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FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

Oestrus ovis: sintomatologia, perdas produtivas, profilaxia e diagnóstico da oestrose em cordeiros naturalmente infestados

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Tese apresentada junto ao Programa de Pós-graduação em Medicina Veterinária para obtenção do título de Doutora.

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DEDICATÓRIA

Dedico a minha família amada, meu pai Antônio, minha mãe Rosimar e meus irmãos Jordana, Glauconita e Jordão.

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RESUMO

Em experimento inicial, os efeitos profiláticos e terapêuticos de ivermectina e closantel foram avaliados em cordeiros com infestação natural de *O. ovis*. O ensaio foi realizado entre o início de Dezembro de 2019 e Março de 2020, com 30 cordeiros machos distribuídos em três grupos de 10 animais cada: controle (sem tratamento), tratado com ivermectina (0,2 mg/kg, via subcutâneo) e tratado com closantel (10 mg/kg, via oral). Os grupos foram tratados em duas ocasiões com 70 dias de intervalo: em 5 de Dezembro de 2019 e em 13 de Fevereiro de 2020. Em 19 de Março de 2020, todos os ovinos foram abatidos. As cabeças dos cordeiros foram retiradas e seccionadas ao longo do seu eixo longitudinal e sagital para recuperação das larvas. As larvas recuperadas de *O. ovis* foram contadas e identificadas de acordo com o seu estádio de desenvolvimento (L1, L2 e L3). Sete dos cordeiros do grupo controle estavam infestados com larvas de *O. ovis* entre seis e 17 larvas. Todas as larvas recuperadas do grupo controle estavam intactas e ativas. Três animais tratados com ivermectina tinham larvas de *O. ovis* (1 a 3 larvas), contudo estavam mortas. Os animais tratados com closantel não tinham quaisquer larvas. Os sinais clínicos sugestivos de oestrose foram escassos durante o período experimental. As médias de ganho de peso diário foram semelhantes ($p > 0,05$) entre os grupos. Closantel e ivermectina tiveram alta eficácia contra a oestrose e o parasitismo por *O. ovis* não prejudicou o desempenho dos cordeiros. Um segundo experimento com cordeiros, naturalmente infestadas por larvas de *O. ovis*, foi realizado com o objetivo de avaliar a variabilidade dos sinais clínicos de oestrose e a adequação da PCR e ELISA como método de diagnóstico. O experimento foi realizado de Dezembro de 2020 a Abril de 2021 com 39 animais divididos em dois grupos: grupo infestado (n=26) e grupo controle tratado (n=13). O grupo infestado não recebeu tratamento contra a oestrose, e o grupo controle foi tratado com closantel (10 mg/kg, via oral), a cada 28 dias, a fim de manter os animais tão livres, quanto possível, da infestação por *O. ovis*. Os sinais clínicos variavam entre os animais independentemente do número de larvas recuperadas, no entanto, os escores de secreção nasal mucosa espessa e de mucopurulenta eram menos frequentes nos ovinos do grupo tratado. Não houve correlação entre o escore de descarga nasal e o número de larvas

recuperadas de *O. ovis* (R^2 = 0,012, P = 0,165). Três animais tratados apresentaram larvas de primeiro estádio (L1) (1 - 4 larvas/animal) as quais eram menores do que as L1 encontradas nos cordeiros do grupo infestado. Noventa e dois por cento dos cordeiros do grupo infestado (24/26) estavam parasitados por larvas de *O. ovis* (número variando de 1 a 54 larvas por animal). Foi observado um aumento gradual dos níveis de IgG plasmático (anti-antígeno das larvas de *O. ovis*) nos animais do grupo infestado enquanto que os ovinos do grupo controle apresentaram níveis baixos de IgG ao longo do experimento. A PCR teve baixa sensibilidade (26%) e alta especificidade (100%), e apresentou leve concordância (k = 0,177) com a detecção das larvas após o abate dos cordeiros. Os sinais clínicos de oestrose não tiveram relação com a intensidade de infestação e o ELISA mostrou-se superior em comparação com a técnica de PCR para a identificação de animais parasitados por *O. ovis*.

Palavras-chaves: antiparasitário, ELISA, Oestridae, ovino, PCR.

ABSTRACT

In an initial experiment, the prophylactic and therapeutic effects of ivermectin and closantel were evaluated in lambs with natural *O. ovis* infestation. The trial was conducted between early December 2019 and March 2020 with 30 male lambs distributed into three groups of 10 animals each: control (no treatment), treated with ivermectin (0.2 mg/kg, subcutaneously) and treated with closantel (10 mg/kg, orally). The groups were treated on two occasions 70 days apart: on December 5th, 2019 and on February 13th, 2020. On March 19th, 2020, all lambs were slaughtered. The heads of the lambs were removed and sectioned along their longitudinal and sagittal axis to larval recovery. The recovered *O. ovis* larvae were counted and identified according to their stage of development (L1, L2 and L3). Seven of the lambs in the control group were infested with *O. ovis* larvae, ranging from six to 17 larvae. All larvae recovered from the control group were intact and active. Three animals treated with ivermectin had *O. ovis* larvae (1 to 3 larvae), however they were dead. The animals treated with closantel did not have any larvae. Clinical signs suggestive of oestrosis were scarce during the experimental period. The mean daily weight gain was similar ($p > 0.05$) among the groups. Closantel and ivermectin had high efficacy against oestrosis and *O. ovis* parasitism did not impair lamb performance. A second experiment with lambs, naturally infested with *O. ovis* larvae, was performed to evaluate the variability of clinical signs of oestrosis and the suitability of PCR and ELISA as a diagnostic method. The experiment was conducted from December 2020 to April 2021 with 39 animals divided into two groups: infested group (n=26) and treated control group (n=13). The infested group received no treatment against oestrosis, and the control group was treated with closantel (10 mg/kg, orally) every 28 days in order to keep the animals as free as possible from *O. ovis* infestation. Clinical signs varied among animals regardless of the number of larvae recovered, however, thick mucus and mucopurulent nasal discharge scores were less frequent in sheep in the treated group. There was no correlation between nasal discharge score and the number of recovered *O. ovis* larvae ($R^2 = 0.012$, $P = 0.165$). Three treated animals had first instar (L1) larvae (1 - 4 larvae/animal), which were smaller than the L1 found in the lambs of the infested group. Ninety-two percent of the lambs in the infested group (24/26) were parasitized by *O. ovis* larvae (number ranging from 1 to 54 larvae per animal). A gradual increase in plasma IgG (anti-antigen of *O. ovis* larvae) levels was observed in the animals of the infested group, while the control sheep showed low IgG levels even throughout the experiment. PCR had low sensitivity (26%) and high specificity (100%), and showed slight agreement ($k = 0.177$)

with the detection of the larvae after the lambs were slaughtered. The clinical signs of oestrosis were not related to the intensity of infestation, and the ELISA was superior compared to the PCR technique for the identification of *O. ovis* parasitized animals.

Keywords: antiparasitic, ELISA, Oestridae, sheep, PCR.

CAPÍTULO 1

Introdução

A criação de ovinos é realizada em todos os continentes (EMBRAPA, 2016), a ampla difusão da espécie se deve principalmente a seu poder de adaptação a diferentes climas, relevos e vegetações. A criação ovina está destinada tanto à exploração econômica como à subsistência das famílias de zonas rurais (VIANA, 2008). Assim como em outras atividades, a ovinocultura possui alguns entraves, dentre estes o parasitismo merece destaque.

Oestrus ovis L. (Diptera: Oestridae) é um parasita cosmopolita causador de miíase cavitária e suas larvas são parasitas obrigatórios da cavidade nasal e seios paranasais de ovinos e caprinos (ZUMPT, 1965). Devido à localização das larvas, é conhecido popularmente como bicho da cabeça (SILVA, 2015).

A mosca da espécie *O. ovis* é larvípara e deposita larvas de primeiro estádio (L1) diretamente nas narinas de ovinos e caprinos. Estas colonizam as cavidades nasais, septo, conchas e etmóide, e em seguida mudam para larva de segundo estádio (L2) no qual migram para os seios frontais, onde irá completar seu desenvolvimento em larva de terceiro estádio (L3). As L3 maduras são expelidas para o ambiente para o período de pupação que acontece no solo. A pupa dará origem à mosca, e após o acasalamento, as fêmeas grávidas depositam as larvas nas narinas dos ovinos, reiniciando o ciclo (ZUMPT, 1965).

O vôo da mosca adulta sobre as narinas dos animais, para realização da larviposição, irrita os ovinos, que podem deixar de se alimentar adequadamente ao esconderem o focinho no solo ou entre a lã de outros animais, balançando a cabeça ou espirrando na tentativa de se protegerem (ZUMPT, 1965). As larvas possuem ganchos e espinhos em sua morfologia que irritam a mucosa nasal, provocando inflamação e produção de exsudato mucoso que favorecem sua alimentação, tendo em vista que se alimentam de muco, exsudato mucoso e seroso (ANGULO-VALADEZ et al., 2010).

A terapia bem-sucedida contra a infestação por *O. ovis* baseia-se na eliminação das larvas com o uso de antiparasitários. Terapias amplamente utilizadas incluem closantel e lactonas macrocíclicas, como a ivermectina oral ou injetável (DORCHIES et al., 1992; DORCHIES et al., 1997).

Estudos têm demonstrado uma interação negativa entre o parasitismo por *O. ovis* e nematódeos gastrintestinais (SILVA et al., 2012a; TEREFE et al., 2005; YACOB et al.,

2008). A presença de larvas de *O. ovis* está correlacionada com a redução significativa da eliminação de ovos de nematódeos, da fecundidade e da população dos vermes adultos (SILVA et al., 2012a). No entanto, foi observado que a presença de nematódeos não interfere na biologia e população de *O. ovis* (TEREFE et al., 2005).

