions (Duranton et al., 1999). It is well known that larvae stimulate the mucus production, which is utilized in their nutrition. Salivary gland products of *O. ovis* contain thermostable proteases, which appear to be important in larval nutrition and host–parasite interaction (Angulo-Valadez et al., 2007a).

In the present study, animals with the highest levels of IgG and IgA anti-*O. ovis* had the highest numbers of *O. ovis* larvae, while inflammatory cell numbers did not

present any consistent association with *O. ovis* larval burden. Apparently, the presence of antibodies in serum or nasal mucus as well as inflammatory cells, were not efficient in the protection against *O. ovis* infestation. However, it is likely that mucus IgA associate with humoral and cellular immune response promote the regulation of *O. ovis* burden in host (Jacquiet et al., 2005) and also can have influence in larval weight and consequently in the viability of adults flies (Cepeda-Palacios et al., 2000), i.e., although the immune response is not enough to limit the parasite establishment, this can at least maintain the *O. ovis* population under control in the host.

Regarding GIN, the levels of immunoglobulins and the inflammatory cell numbers in gastrointestinal mucosa presented significant inverse relationship only with *H. contortus* worm burden in Santa Ines animals. Probably this was one of the reasons why these animals showed the lowest FEC and worm burden compared to IF, since *H. contortus* was the more prevalent GIN during the experiment. In addition, the SI sheep showed higher blood eosinophils and higher level of total serum IgE which indicated that the immune responses of these animals against GIN parasites were more pronounced than that of IF and these variables were also negatively correlated with nematode FEC (Silva et al., 2011b). In other studies, inflammatory cells and IgA-parasite-specific in abomasum were also inversely associated with *H. contortus* worm burden and FEC indicating that they may impair parasite development or fecundity (Strain and Stear, 2001; Amarante et al., 2005; Bricarello et al., 2005).

The lambs arrived in the experimental site already parasitized by GIN, and consequently, serum IgG anti-GIN was observed since the beginning of the study. The serum IgG levels anti-L3 of *H. contortus* and anti-L5 of *T. colubriformis* had a significant rise until the end of experiment in lambs of both breeds as a consequence to continue exposure to nematode larvae on pasture.

It was also observed that animals with more nasal bot fly larvae tended to display smaller worm burden (Silva et al., 2011b). In other trials, it was demonstrated that nematode egg production and worm burden were significantly depressed in mixed infections with *O. ovis*, as well, the clinical signs of GIN infections (Dorchies et al., 1997; Terefe et al., 2005; Yacob et al., 2006). *O. ovis* infestation stimulates the immune response which possibly had a negative influence in the GIN parasitism. This influence was probably through the enhanced recruitment of activated inflammatory cells (eosinophils, mast cells and globule leucocytes) and/or their products towards the gut mucosa that finally created an unfavourable environment to the nematodes, thereby reducing worm length and fecundity (Terefe et al., 2005), fact that could explain the low FEC and worm burden in animals of both breeds in this study. Eosinophils are considered very important in the response against helminth infections and are frequently associated with the expression of resistance to parasites (Dawkins et al., 1989; Stear et al., 2002; Balic et al., 2006; Shakya et al., 2011) as well as immunoglobulin E (Sayers et al., 2008; MacKinnon et al., 2010).

Although the IF seems to have had the immune response less effective than SI lambs, the former breed showed greater productivity compared to SI lambs, with higher body weight gain, fact that could be attributed to the high quality of the diet offered to animals during the study (Silva et al., 2011b). Immunity to parasitic infection was considered as a function which competes for nutrient resources against the requirement of the host to maintain other body functions (Coop and Kyriazakis, 1999). The resistant breeds can present inferior productivity when compared with other breeds selected for higher weight gain and meat quality, for example, SI sheep prioritize survival to the detriment of productive development, while IF animals, which have higher productive capabilities, prioritize growth (Amarante et al., 2009).