Por outro lado a intensificação da ovinocultura e o surgimento de parasitas com resistência aos antiparasitários se constituem em novos desafios para profilaxia e tratamento de parasitoses em pequenos ruminantes. Há escassez de estudos que avaliam a eficácia dos tratamentos contra *O. ovis*, o impacto da oestrose no desempenho de ovinos e os métodos de diagnosticar essa parasitose em pequenos ruminantes. Com isto, o presente estudo teve como objetivo avaliar se o efeito profilático e terapêutico dos fármacos mais usados para o controle de oestrose, closantel e ivermectina, se mantém, bem como avaliar o impacto dessa parasitose no desempenho produtivo, seus sinais clínicos e, os testes de PCR e ELISA como possíveis métodos diagnósticos.

Revisão de Literatura

Oestrose

Oestrus ovis está presente onde se encontram ovelhas e cabras, com maior prevalência em regiões com climas tropicais (GRACIA et al., 2019). No Brasil esta mosca está presente o ano inteiro em regiões tropicais com maior prevalência na primavera e verão (SILVA et al., 2012b; SCHENKEL et al., 2012; MUSTAFA et al.; 2015; VASCONCELOS et al., 2016) e em regiões subtropicais, porém em épocas com inverno mais rigoroso as larvas entram em diapausa nos animais (RAMOS et al., 2006).

Há vários trabalhos sobre a epidemiologia da oestrose em diversos países, tais como Egito (GAABOUB, 1978), Zimbábue (PANDEY, 1989), França (YILMA e DORCHIES, 1991), Itália (CARACAPPA et al., 2000), Espanha (ALCAIDE et al., 2003), porém no Brasil, há poucos estudos. Em Botucatu-SP, foi observado a presença deste parasita em ovinos em todas as estações dos anos avaliados, porém a frequência de infestação foi influenciada por fatores sazonais com maior frequência de infestação nos meses de primavera e verão (SILVA et al., 2012b). Resultado similar foi observado no sul do país, no qual a mosca estava presente em todos os meses do ano, entretanto, as condições mais favoráveis para sua atividade e desenvolvimento foi observado nos meses da primavera,

verão e outono, época em que houve maior infestação dos animais pelas larvas (RIBEIRO et al., 1990; RAMOS et al., 2006).

A fêmea adulta, deposita larvas de primeiro estádio (L1) diretamente nas fossas nasais dos ovinos e caprinos. Estas, colonizam as cavidades nasais, septo, as conchas e o seio etmoidal, e em seguida, mudam para a larva de segundo estádio (L2) que migram para os seios frontais, onde irão 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 à mosca, e após o acasalamento, as fêmeas grávidas procuram os ovinos para depositar as larvas, reiniciando o ciclo (SILVA, 2015).

As moscas adultas têm cerca de 12 mm de comprimento, com corpo castanho-acinzentado, com várias pequenas manchas pretas no tórax, que são cobertas por uma fina camada de pelos castanhos. Elas têm aparelhos bucais rudimentares e não funcionais, o que as torna incapazes de se alimentar, por isso a vida dos adultos é curta (de 2 a 4 semanas) (ANGULO-VALADEZ et al., 2010). As fêmeas emergem do pupário com ovos totalmente desenvolvidos prontos para fertilização e seus olhos grandes facilitam a localização de hospedeiros potenciais, bem como parceiros adequados (ibidem).

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 e WALL, 1995; ALCAIDE et al., 2003; ALCAIDE et al., 2004). Em climas mais frios, as larvas entram em diapausa principalmente como L1, nas passagens nasais e também como pupas no solo (GRACIA et al., 2010, RUGG et al., 1997).

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., 2003). 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 liberação de óxido de nitrogênio (ANGULO-VALADEZ et al., 2010).As biomoléculas excretadas / secretadas pelas larvas e aquelas na glândula salivar larval compreendem uma matriz complexa de enzimas necessárias para degradar o muco e as proteínas plasmáticas para alimentação e nutrição larval (FRUGÈRE et al., 2000; ANGULO-VALADEZ et al., 2007). Assim, quando o desenvolvimento larval aumenta, não apenas a produção de proteína em produtos excretados / secretados aumenta, mas a atividade proteolítica também (TABOURET et al., 2003). Portanto, a patogênese é

amplificada na mucosa etmoidal e sinusal quando L2 e L3 estão presentes (TABOURET et al., 2003).

A patogênese da oestrose está relacionada com os efeitos traumáticos causados pelo movimento das larvas e pelas suas moléculas secretadas/excretadas. Todos estes estímulos induzem uma reação de hipersensibilidade imunológica (JACQUIET et al., 2005), que é responsável pelos sintomas clínicos. No entanto, foi observado que os sinais clínicos podem diferir entre indivíduos independentemente do número de larvas (ANGULO-VALADEZ et al., 2011).

Alguns ovinos e caprinos mesmo com a presença do parasita na cavidade nasal e seios frontais, não apresentam sinais clínicos. Em contraste, alguns indivíduos apresentam sinais clínicos severos mesmo com poucas larvas de *O. ovis* (ANGULO-VALADEZ et al., 2010).

Esses acometimentos patogênicos afetam o bem estar e o desempenho produtivo nos hospedeiros, resultando em perdas significativas (ALCAIDE et al., 2003; EL-TAHAWAY, 2010), como por exemplo na queda de produção de carne e leite (ARSLAN et al., 2009). A infestação com miíases nasais pode ocasionar rinite, sinusite e posterior lesões pulmonares secundárias que podem afetar diretamente a respiração e indiretamente interferir no tempo de pastejo e ruminação, levando a queda no desempenho e até a óbito de animais (ANGULO-VALADEZ et al., 2011)

A infestação por *O. ovis* causa prejuízos na ovinocultura direta e indiretamente. O impacto negativo na produtividade ocorre através de uma combinação de redução de peso dos animais infestados, o que prejudica a eficiência econômica do sistema de criação de ovinos por meio de um aumento nos custos com tratamento do rebanho (EL-TAHAWAY, 2010).

Oestrus ovis também é considerado um parasita zoonótico, ocasionalmente responsável por miíase ocular em humanos (ANGULO-VALADEZ et al., 2010; ANGULO-VALADEZ et al., 2011; GRACIA et al., 2010), principalmente naqueles com contato frequente com ovinos (RUGG et al., 1997).

Enquanto o diagnóstico da grande maioria das ectoparasitoses causada por artrópodes em animais baseia-se na detecção visual de parasitas no corpo do animal (por exemplo, mosca dos chifres, miíase por *Cochliomyia hominivorax*, e infestação por carrapatos) ou em amostras biológicas (ácaros da sarna detectáveis em crostas), este não é o caso da maioria dos parasitas causadores de miíases internas, por exemplo, gasterofilose em equinos e oestrose em ovinos, cujo diagnóstico depende do exame *post mortem* de órgãos

internos (TRAVERSA e OTRANTO, 2006). No entanto, do ponto de vista científico e do bem-estar animal, a utilização da necropsia para diagnosticar e determinar a intensidade do parasitismo limita as possibilidades de investigação (ARIAS, 2014) e é economicamente inviável na criação de ovinos.

O teste sorológico, como o ensaio de imunoabsorção enzimática (ELISA), que detecta anticorpos contra *O. ovis*, poderia ser utilizado para monitorar a ocorrência de oestrose no rebanho (OTRANTO, 2001; ALCAIDE et al., 2005; ANGULO-VALADEZ et al., 2008; SILVA et al., 2012a, 2018; ARIAS et al., 2014). Além disso, a informação relativa à resposta imunitária do hospedeiro às larvas causadoras da miíases é fragmentada em comparação com a resposta contra outros ectoparasitas por exemplo, carrapatos e mosquitos (PRUETT, 1999), e a compreensão da interação hospedeiro-parasita/mecanismos imunológicos é a base para o desenvolvimento de novos métodos de controle e diagnóstico da oestrose.

A prevalência de infestações por *O. ovis* em ovinos têm sido subestimada devido às limitações do diagnóstico *ante-mortem*. Estudo recente mostrou a PCR como uma técnica válida para detectar e confirmar a presença de larvas de *O. ovis ante-mortem* e pode ser útil como teste de diagnóstico alternativo (ÍPEK e ALTAN, 2017). Além disso, o método PCR também pode ser útil para avaliar a eficácia dos medicamentos contra *O. ovis* após tratamento de ovinos com oestrose através do monitoramento da presença de DNA específico de parasitas no muco nasal, evitando assim sacrificá-los para confirmar a presença de larvas.

Assim, devido à sua alta prevalência e à gravidade da infestação, é necessário diagnosticar e tratar os ovinos infestados para prevenir os prejuízos decorrentes do parasitismo.

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

Infecções mistas por larvas de dípteros e helmintos são bastante comuns nos ruminantes. Os ovinos são frequentemente parasitados simultaneamente por nematódeos gastrintestinais e por larvas de *O. ovis*. Estas infecções parasitárias estimulam mecanismos imunitários de defesa que podem ser mediado por anticorpos ou células, mas a eficiência desta resposta imunitária depende do genótipo animal, idade, sexo, estado fisiológico, exposição prévia ao patógeno, capacidade de reconhecimento do antígeno, estado de saúde e nutrição, parasita e fase de infecção (COLDITZ, 2008).

O parasitismo simultâneo pode resultar em diminuição na eliminação de ovos e na população de helmintos, mas a presença de helmintos parece não interferir no estabelecimento e crescimento das larvas de *O. ovis* (DORCHIES et al., 1997; YACOB et al., 2004a). Acredita-se que essa interação entre as populações de parasitos ocorra devido à reação imunitária característica da infestação por *O. ovis* que envolve o recrutamento de células (mastócitos, eosinófilos, macrófagos, linfócitos T e B) e a secreção de imunoglobulinas, sugerindo uma resposta imunitária de tipo Th2 (ANGULO-VALADEZ et al., 2011), semelhante à resposta imunitária contra o parasitismo por nematódeos gastrintestinais (ANTHONY et al., 2007; ROWE et al., 2008). Em estudo desenvolvido para avaliar o parasitismo entre as raças Santa Inês e Ile de France e a interação entre a infestação por *O. ovis* e nematódeos gastrintestinais, não foi observado diferença entre raças, porém, os animais com maior quantidade de larvas de *O. ovis* tenderam a apresentar menos nematódeos gastrintestinais (SILVA et al., 2012c).

Tratamento antiparasitário

A maioria dos antiparasitários disponíveis no mercado foram desenvolvidos a partir da década de 60 e se tornaram peças “chave” no controle parasitário em ovinos. A medicina veterinária é pioneira no desenvolvimento de vários princípios ativos antiparasitários (SPINOSA, 2017). Atualmente já existem diversos fármacos disponíveis para o tratamento de endoparasitas e ectoparasitas, que podem possuir pequeno ou amplo espectro, como exemplo dos grupos de parasiticidas sistêmicos salicilanilidas e lactonas macrocíclicas, que tem ação contra ambos tipos de parasitas (AMARANTE, 2015; SPINOSA, 2017). No entanto, o uso excessivo, frequente e indiscriminado de antiparasitários da mesma classe de medicamentos predispõe ao desenvolvimento de resistência parasitária (HABELA et al., 2006). Por isso, a preservação da atividade e eficácia dessas drogas se torna imperativa (AMARANTE e OLIVEIRA, 2007).

Closantel

Closantel é um antiparasitário derivado da salicilanilida eficaz contra *Haemonchus contortus*, *Fasciola hepatica* e *Oestrus ovis*, utilizado principalmente em bovinos, ovinos e caprinos (COSTA et al., 2011; BORGSTEEDE et al., 2008; PANGUI et al., 2001, DORCHIES et al., 1997). Sua ação se dá por provocar alteração funcional em mitocôndrias causando desacoplamento do processo de oxidação e à suspensão

fosforilação oxidativa, impedindo assim a produção de energia a partir do catabolismo dos hidratos de carbono (DORCHIES et al., 1997).

O closantel é um dos principais produtos recomendados para o controle de oestrose em ovinos (GUERRERO et al., 1984). Em estudo realizado no Rio Grande do Sul, durante a primavera e verão, foi observado que a dose de 5 mg/kg matou as L2 e L3 porém não foi suficiente para matar larvas de primeiro estádio enquanto que a dose de 10 mg/kg eliminou 100% das larvas de *O. ovis* em todos os estádios (SANTIAGO et al., 2008).

Na França, Pangui et al. (2001) compararam a eficácia entre closantel e doramectina contra oestrose nas doses, respectivamente, de 10 mg/kg (via oral) e 0,2 mg/kg (via intramuscular). Neste estudo, observou-se que os animais que não receberam tratamento apresentaram sinais clínicos de oestrose durante todo período experimental, enquanto os animais dos grupos tratados já se apresentavam assintomáticos três dias após serem tratados. Adicionalmente, a partir do 3° dia após os tratamentos, foram abatidos 6 animais a cada 3 dias e após o 9° dia, os intervalos eram de 6 dias entre os abates. Os autores observaram que após o 6° dia pós-tratamento, os ovinos já não apresentavam larvas de *O. ovis*, com manutenção da eficácia terapêutica dos antiparasitários até o 30° dia, momento em que todos foram abatidos.

O closantel possui um elevado peso molecular, se liga a proteínas plasmáticas como a albumina. Esta ligação prolonga os níveis de closantel no plasma, aumentando consequentemente o efeito residual desta formulação contra re-infecções (MICHIELS, 1987).

Embora este princípio ativo apresente, dependendo da região, uma elevada eficácia, não é difícil encontrar relatos de intoxicação de animais pelo closantel. Casos de intoxicação foram descritos em bovinos (PEREZ et al., 1988), caprinos (OBWOLO et al., 1989; ECCO et al., 2008) e ovinos (BARLOW et al., 2002; FURLAN et al., 2009; LOPES et al., 2014). Porém, nos casos supracitados, os autores enfatizam que a intoxicação com closantel aconteceu quando se utilizou de duas até dez vezes a dose terapêutica recomendada em bula para o princípio ativo em questão. Histologicamente as lesões mais evidentes são edema mielínico no sistema nervoso central (status spongiosis) e nervo óptico, degeneração e depleção da camada fotorreceptora e/ou camadas nucleares da retina (PÉREZ et al., 1988, OBWOLO et al., 1989, BORGES et al., 1999, GILL et al., 1999, BARLOW et al., 2002, ECCO et al., 2008, VAN DER LUGT e VENTER, 2007).

A eficácia do closantel contra ectoparasitas e nematódeos gastrintestinais é descrita por alguns autores (DORCHIES et al., 1997; COSTA et al., 2011), entretanto já foi

relatado caso de resistência anti-helmíntica a esse princípio ativo (ALMEIDA et al., 2010; ECHEVARRIA et al., 1996).

Ivermectina

Na década dos 1980, foi descoberto o grupo das lactonas macrocíclicas, que compreende as avermectinas e milbemicinas, e constituem um dos grupos de anti-helmínticos mais administrados em ruminantes (LEATHWICK et al., 2016). A ação do fármaco é explicada pela sua característica em atingir elevados níveis de concentração nos sítios de localização dos parasitas devido à sua lipofilicidade. Seu mecanismo de ação se dá pela hiperpolarização da musculatura e inibição da transmissão nervosa, por meio da potencialização da ação do neurotrasmissor ácido γ-amino butírico (GABA) e do aumento da permeabilidade da membrana ao cloro. Desta forma, provocam paralisia do tônus muscular do parasita, levando a ataxia e morte (WOLSTENHOLME e ROGERS, 2005). É um grupo de anti-helmínticos que pode ter ação endectocida (MOYA-BORJA et al., 1997; RUGG et al., 1997; ANDRADE et al., 2017; BARBOSA et al., 2018; BELLO et al., 2022).

Na Espanha, foi reportado eficácia de 100% da ivermectina administrada na dose de 0,2 mg/kg por via oral em animais que apresentavam sintomas de oestrose. Nesse estudo, os animais do grupo tratado não apresentaram nenhuma larva viva, enquanto que os animais não tratados abrigavam os três ínstares larvais. Também foi observado que os sinais clínicos no grupo tratado não desapareceram completamente, porém diminuíram consideravelmente 12 dias após o tratamento (LUCIENTES et al., 1998).

Rugg et al. (1997) verificaram a eficácia terapêutica e profilática de cápsulas de liberação lenta de ivermectina administradas por via oral em animais infestados naturalmente e livres de infestação, que se tornaram parasitados de forma natural ao longo do experimento. Esta formulação de ivermectina apresentou 100% de eficácia e efeito profilático no tratamento de oestrose.

São antiparasitários amplamente comercializados no mercado por razões econômicas, por apresentarem um amplo espectro de ação em diversas espécies de animais e pela sua elevada segurança clínica. No entanto, a falta de orientação profissional e a facilidade de acesso aos anti-helmínticos têm levado a uma má utilização desses medicamentos de forma frequente e indiscriminada, o que compromete o controle das infecções por nematódeos gastrintestinais (GILLEARD, 2006; AMARANTE, 2014;

BARTLEY et al., 2015) e das infestações por ectoparasitas (LOPES et al., 2013; RECK et al., 2014; NEVES et al., 2015; CONDE et al., 2020).

Ainda não foram descritos relatos de resistência de *O. ovis* a ivermectina, porém esse princípio ativo apresenta diversos relatos de resistência parasitária em ruminantes tanto a parasitas gastrintestinais (SOUTELLO et al., 2007; SOUZA et al., 2008; COSTA et al., 2011; NEVES et al., 2014; ALMEIDA et al., 2010) quanto a outros ectoparasitas (LOPES et al., 2013, CONDE et al., 2020; NEVES et al., 2015).

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CAPÍTULO 2 – Prophylactic effects of ivermectin and closantel treatment in the control of *Oestrus ovis* infestation in sheep

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Prophylactic Effects of Ivermectin and Closantel Treatment in the Control of *Oestrus ovis* Infestation in Sheep

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The sheep nasal bots *Oestrus ovis* is parasite of the nasal cavities and sinuses of small ruminants causing oestrosis, one of the most frequent parasitic diseases in sheep and goats. The widely use of ivermectin and closantel by the sheep breeders in the treatment and prophylaxis of gastrointestinal nematodes resulted in widespread cases of anthelmintic resistance. However, there is no report about cases of *O. ovis* with drug-resistance. In this study, we evaluated the prophylactics and therapeutic effects of both antiparasitics in sheep with *O. ovis* natural infestation. The trial was carried out from early December 2019 to March 2020, with 30 crossbred males lambs allocated into three groups of 10 animals each: control (without treatment), treated with ivermectin (0.2 mg/kg subcutaneously) and treated with closantel (10 mg/kg orally). The animals were kept together grazing the same pasture area. The treatment groups were drenched in two occasions 70 days apart: on 5th December 2019 and on 13th February 2020. On 19th March 2020, all lambs were slaughtered. The lamb heads were removed and sectioned along their longitudinal and sagittal axis to search for larvae. Recovered *O. ovis* larvae were counted and identified according to their developmental stage (L1, L2, and L3). Seven of the control lambs were infested with *O. ovis* larvae ranging from six to 17 larvae (11.6 mean infestation intensity). All recovered larvae from control group were intact and active. Three animals treated with ivermectin had *O. ovis* larvae (1–3 larvae), however they were dead and in degeneration. The animals treated with closantel did not have any larvae. The clinical suggestive signs of oestrosis were scarce over the experimental period. The averages of daily weight gain were similar ($p > 0.05$) among groups. Closantel and ivermectin had high efficacy against oestrosis and *O. ovis* parasitism did not hinder the performance of lambs.