In conclusion, the immune response against *O. ovis* and GIN are very similar and involve the recruitment of inflammatory cells and production of immunoglobulins against the parasites that can be detected in serum and mucus. However, the immune response seems to be more efficient against GIN in comparison with bot fly. *O. ovis* seems to be very well adapted to sheep and the immune response against their larvae promotes just the delay in larval development but not the expulsion of the parasite. More studies are necessary to prove if the immune response of SI sheep can became more efficient and able to limit *O. ovis* larval establishment when animals are exposed to infestation for longer periods of time.

Acknowledgments

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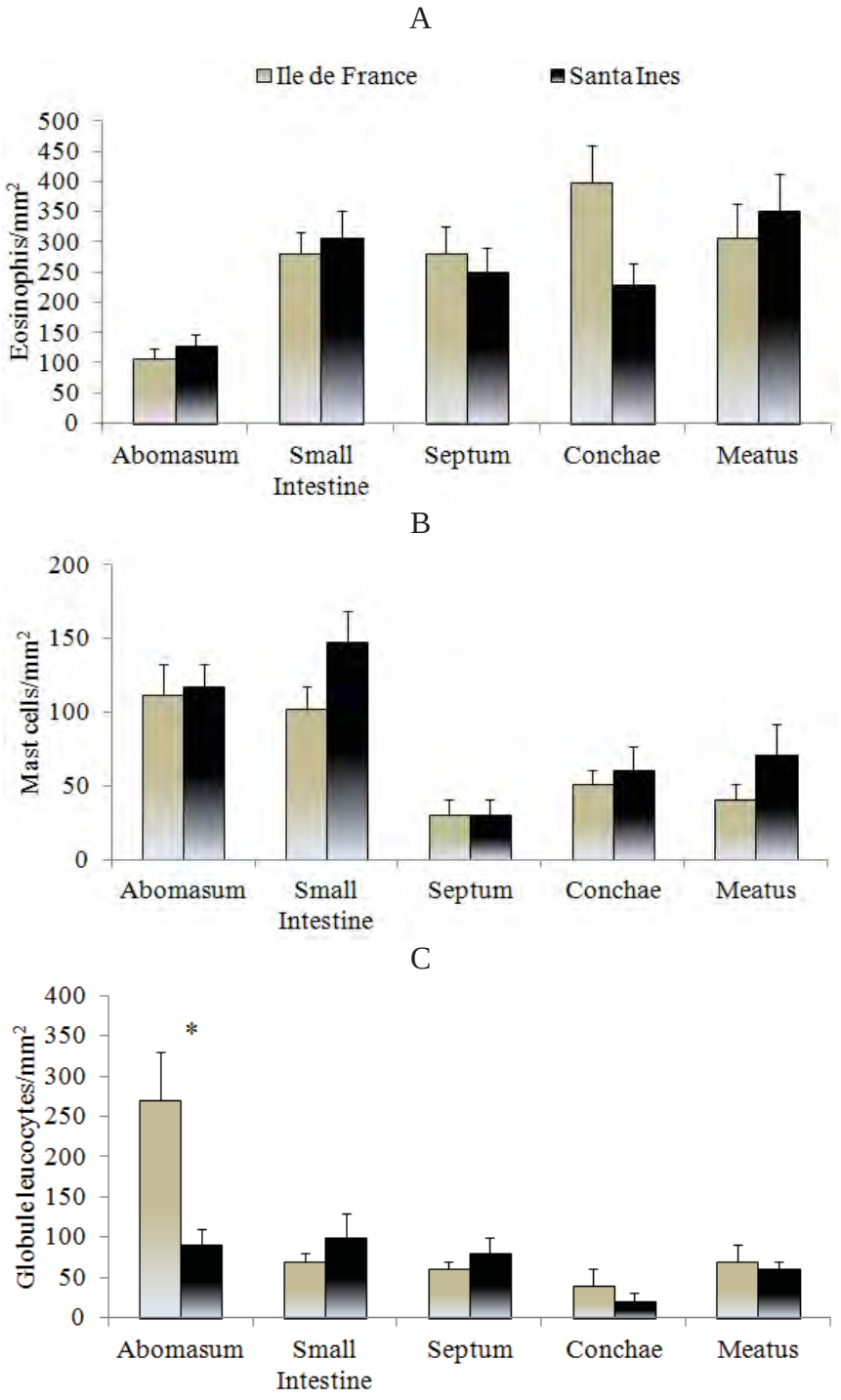