Keywords: myiasis, Ovis aires, lambs, bot fly, Oestridae

INTRODUCTION

Oestrus ovis L. (Diptera: Oestridae) is a cosmopolitan parasite with a higher prevalence in tropical regions. It causes cavitary myiasis in small ruminants, once the larvae are an obligate parasite of the nasal and sinus cavities of sheep and goats (1).

In endemic areas, the *O. ovis* larvae have been found in humans' eyes and nasopharyngeal airway. Cases of ocular affections are likely to occur more frequently than currently notified, because many cases without complications are unreported (2).

The female fly flies around the head of its host to deposits larvae at a few centimeters from sheep nostrils (3). In case of the fly presence, the animals hide the muzzle on the soil or in the others sheep's wool, swing head and sneezing, which leads to distress. The larvae hooks and spine irritate the nasal mucosal, provoking inflammation and mucous nasal discharge. At the same time, this irritating action secures the production of an inflammatory exudate, which count for larva's feeding (4). Besides frequent sneezes, sheep infested with *O. ovis* larvae may also present difficulty in breathing, hyporexia, and weight loss. In heavy infestation, animals may have related secondary bacterial infection in the lungs (5).

In a trial conducted in Brazil for three consecutive years, the prevalence of *O. ovis* was 50% with the occurrence of the parasite in all seasons. Nevertheless, the highest prevalence (61.1%) were observed in the spring and summer. The climatic conditions of this region during this period are optimal for fly activity, and consequently for a high rate of sheep infestation (6).

The oestrosis therapy is based on the use of antiparasitic drugs that target the larvae. Many systemic antiparasitic drugs may be used for the oestrosis treatment. The most widely used antiparasitics to control *O. ovis* are closantel and the macrocyclic lactones, such as orally or injectable ivermectin (7, 8). Sheep treated with closantel, injectable and oral ivermectin had 100, 100, and 98% of efficacy, respectively considering all instars (7). In a survey conducted by Oliveira et al. (8) the injectable doramectin was 100% effective against all larval stages. Nevertheless, the first instar larvae (L1) is considered less susceptible to these drugs in comparison with the second instar larvae (L2) and third instar larvae (L3) (9) because the L1 larvae feed less thus they are less prone to ingest the systemic parasiticides (10). Martínez-Valladares et al. (11) observed that the long-acting injectable moxidectin had 90.2% of efficacy against L1.

The intensification of sheep farming and emergence of drug-resistant parasites have brought new challenges regarding the prophylaxis and treatment of small ruminant parasitism. Frequent cases of anthelmintic resistance have been reported (12–14) as well as ectoparasites, such as *Dermatobia hominis* (15, 16), *Rhipicephalus microplus* (17) and *Cochliomyia hominivorax* (18), with resistance to the treatments with macrocyclic lactones. For this reason, the aim of this work was to verify whether closantel and ivermectin that have been used for decades are still effective in sheep naturally infested with *O. ovis*. Because animals rarely die due to oestrosis, the economic losses caused by the parasitism may be underestimated. Therefore, the present study aimed also to assess the prophylactic effects of closantel and ivermectin on the productive performance of lambs.

METHODS

Statement of Ethics

The study was carried out according to the standards established by the local Ethics Committee on Animal Use (FMVZ/UNESP protocol number 0159/2019).

Study Area

The experiment was conducted in the experimental area of the São Paulo State University (UNESP), Department of Biostatistics, Plant Biology, Parasitology and Zoology of the Bioscience Institute, Botucatu, SP, Brazil.

The lambs were kept in an area of 3.840 m² divided in 5 paddocks, in rotational grazing on *Urochloa decumbens,* exposed to natural infestation with *O. ovis*, and infection with gastrointestinal nematodes. The area had been grazed previously by sheep with natural infestation by those parasites. The animals had free access to tap water and mineral salt (Supre Ovinos - Coopermota®) and were fed daily with supplement with 16.5% of crude protein (Ração Ovinos Confinamento - Coopermota®) with an amount corresponding to 2% of their body weight.

Experimental Design

The experiment was conducted from December 2019 to March 2020, during the rainy season. Thirty 2 to 3-month-old uncastrated male lambs crossbred Santa Ines x White Dorper were purchased from a local commercial farm. The lambs arrived in the experimental area on October 29th, and underwent an adaptation period of 37 days, before the beginning of trial on December 5th. Upon the arrival, blood and fecal samples from all the animals were collected for hematological and parasitological procedures, and in addition, all lambs were vaccinated against clostridiosis (Sintoxan Polivalente® - Merial), and were treated with Toltrazuril (Farmacox® - Farmabase, 20 mg/kg, orally) in order to prevent coccidiosis.

The animals were randomly distributed into three groups that were as homogenous as possible regarding body weight: group 1 ($n = 10$), control animals that did not receive any antiparasitic treatment against oestrosis; group 2 ($n = 10$), animals treated with ivermectin (Ivomec® - Merial), 0.2 mg/kg, subcutaneous injection; and group 3 ($n = 10$), animals treated with closantel (Diantel® - Merial), 10 mg/kg, orally. Treatments against oestrosis with closantel or ivermectin were performed on 5th December 2019 and on 13th February 2020.

Additionally, all experimental animals were treated with an anthelmintic combination with monepantel (2.5 mg/kg, Zolvix® - Novartis), albendazole (10 mg/kg Endazol® - Hipra) and levamisole phosphate (9.4 mg/kg Ripercol – Zoetis) for three consecutive days, which were administered in three different occasions: on 12th December, 16th January and 13th February. These anthelminitic treatments were performed to prevent losses due to nematode infections, keeping helminth infection degree similar among groups.

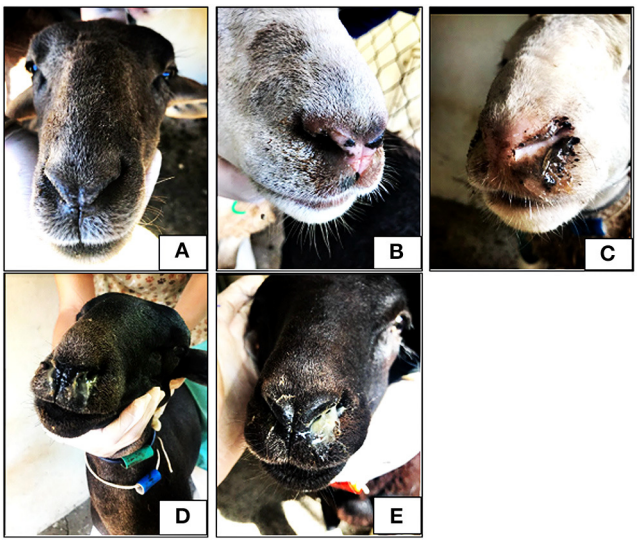

FIGURE 1 | Photos of lambs with different nasal discharge score. No discharge **(A)**, serous discharge **(B)**, sero-mucous discharge **(C)**, thick mucous discharge **(D)** and mucopurulent thick discharge **(E)**.

TABLE 1 | Nasal discharge score.

Score	Nasal discharge
1 – **Figure 1A**	No discharge
2 – **Figure 1B**	Serous discharge
3 – **Figure 1C**	Sero-mucous discharge
4 – **Figure 1D**	Thick mucous discharge
5 – **Figure 1E**	Mucopurulent thick discharge

Animals Performance and Clinical Observation

Weekly, the lambs were weighed and examined looking for clinical signs of infestation by *O. ovis*, which included their nasal discharge degree (**Figure 1**; **Table 1**), recorded according to Dorchies et al. (7).

Parasitological Analysis

Feces samples were obtained directly from the rectum of each animal weekly to perform fecal egg counts by modified McMaster technique, in which each worm egg counted represented 100 eggs per gram (EPG) (19). In addition, fecal cultures were performed for each group to obtain infective larvae (L3) of gastrointestinal nematodes, which were identified according to descriptions of Ueno and Gonçalves (19).

Recovery of *Oestrus ovis* Larvae

The animals were slaughtered 35 days after the last oestrosis treatment. They had their heads removed and then sectioned along their longitudinal and sagittal axis. Nasal cavity (nasal passage, septum, middle meatus, and conchae) (**Figure 2A**) and frontal sinus (**Figure 2B**) were carefully examined, and all larvae found were collected, counted, and identified with regards to their stage of development (1, 20).

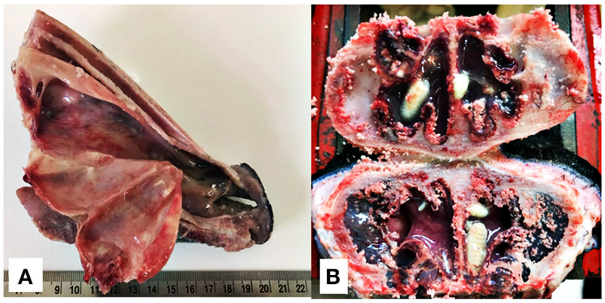

FIGURE 2 | Longitudinal section of the rostral portion of the head without the mandible **(A)** and cross section of the nasal sinuses for the recovery of *O. ovis* larvae from naturally infested lambs **(B)**.

Recovery of Gastrointestinal Nematodes

One lamb from each group, with the highest EPG in the last sampling, was chosen for worm counts. After slaughter, the lambs had their abomasum and small intestine removed and opened longitudinally for content recovery. Their gastrointestinal contents were placed in graduated buckets to obtain aliquots of 10% of the total contents from each organ. The aliquots of 10% were stored in identified plastic flasks and preserved with 5% formaldehyde. All nematodes present in the 10% preserved material were morphologically identified and quantified according to their developmental stages (19).

Hematological Analyses

Blood samples (5 ml) were obtained every 14 days by jugular vein puncture into Vacutainer® tubes containing anticoagulant (K2 EDTA 7.2 mg, BD, Brazil). Packed cell volume (PCV) was determined by microhematocrit centrifugation (5 min/15,000 g, Fanem 211 microhematocrit centrifuge), and Total Protein Plasmatic (TPP) concentrations were estimated using a handheld refractometer (Refractometer SPR-N, Atago) (21, 22). Eosinophil counts were performed in a Neubauer's chamber after staining with Carpentier's solution, and the counts were expressed as the number of eosinophil cells per μL of blood (23).

Enzyme-Linked Immunosorbent Assay

Plasma samples collected at 8 time-points were used to determine IgG levels against L2-soluble extract of *O. ovis*. The L2 extract was prepared as described by Silva et al. (24). A previously described protocol was applied to determine the parasite-specific plasma IgG levels (24) with some modifications: the plates were coated with 2 μg of antigen/mL, each wash was done six times which were incubated with peroxidase-conjugated rabbit anti-sheep IgG diluted at 1:5,000 then the plates were read at 450 nm by using an automated ELISA reader (Biotrak II; Amersham Biosciences, Little Chalfont, UK). For the negative control, plasma samples were obtained from young animals that were kept indoors without any contact with adult bot flies as previously described by Silva et al. (24). The plasma positive control sample

used was from a sheep naturally infested with *Oestrus ovis*. Results were expressed as the percentage of plasma sample optical density (OD) value divided by OD of positive control.

Therapeutic Efficacy

The therapeutic efficacy of each antiparasitic was based on the mean number of *O. ovis* larvae recovered from lambs of each group at necropsy and calculated according to the following Abbot's formula (25).

$$Efficacy = (1 - \text{Treated group/Control group}) \times 100$$

For a therapeutic claim a reduction of larval counts should be at least 90% (25).

Meteorological Data

The meteorological data was registered daily (temperatures, precipitation and relative humidity). The climatic conditions during the experiment period were similar to those reported in the last years, except for February with total rainfall of 552.2 mm. This high value occurred because on the 9th it rained 299 mm, more than expected for the whole month. Data was obtained from the Lageado Experimental Farm weather station belonging to Unesp - Campus Botucatu at a distance of 8 km from the experimental area.

Statistical Analyses

All data were submitted to the normality test Shapiro-Wilk and transformed using log10 (x + 1) when necessary, which was the case of EPG, *O. ovis* larvae stages, eosinophils and IgG values. Data were analyzed by one-way analysis of variance (ANOVA) in the case of variables measured only once (carcass weight, daily BWG and larvae stages of *O. ovis*) and by ANOVA with repeated measures in the case of variables measured at several time points (body weight, PCV, TPP, OD and eosinophils) using the General Linear Model (GLM) of the Statistical Analysis System, version 9.2 (SAS Institute, Inc., Cary, NC, USA). The group means were compared by Tukey's test and chi-square test was used for categorical variable (nasal discharge). Values of $p < 0.05$ were considered statistically significant.

Descriptive statistical analyses were used to summarize the data on larval burden, in agreement with Bush et al. (26), using the following terms:

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

During the study none of sheep showed adverse effects as a consequence of the treatments. Seven animals of the control group were infested with *O. ovis* larvae, which were alive. The control group average, considering also three animals with zero value, was 8.1 larvae per sheep (**Table 2**), which were 1.4 L1, 4.3 L2 e 3.0 L3 per head. An animal from control group with the highest intensity of infestation had 17 larvae (four L2 and 13 L3).

TABLE 2 | First (L1), second (L2) and third stage (L3) larvae of *Oestrus ovis* in naturally infested lambs of the control, ivermectin and closantel groups.

Group	Infested animal	Mean	Min–max	Total		
				L1	L2	L3
Control ($n = 10$)	7	8.1[b]	0–17	14 (17%)	41 (51%)	26 (32%)
Ivermectin ($n = 10$)	3	0.6[a]	0–3	0	2 (33%)	4 (67%)
Closantel ($n = 10$)	0	0[a]	0	0	0	0
p-value		<0.001				

Different letters in the column indicate a significant difference among means (Tukey test, $p < 0.05$).

The treatment with closantel was 100% effective against oestrosis, once there were no larvae on animals' heads. The treatment with ivermectin was 93% effective with three lambs presenting two larvae each in total six *O. ovis* larvae (**Table 2**). However, the larvae found were dead and had altered morphology, suggestive of the beginning of larva degeneration process. Differently from the lambs treated with ivermectin, the larvae identified in animals from the control group were active and without any morphological changes. Therefore, considering only alive larvae counting, the efficacy of ivermectin was 100%.

All the L1 observed in control group were in nasal conchae, 56% of the L2 were in ethmoidal conchae, and 44% in frontal sinus, while 23% of the L3 were in ethmoidal conchae and 77% in frontal sinus. Regarding the six dead larvae found in lambs treated with ivermectin, one L2 were in ethmoidal conchae, two L3 were in ethmoidal conchae and two L3 were in frontal sinus.

The clinical suggestive signs of oestrosis were scarce over the experimental period (**Table 3**) and there was no influence of treatment in the characterization of the lamb nasal discharge ($p = 0.060$). Each animal was examined 15 times over the trial. From the 450 nasal discharge exams conducted, 88% of them were serous, 9% were sero-mucous, 1% were very tick mucous, and 2% were mucopurulent thick (**Table 3**). This last score was observed in six animals, two from each group. Two weeks post the second oestrosis treatment, one animal from control group and one from group treated with closantel had muco-purulent discharges. Because lambs treated with closantel did not present larvae, it was possible to deduce that such discharge had no relation with *O. ovis* infestation.

At the beginning of the trial, the average (±standard error) body weight were 23.1 (±1.3) kg, 22.8 (±1.4) kg and 22.9 (±1.6) kg, for non-treated control, ivermectin and closantel groups, respectively. In the last sampling, lambs were about 7–8 months old, and before slaughter, they had the following weight averages: 40.8 (±1.7), 40.5 (±2.0) kg and 40.6 (±1.6) kg for non-treated control, ivermectin and closantel groups, respectively. There was no significant difference among groups ($p = 0.971$) and also no significant group × time interaction ($p = 0.615$). The

control group had daily gain mean (0.193 ± 0.016 kg), similar to ivermectin group (0.192 kg ± 0.01) and closantel group (0.192 kg ±0.02) ($p = 0.994$). In the same way, the means of carcass weight were similar ($p = 0.999$) among the different groups: 19.16 kg (±0.80), 19.11 kg (±1.01), 19.13 kg (±0.92), for control, ivermectin and closantel groups, respectively (**Table 4**).

Because the animals had been kept in feedlot on their commercial farm of origin, EPG values were low and relatively homogeneous with averages similar between groups ($p = 0.611$) at the arrival in the experimental facilities. Lambs from control group had 70 (±39.58) EPG, lambs from ivermectin group had 740 (±322.90) EPG, and lambs from closantel group had 370 (±247.68) EPG.

After the first treatment for oestrosis, closantel group had significantly lower ($p < 0.001$) EPG mean (300 ± 84) than the control (2,220 ± 424) and ivermectin (2,730 ± 925) groups. In order to avoid interference of helminthiasis in the animals' performance, it was chosen to drench all the animals with the combination of three anthelmintics (levamisole + albendazole + monepantel) on three occasions as previously described. By adopting this procedure, the EPG counts remained similar in all groups until the day of slaughter (**Figure 3**). Despite the low EPG values, group mean differences were observed on 19th December 2019, when the EPG average of the control group (390 ± 55) was higher ($p < 0.001$) than the means of the ivermectin (110 ± 48) and closantel (40 ± 22) groups; and on the last evaluation (12th March 2020), when once again the control group presented EPG mean (170 ± 49) significantly higher ($p = 0.024$) than the closantel (40 ± 40) and ivermectin (70 ± 30) groups. There was no significant interaction between the group and time ($p = 0.281$), but there was significant effect of time ($p < 0.001$) in the EPG counts.

It was also observed eggs of *Strongyloides* spp. and *Moniezia* spp. and oocysts of *Eimeria* spp. in fecal exams, however in small amounts. None of lambs had symptoms of parasitic gastroenteritis during the trial.

In fecal cultures, larvae of *Haemonchus* spp., *Cooperia* spp., and *Trichostrongylus* spp. were detected. However, *Haemonchus* spp. predominated during the experiment, being the only genus recorded from February until the end of experiment period (**Table 5**).

In the examination of the gastrointestinal contents, *Haemonchus* spp. was the major genus found, with a high number of immatures stages (**Table 6**). Additionally, ten adult females of *Trichostrongylus* spp. were recovered from a lamb

TABLE 3 | Total and percentage of nasal discharge classification of lambs naturally infested with *Oestrus ovis* of the control, ivermectin and closantel groups, in 15 evaluations from December 2019 to March 2020.

Group	Serous	Sero-mucous	Thick mucous	Mucopurulent	Total
Control	140 (94%)	8 (5%)	0	2 (1%)	150
Ivermectin	124 (83%)	19 (12%)	3 (2%)	4 (3%)	150
Closantel	133 (89%)	14 (9%)	0	3 (2%)	150
Total	397 (88%)	41 (9%)	3 (1%)	9 (2%)	450 (100%)

TABLE 4 | Initial and final body weight, daily body weight gain and carcass weight averages (±S.E.) of lambs naturally infested with *Oestrus ovis* of the control, ivermectin and closantel groups.