Figure 1. Mean number of eosinophils, mast cells and globule leucocytes per mm^2 from digestive tract: abomasum and small intestine; and from upper respiratory tract: septum, ventral nasal conchae and middle meatus in Ile de France and Santa Ines male lambs naturally infested by *Oestrus ovis* and gastrointestinal nematodes. Bars are standard error. Data on which a significant difference ($P < 0.05$) was found between the groups are indicated with an asterisk (*).

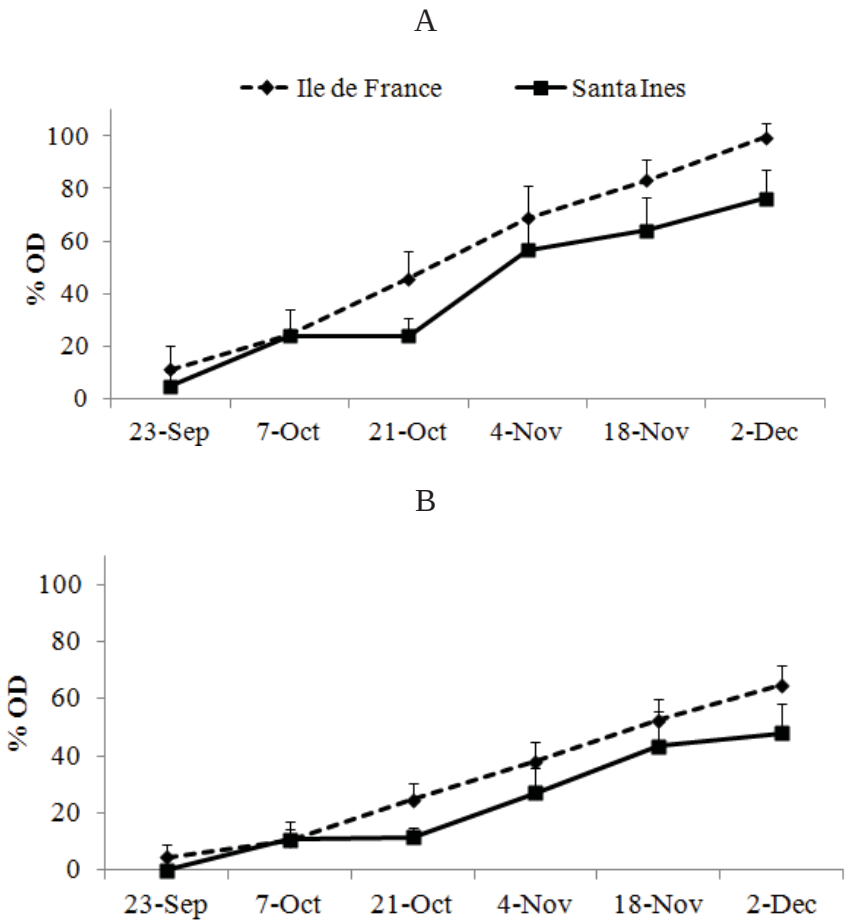

Figure 2. Mean of levels of serum IgG against crude extract (CE) (A) and excretory and secretory products (ESP) (B) of *Oestrus ovis* second larval instar (L2) in Ile de France and Santa Ines males lambs naturally infested by *O. ovis* and gastrointestinal nematodes. Bars are standard error.