Variable	Control	Ivermectin	Closantel	*p*-value
Initial	23.1 (±1.3)	22.8 (±1.4)	22.9 (±1.6)	0.971
Final	40.8 (±1.7)	40.5 (±2.0)	40.6 (±1.6)	0.990
Daily gain	0.193 (±0.02)	0.192 (±0.01)	0.192 (±0.02)	0.994
Carcass	19.16 (±0.80)	19.11 (±1.01)	19.13 (±0.92)	0.999

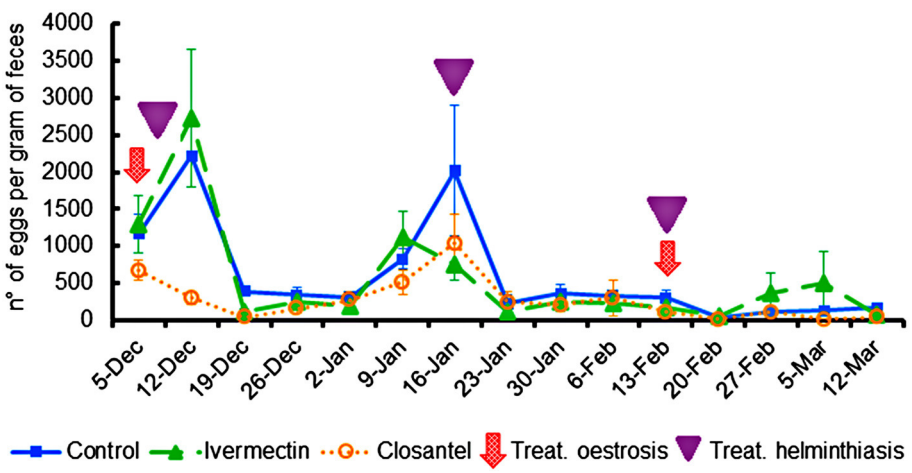

FIGURE 3 | Averages of eggs per gram feces (±standard error) of lambs naturally infected with gastrointestinal nematodes and infested with *O. ovis*. The moments of treatments against oestrosis (with ivermectin or closantel) are indicated by arrow and treatments against helminthiasis (with a combination of levamisole + albendazole + monepantel) are indicated by triangle.

TABLE 5 | Percentage of infective larvae of *Haemonchus* spp. (*Haem*), *Trichostrongylus* spp. (*Trich*) e *Cooperia* spp. (*Coop*) in fecal cultures from control, ivermectin and closantel groups.

Date	Control			Ivermectin			Closantel		
	Haem	*Trich*	*Coop*	*Haem*	*Trich*	*Coop*	*Haem*	*Trich*	*Coop*
5/Dec	99	1	0	100	0	0	98	1	1
12/Dec	95	1	4	100	0	0	100	0	0
19/Dec	91	0	9	97	0	3	100	0	0
26/Dec	92	0	8	96	0	4	96	0	4
2/Jan	91	0	9	98	0	2	99	0	1
9/Jan	84	0	16	93	0	7	97	0	3
16/Jan	90	0	10	96	1	3	94	1	5
23/Jan	100	0	0	100	0	0	100	0	0
30/Jan	100	0	0	99	0	1	100	0	0
6/Feb	100	0	0	100	0	0	100	0	0
13/Feb	100	0	0	100	0	0	100	0	0
20/Feb	100	0	0	100	0	0	100	0	0
27/Feb	100	0	0	100	0	0	100	0	0
5/Mar	100	0	0	100	0	0	100	0	0
20/Mar	100	0	0	100	0	0	100	0	0

TABLE 6 | Total *Haemonchus contortus* and *Trichostrongylus* spp. worm burden from one lamb of each group naturally infected with gastrointestinal nematodes.

Genera	Stage	Control	Ivermectin	Closantel
Haemonchus spp.	Early L4	2,360	1,000	1,620
	Female late L4	2,540	10	180
	Male late L4	2,630	330	0
	Female early L5	370	0	0
	Male early L5	910	0	0
	Adult female	20	100	0
	Adult male	0	50	0
Trichostrongylus spp.	Adult female	10	10	0
	Total worm burden	8,840	1,500	1,800

treated with ivermectin and ten in an animal of the control group (**Table 6**).

The values of eosinophils, PVC and TPP (**Figure 4**) were similar among the three groups throughout the trial, and there were no significant interactions between group and time for these variables. The only exception occurred on 19th December 2019, at the beginning of the experiment, when the TTP mean of ivermectin group (6.02 ± 0.109) was significantly higher than the closantel group (5.60 ± 0.126) ($p = 0.017$). The lowest means of PCV (**Figure 4B**) were registered on 16th January 2020 ($p = 0.097$), that were 21.7% (±3.65), 25.2% (±4.10) and 24.2% (±2.86), for the control, ivermectin and closantel groups, respectively. This reduction in the values of PCV coincided with the increase in the EPG means (**Figure 3**). Additionally, in the same date, it was observed the highest means of blood eosinophils (**Figure 4A**).

The mean levels of anti-*O. ovis* L2 IgG in plasma showed changes over time, resulting in significant interaction between time and treatment ($p = 0.007$). The antibody levels increased in the control group on the last 2 weeks of the trial, being significantly higher than values of the treated groups ($p = 0.004$) on 27th February and 12th March (**Figure 4D**). The three animals from control group that did not have any larvae of *O. ovis* presented the lowest averages of antibodies in their group (lamb 1: 49%; lamb 2: 49%; and lamb 4: 45%).

DISCUSSION

The results of this trial confirmed the high effectiveness of closantel in animals naturally infested by *O. ovis*, with the absence of larvae 35 days after the last treatment. Regarding the ivermectin, some treated animals still hosted larvae of second and third stages. Nevertheless, the larvae recovered from these animals were already dead presumably because of ivermectin action. However, it was not possible to determine when the larvae died. It is possible that the larvae have died soon after the treatment, but, somehow, they remained in the nasal cavities. The dead larvae are possibly expelled by sneezes that combined with the force of gravity cause nasal discharge.

Therefore, the results of the present study were in accordance with Dorchies et al. (7) that reported 100% of efficacy of oral closantel and injectable ivermectin against oestrosis, in sheep treated twice with an interval of 60 days. Likewise, oral ivermectin resulted in 100% efficacy against all stages 12 days after the treatment (9). Different of other Diptera that cause myiasis (*D. hominis* and *C. hominivorax*) with cases of drug-resistance (15-16-18), to the best of our knowledge, there is no report of populations of *O. ovis* with resistance to ivermectin or closantel.

The typical clinical signs of oestrosis were uncommon over trial and during the examination performed at necropsy there were no macroscopic morphological changes in the nasal cavities. There was only a small amount of secretion at the site where parasites were located and around the larvae. The absence of clinical sings in most of the evaluations may be a consequence of the low infestation rate. *Oestrus ovis* clinical manifestation is induced partially by spines on the larval cuticle and oral hooks used by L1 to move and progress toward nasal cavities and to anchor to the mucous surface. In addition, the L3 has large hooks, stout spines and dorsal plates which serve to support its gradual descend in the nasal cavities to the outside environment (4), whereupon this mechanical action provokes irritation in nasal mucosa (27). The pathogenesis of *O. ovis* is also induced by biomolecules (enzymes and antigens) excreted or secreted by the larvae onto the mucosa. These biomolecules comprise a complex array of enzymes necessary to degrade mucus and plasma proteins for larval feeding and nutrition (28). In agreement with our results, Silva et al. (6) observed that most tracer lambs in their study did not show any clinical signs typical of oestrosis after exposed to infestation during 4 weeks, although they hosted among 12 and 66 larvae, mainly L1 and L2. Like in the trial conducted by Silva et al. (6), possibly in our trial the infestation was recent, without enough time to manifestation of the immunopathological changes typical of the disease. Although not all infested sheep show clinical signs of

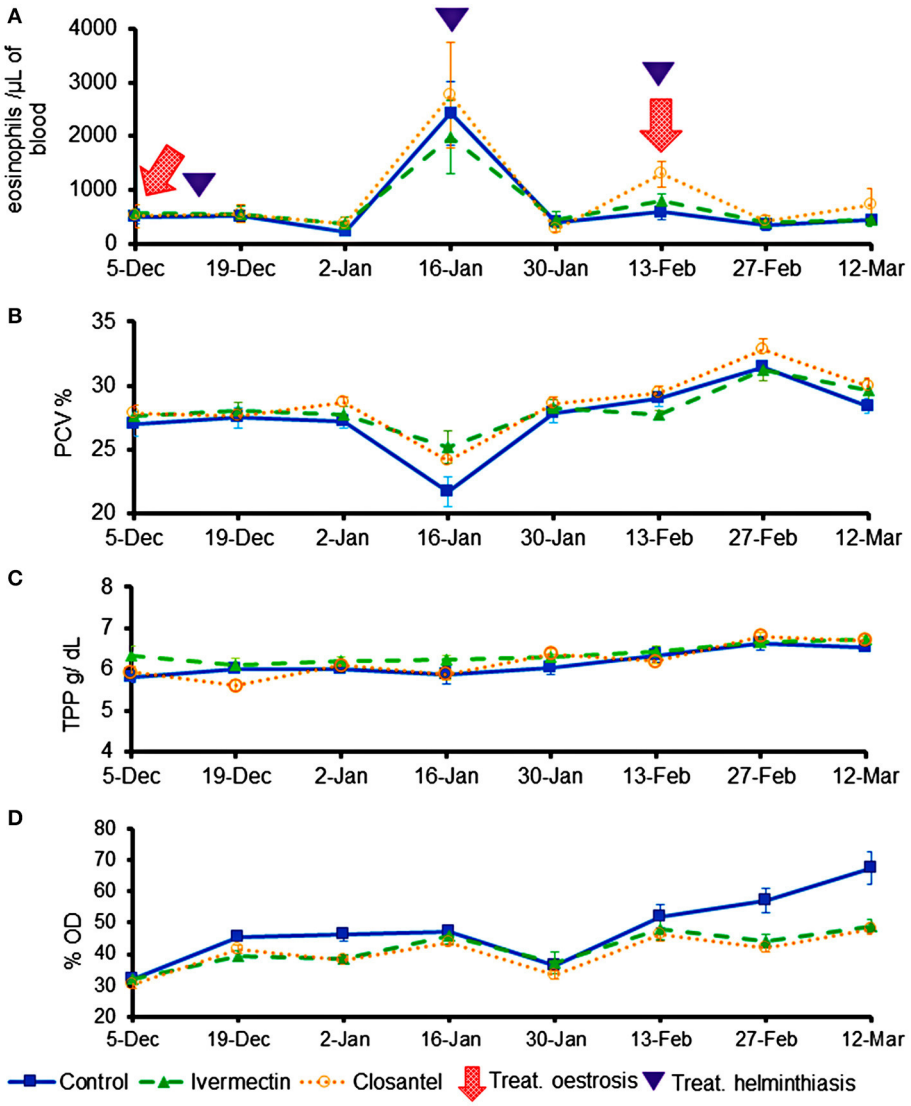

FIGURE 4 | Averages (±standard error) of eosinophils (cells/µL) **(A)**, packed cell volume (%) **(B)**, total plasma protein (g/dL) **(C)**, and optical density (%OD) of IgG anti-*O. ovis* L2 **(D)** of lambs treated with ivermectin or closantel. The moments of treatments against oestrosis (with ivermectin or closantel) are indicated by arrow and treatments against helminthiasis (with a combination of levamisole + albendazole + monepantel) are indicated by triangle.

oestrosis, the major symptoms of infestation (nasal discharge and frequent sneezing) are immune-mediated depending on acquisition of an immune response against the parasite (6, 29). Infestation induces a great recruitment of inflammatory cells such as mast cells and eosinophils, to site of host-parasite interaction and also increases the immunoglobulin production (29).

If the trial had a longer duration, perhaps the typical clinical signs would occur, because the ability of sheep to become resistant to oestrosis is limited, different from what usually happens in infection by helminths (24). Also drew attention the fact that the treatment against oestrosis did not influence the weight gain of the animals, demonstrating the benign character of the disease in lambs. The treatment with anthelminthics, including in those lambs from control group, avoided the interference of helminthiasis in the body weight gain of the lambs, which were around 190 g per day, within the expected for grass fed male lambs supplemented with concentrate (30). However, more studies are necessary to evaluate the influence of oestrosis on sheep productivity in our environmental conditions, especially in older animals continuously exposed to the parasitism.

Lopes et al. (31) reported a case of poisoning by closantel in three animals from a group of 15 sheep treated with closantel twice with interval of 28 days. These lambs showed apathy, anorexia, diarrhea and blindness after the second treatment

with 7.5 mg/kg orally. In our trial, lambs did not show any adverse effects post treatment with closantel or ivermectin. Some studies have shown that well-nourished animal, which was the case of our lambs, has less chances of poisoning because the closantel binds to plasmatic proteins, mainly albumin and healthy animals tend to have higher plasmatic protein level that reduce the amount of drug available in tissues (32, 33).

There was an increase in EPG means and a reduction in PCV values caused by *Haemonchus* spp. in all groups on 16th January 2020. After the anthelmintic treatment, PCV means increased and remained within the reference interval for sheep until the end of the study. Concomitantly, it was observed a significant increase in blood eosinophils counts on 16th January. Eosinophilia has been reported as one of the defense mechanisms against the gastrointestinal nematode parasitism (34, 35) and against *O. ovis* infestation (36).

While IgG-anti *O. ovis* means remained low in treated groups, the control lambs showed an increase in averages at the end of the trial, thus indicating that the animals were responding to parasite infestation. Previous studies demonstrated that detection of *O. ovis* antibodies in sheep is a valid diagnosis technique (24, 37–39).

CONCLUSIONS

Closantel oral drench at a dose rate 10 mg/kg and ivermectin subcutaneously at 0.2 mg/kg remain effective in the prophylaxis of oestrosis. These drugs are effective at the same concentrations used in the previous studies and there is no evidence of increasing trends of resistance. At the early stage of infestation, *O. ovis* parasitism did not cause a negative impact on the performance of lambs.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee on Animal Use (FMVZ/UNESP Protocol Number 0159/2019).

AUTHOR CONTRIBUTIONS

HB performed all experiment, collected and analyzed the data, and completed the manuscript preparation. JL, ACA, and GF helped in the collection of data. AFA, JL, ACA, and MA participated in its design and coordination and helped to interpret the results. All authors interpreted the results and substantively revised the manuscript, read, and approved the final manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

CAPÍTULO 3 – Diagnosis of *Oestrus ovis* infestation in sheep by PCR and enzyme-linked immunosorbent assay

Nas páginas a seguir encontra-se o manuscrito que foi submetido à publicação no formato de “Original Research” no periódico Veterinary Parasitology.

Normas de publicação do periódico: https://www.elsevier.com/journals/veterinary-parasitology/0304-4017/guide-for-authors.

Diagnosis of *Oestrus ovis* infestation in sheep by PCR and enzyme-linked immunosorbent assay

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ABSTRACT

The aim of this study was to assess the variability of clinical signs of oestrosis and the suitability of PCR and ELISA as diagnostic method in young sheep naturally infested by *Oestrus ovis* larvae. The experiment was carried out from December 2020 to April 2021 with 39 lambs divided into two groups: infested (n=26) and control treated group (n=13). The infested group did not receive treatment against oestrosis, and the control group was treated with closantel (10 mg/kg orally) every 28 days in order to keep the animals as free as possible of *O. ovis* infestation. The clinical signs varied among animals regardless of the number of recovered larvae of each lamb, however, the thick mucus and mucopurulent nasal discharge scores were less frequent in lambs from treated group. There was no correlation between the nasal discharge score and the number of *O. ovis* recovered larvae ($R^2 = 0.012$, $P = 0.165$). Three control treated animals only presented first instar larvae (L1) (1 – 4 larvae/animal) which were smaller than L1 found in the lambs of the infested group. Ninety-two percent of the lambs from infested group (24/26) were parasitized by *O. ovis* with number ranging from 1 to 54 larvae per animal. A gradual increase in plasma IgG (anti-antigen of *O. ovis* larvae) levels of animals from infested group after the third week of the trial was observed, whereas the control lambs had low levels of IgG until the end of the experiment. The PCR had low sensibility (26%) and high specificity (100%), and it presented slight agreement ($k = 0.177$) with the larvae detection after the lamb slaughter. The oestrosis clinical signs were not related to larvae infestation intensity and

ELISA showed a greater advantage over the PCR technique in identifying animals that are carrying *O. ovis.*

Keywords: oestrosis, bot fly, molecular diagnostic, lamb, ELISA

Introduction

Oestrus ovis (Linnaeus 1761, Diptera, Oestridae) is responsible for causing cavitary myiasis (oestrosis) in sheep and goats. The larvae of this fly are obligate parasites of the nasal cavities and paranasal sinus of these animals (Zumpt, 1965). The larval infestation can cause breathing difficulties due to thick mucus and even mucopurulent nasal discharge. Streaks of blood can be found in the nasal discharges, moreover the sheep may present frequent sneezing, dyspnea and cough (Butterfield, 1900; Dorchies et al., 1996; Dorchies, 1997), which impair the health and welfare of infested animals (El-Tahawy, 2010). The oestrosis symptoms are commonly associated with the clinical signs observed in other respiratory diseases (Dorchies et al., 1998). However, this disease usually affects a high number of individuals in the flock being considered a collective pathology, an aspect that differentiates it from other important upper respiratory tracts pathologies, which are more commonly individual (Gracia et al., 2019).

Oestrosis is also considered a zoonotic disease causing ophthalmomyiasis and nasopharingeal myiase in humans (Panadero-Fontán and Otranto, 2015) reported in many parts of the world (Al-Antary et al., 2018; Velev and Mikov, 2018; Jenkins and Layton, 2018; D'Assumpcao et al., 2019). In endemic areas, several cases remain unreported (Panadero-Fontán and Otranto, 2015).

Infestation by *O. ovis* starts when the female flies deposit tiny first stage larvae (L1) into the nostrils of their hosts. The larvae have buccal curved hooks and rows of spines throughout the body in all developmental stages (L1, L2 and L3) that enable them to move into nasal passages and to anchor on mucosal surface. Such morphological larvae adaptations avoid larvae to be expelled by strong breath or sneezing before completing their development and also favor larvae feeding on the mucosal and mucus secretion (Cepeda-Palacios and School, 1999; Tabouret et al., 2003a; Kamal et al., 2021). Afterwards, mature L3s migrate back to nasal passages and they are expelled by the sneezing onto the ground and then pupate (Giannetto et al., 1999). During their development these parasite excrete/secrete biomolecules that degrade in smaller substrate units the mucosal components, such as mucin, albumin and immunoglobulin G (Tabouret et al., 2003b; Angulo-Valadez et al., 2007, 2011).

The larvae behavior and their expelled molecules induce an immunological hypersensitivity reaction (Jacquiet et al., 2005) to the manifestation of the clinical symptoms, which may differ among individuals regardless of the number of larvae (Ângulo-Valadez et al., 2011).

While the diagnosis of the vast majority of the animal ectoparasitosis caused by arthropodes is achieved by the visual detection of parasites on the animal's body (e.g. horn fly, myiasis by *Cochliomyia hominivorax*, and tick infestation) or in biological samples (e.g. mange mites detectable in scabs), this is not the case for the majority of oestrids causing internal myiases, e.g. horse gasterophilosis and sheep oestrosis, whose diagnosis depends on the post-mortem examination of internal organs (Traversa and Otranto, 2006). However, from a scientific and animal welfare point of view, the use of necropsy to diagnose and determine the intensity of parasitism limits the research possibilities (Arias, 2014) and is economically unfeasible in sheep farming.