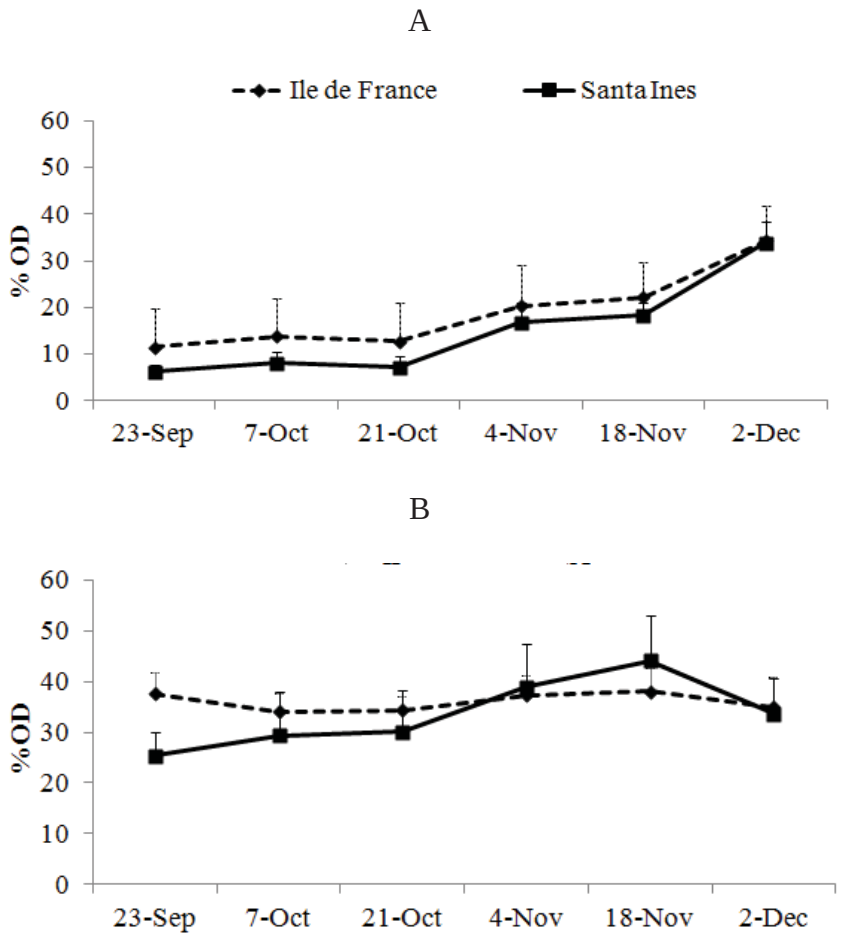

Figure 3. Mean of levels of serum IgG against third stage larvae (L3) (A) and against adult (L5) (B) of *Haemonchus contortus* in Ile de France and Santa Ines males lambs naturally infested by *Oestrus ovis* and gastrointestinal nematodes. Bars are standard error.

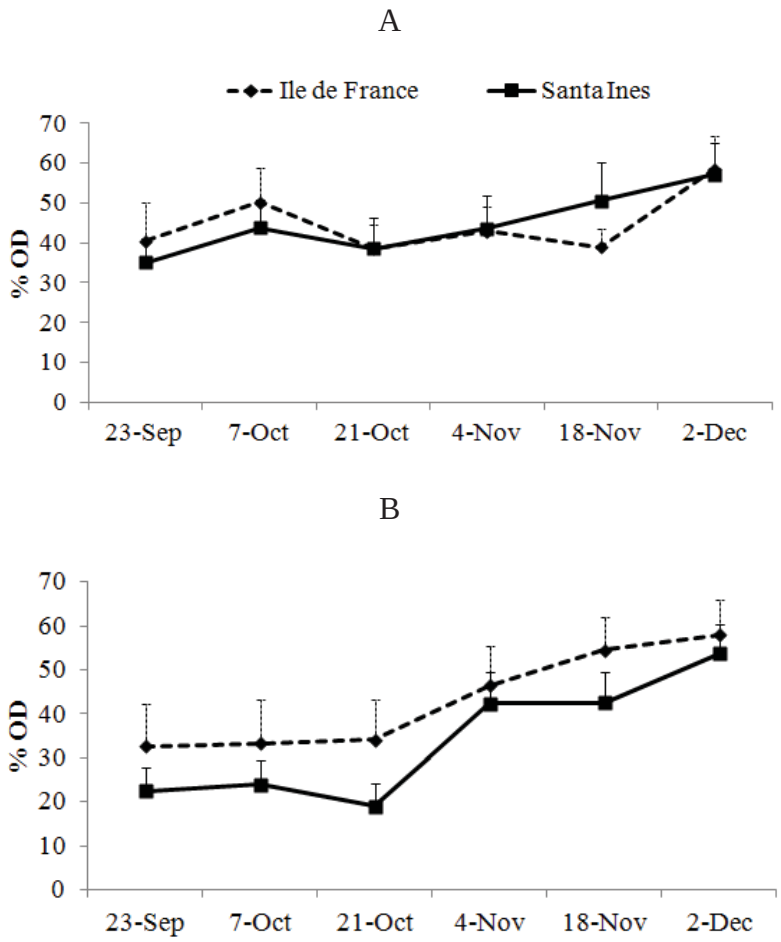

Figure 4. Mean of levels of serum IgG against third stage larvae (L3) (A) and against adult (L5) (B) of *Trichostrongylus colubriformis* in Ile de France and Santa Ines males lambs naturally infested by *Oestrus ovis* and gastrointestinal nematodes. Bars are standard error.

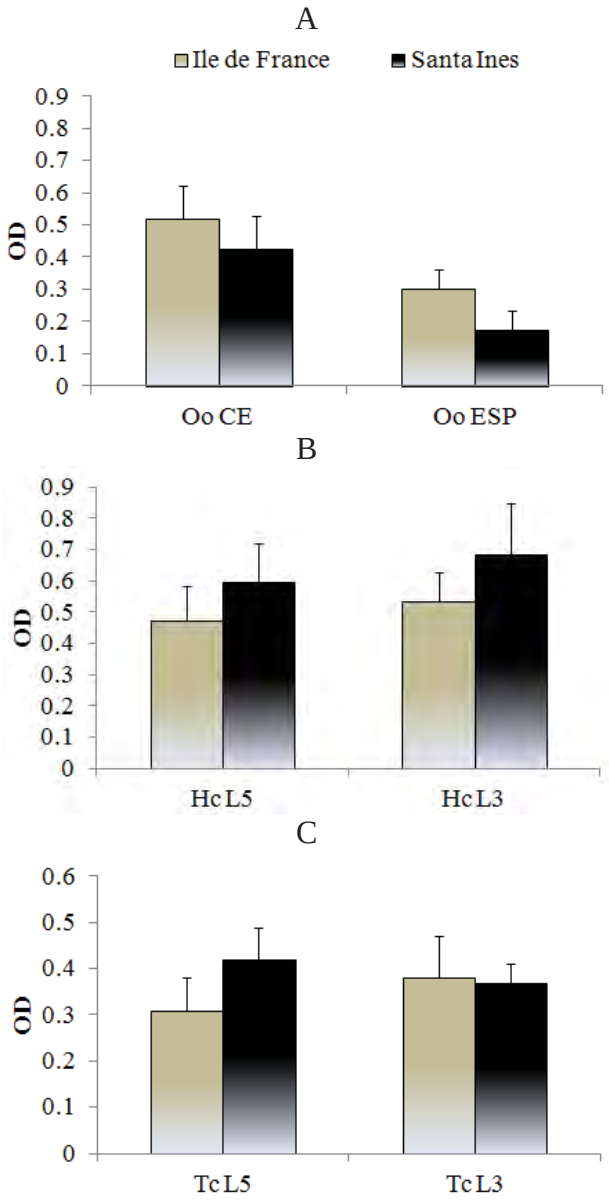

Figure 5. Mean level of mucus IgA against crude extract (CE) and excretory and secretory products (ESP) of *Oestrus ovis* (Oo) second larval instar (L2) (A); against third stage larvae (L3) and against adult (L5) of *Haemonchus contortus* (Hc) (B); against third stage larvae (L3) and against adult (L5) of *Trichostrongylus colubriformis* (Tc) (C) in Ile de France and Santa Ines males lambs naturally infested by *O. ovis* and gastrointestinal nematodes. Bars are standard error. There was no significant difference between means ($P > 0.05$).

Table 1. Correlation coefficients between *Oestrus ovis* burden x inflammatory cells in nasal mucosa of septum, conchae and meatus; and seric IgG and mucus IgA against crude extract (CE) and excretory and secretory products (ESP) of *O. ovis* in Ile de France male lambs naturally infested.

	Oestrus ovis burden	
	Ile de France	Santa Ines
IgG anti-CE	0.58*	0.66*
IgG anti-ESP	0.59*	0.63*
IgA anti-CE	0.11	0.54
IgA anti-ESP	0.24	0.35
Eosinophils		
Septum	0.17	0.03
Conchae	-0.18	-0.18
Meatus	-0.13	0.31
Mast Cells		
Septum	0.45	-0.29
Conchae	0.06	0.14
Meatus	0.29	-0.01
Globule leucocytes		
Septum	-0.26	0.17
Conchae	-0.16	-0.32
Meatus	0.71*	-0.33

* $P < 0.05$

Table 2. Correlation coefficients between *Haemonchus contortus* worm burden x inflammatory cells in abomasal mucosa and seric IgG and abomasal mucus IgA against antigens from infective larvae (L3) and adults (L5) of *H. contortus* in Ile de France and Santa Ines male lambs naturally infected.

	H. contortus worm burden	
	Ile de France	Santa Ines
Eosinophils	-0.13	-0.24
Mast Cells	-0.38	-0.73*
Globule Leucocytes	-0.41	-0.46
IgG anti-L3	0.10	-0.72*
IgG anti-L5	0.14	-0.56
IgA anti-L3	-0.09	-0.36
IgA anti-L5	0.01	-0.61**

* $P < 0.01$ ** $P = 0.07$

Table 3. Correlation coefficients between *Trichostrongylus colubriformis* (Tc) and *Strongyloides papillosus* (Sp) worm burden x inflammatory cells in intestinal mucosa and seric IgG and intestinal mucus IgA against antigens from infective larvae (L3) and adults (L5) of *T. colubriformis* in Ile de France and Santa Ines male lambs naturally infected.

	Ile de France		Santa Ines	
	Tc	Sp	Tc	Sp
Eosinophils	-0.02	-0.30	0.44	-0.56
Mast Cells	-0.02	-0.07	0.60*	0.17
Globules Leucocytes	-0.02	-0.09	0.18	-0.20
IgG anti-L3	-0.09		0.40	
IgG anti-L5	-0.20		0.27	
IgA anti-L3	0.04		0.12	
IgA anti-L5	0.00		0.12	

* P = 0.07

Table 4. Correlation coefficients between inflammatory cells (eosinophils, mast cells and globule leucocytes) in nasal mucosa (septum, conchae, meatus) and gastrointestinal mucosas (abomasum and small intestine) in Ile de France and Santa Ines male lambs naturally infested by *Oestrus ovis* and with nematode infections.

Cell	Breed		Abomasum	Small Intestine	Septum	Conchae
Eosinophils	Ile de France	Small Intestine	0.74*			
		Septum	-0.51	-0.03		
		Conchae	0.53	0.23	-0.07	
		Meatus	0.20	0.30	0.44	0.46
	Santa Ines	Small Intestine	0.19			
		Septum	-0.34	0.33		
		Conchae	-0.17	0.21	0.19	
		Meatus	0.05	-0.11	-0.34	0.35
Mast cells	Ile de France	Small Intestine	0.15			
		Septum	-0.06	0.28		
		Conchae	-0.33	-0.23	0.62*	
		Meatus	-0.25	0.39	0.71*	0.50
	Santa Ines	Small Intestine	0.34			
		Septum	0.10	-0.10		
		Conchae	-0.08	-0.54	0.67*	
		Meatus	0.25	0.03	0.87*	0.62*
G. leucocytes	Ile de France	Small Intestine	0.25			
		Septum	0.13	0.17		
		Conchae	0.03	0.63*	-0.14	
		Meatus	0.09	0.07	-0.11	0.08
	Santa Ines	Small Intestine	0.64*			
		Septum	0.57	0.45		
		Conchae	-0.02	-0.02	0.20	
		Meatus	-0.09	0.12	0.20	0.75*

*$P < 0.05$