Serological test, such as the enzyme-linked immunosorbent assay (ELISA), that detects antibodies against *O. ovis*, could be used for monitoring the occurrence of oestrosis in the herd (Otranto, 2001, Alcaide et al., 2005; Angulo-Valadez et al., 2008; Silva et al., 2012b, 2018; Arias et al., 2014). However, information regarding the host immune response to myiasis-causing larvae is fragmented in comparison to the response against other ectoparasites (e.g., ticks and mosquitoes) (Pruett, 1999), and understanding such host-parasite interaction/immunological mechanisms is the basis for the development of new methods for controlling and diagnosing oestrosis.

The prevalence of *O. ovis* infestations in sheep has been underestimated due to limitations of ante-mortem diagnosis. Recent studies have shown PCR as a valid technique to detect the presence of *O. ovis* larvae ante-mortem in the herd and may be useful as an alternative diagnostic test (Ípek and Altan, 2017), however this method should be validated in a new trial or biological samples. In addition, the PCR method may also be useful to assess the efficacy of drugs against *O. ovis* via monitoring the decline or absence of parasite-specific DNA in nasal mucus after treatment of sheep with oestrosis, thus avoiding sacrificing them to confirm the presence of larvae.

In a previous trial, we observed that closantel had high efficacy against oestrosis in sheep allowing protection against re-infestation for several weeks (Bello et al., 2022). This observation allowed us to set up an experiment with grazing lambs in order to compare a non-infested control group, treated with closantel, with an infested group of young sheep, without treatment. This approach allowed to elucidate inconsistencies in the

diagnosis of clinical oestrosis and evaluate the accuracy and the suitability of PCR and ELISA as diagnostic methods for detecting this parasitosis.

Methods

Study area and experimental design

All the procedures involving the animals in this study were carried out according to the standards established by the local Ethics Committee on Animal Use (FMVZ/UNESP protocol number 0159/2019).

The experiment was conducted from 22nd December to 14th April 2021, during the rainy season, in the experimental area of the Department of Biostatistics, Plant Biology, Parasitology and Zoology of the Bioscience Institute, São Paulo State University (UNESP), Botucatu, SP, Brazil.

Thirty-nine 4-5 months-old crossbred lambs, with the predominance of the Santa Ines breed, were purchased from a local commercial farm. The lambs were randomly allocated into two groups balanced by sex and weight: infested group with 14 males and 12 females (n=26) without treatment against oestrosis; and a control treated group with seven males and six females (n=13), that were treated with closantel (10 mg/kg, orally, Diantel® - Hipra) every twenty-eight days, in order to keep the animals as free as possible of *O. ovis* infestation (Bello et al., 2022).

Upon the arrival, all lambs were vaccinated against clostridiosis (Sintoxan Polivalente® - Merial), and were treated with toltrazuril (Farmacox® - Farmabase, 20 mg/kg, orally) in order to prevent coccidiosis. Before the beginning of the experiment, the lambs underwent an adaptation period of fifty-three days. The lambs were kept in an area of 3.840 m2 divided in 5 paddocks, in a rotational grazing on *Urochloa decumbens* grass. The same area had been grazed previously by sheep with natural *O. ovis* infestation, thus, all lambs were exposed to natural infestation with this parasite. The animals had free access to tap water and mineral salt (Supre Ovinos - Coopermota®) and were fed daily with supplement with 16.5% of crude protein (Ração Ovinos Confinamento - Coopermota®) with an amount corresponding to 2% (weekly adjusted) of their body weight.

Clinical signs and *post-mortem* larvae count

The clinical signs and their severity were classified weekly by nasal discharge score visualization (Dorchies, 1997; Bello et al., 2022), as follows: (1) absent nasal discharge: wet nostrils; (2) serous: transparent and watery nasal discharge; (3) mucus: transparent to translucent nasal discharge; (4) thick mucus: slimy, thick and translucent nasal discharge, with some difficulty breathing and nasal noises when breathing; (5) mucopurulent: thick nasal discharge, opaque with mucus and pus. For the recovery of *O. ovis* larvae, lambs were slaughtered, their heads were removed and three cuts were made on each head: first frontal cut immediately posterior to the eyes, without cutting the mandible (Figure 1A); second cut in the frontal bone (Figure 1B); and the third sagittal cut along the nasal bone and palate of the rostral portion that was removed in the first cut (Figure 1C). When the heads were cut, the presence or absence of thick or mucopurulent mucus secretion in the nasal cavities and frontal sinuses were observed and recorded. All the larvae in the nasal cavities (septum and conchae) and frontal sinus were collected, counted and identified according to their developmental stage based on description of Zumpt (1965). First stage larvae (L1) were measured using a micrometric "eye piece" graticule at 100x magnification in a Leica® DMS2 microscope.

Enzyme-Linked Immunosorbent Assay

Blood samples were collected individually (5 ml) every fourteen days by jugular vein puncture into Vacutainer® tubes containing anticoagulant (K2 EDTA 7.2 mg, BD, Brazil) and were centrifugated for 15 min at 590 ×g at 4°C to allow plasma separation. Aliquots of plasma samples were stored at −20 °C prior to ELISA.

Plasma samples collected at 9 time-points were used to determine IgG levels against second stage larvae (L2) soluble extract of *O. ovis*. The L2 extract was prepared as described by Silva et al. (2012). A previously described protocol was applied to determine the parasite-specific plasma IgG levels (Silva et al., 2012) with some modifications: the plates were coated with 2 μg of antigen/mL, each wash was done three times, rotating through 180º and re-washing three more times; the peroxidase-conjugated rabbit anti-sheep IgG was diluted at 1:5,000; and the plates were read at 450 nm by using an automated ELISA reader (Biotrak II; Amersham Biosciences, Little Chalfont, UK). For the negative control, plasma samples were obtained from young animals that were kept indoors without any contact with adult bot flies as previously described by Silva et al.

(2012). The plasma positive control sample used was from a sheep naturally infested with *O. ovis*. Results were expressed as the percentage of plasma sample optical density (OD) value divided by OD value of positive control (Kanobana et al., 2001).

Collection of swab samples and DNA extraction

Mucus samples were collected from the caudal regions of both the ventral nasal and common nasal conchae from left (L) and right (R) nasal cavities, one day before the slaughter using sterile cotton swabs (Olen®). All samples were cataloged and swabs stored at – 20°C until use in molecular analysis.

Cotton Swabs L and R of each lamb were placed in only one swab tube and 400 µl of phosphate-buffered saline (PBS) was employed to wash the empty swab tube and poured to the one containing L and R swabs. Additionally, 200 µl of QIAGEN buffer ATL at 56°C was added and mixed. Then, 20 µl QIAGEN protease stock solution was added and mixed and 400 µl QIAGEN buffer AL was also added. This solution was mixed by pulse-vortexing for 15 seconds and incubated for 10 minutes at 56ºC. The cotton tips of the swabs and the solution were placed in a 2 ml tube and 400 µl ethanol (96–100%) was added and mixed by pulse-vortexing for 15s. 510 µl of that mixture was applied to the QIAamp Mini spin column and centrifuged at 6000 ×g for 1 minute. The QIAamp Mini spin column was placed in a clean 2 ml collection tube and the remaining 510 µl of the mixture was applied to the same QIAamp Mini spin column followed by centrifugation (6000 ×g for 1 minute). The filtrate was discarded and the remaining extraction procedure followed the manufacturer's instructions for Bucal Swab spin protocol (QIAamp DNA Mini kit, QIAGEN, Hilden, Germany).

Finally, all extracted DNA samples were purified using a cleanup and concentration kit (Microcon DNA fast flow, Merck Millipore Ltd. Cork, Ireland).

Obtaining new forward primer OoBotu_F and PCR reaction

Forward primer OoBotu_F (5'-GCAGGCTTTGTACATTGATATCC-3') was designed from the Genbank Locus AF497767 and using Primer3 (Rozen and Skaletsky, 2000).

All DNA samples were subjected to PCR analysis using the designed forward primer OoBotu_F and the reverse primer UEA10 (5' TCCAATGCACTAATCTGCCATATTA-3) described by Otranto et. al. (2003) and a

406 base pairs (bp) sequence was amplified in order to identify the presence of *Oestrus ovis* in mucus samples.

The Gene Amp PCR System 9700® thermocycler (Applied Biosystems) was employed to perform PCR reactions. Amplification was carried out in a 10 µl reaction mix containing 10 mM Tris-HCl, 1.5 mM MgCl2, 50 mM KCl (pH 8.0), 100 µM each deoxynucleoside triphosphate (dNTP), 10–20 ng genomic DNA and 0.5 U Taq polymerase (Invitrogen, São Paulo, Brazil). The cycling conditions for the primer pair were as follows: 95°C for 5 min; followed by 40 cycles of 95°C for 30 s, 58° C for 30 s and 72°C for 30 s; followed by 5 min at 72°C and 4°C to finalize.

Genomic DNA from the L2 larvae and sheep blood DNA samples were included in all PCR reactions, as positive and negative controls, respectively. All PCR products were electrophoresed on 2% agarose gels in 1% Tris–acetate–EDTA (TAE) buffer containing ethidium bromide and photographed under UV light using a Sony Cyber-shot DSC-HX1 camera (Sony Electronics, California, USA).

Meteorological data

Temperature means, the average relative humidity and rainfall were measured at meteorological station of Faculdade de Ciências Agronômicas – UNESP located at Fazenda Lageado, Botucatu, Brazil, with 8 km far from the experimental paddock. The results were expressed in temperature (ºC) and relative humidity (%) means, and accumulated rainfall precipitation (mm) of experimental period.

Statistical analysis

All data were submitted to normality test Shapiro-Wilk. It was necessary the transformation using $\sqrt{(x + 0.5)}$ of IgG values and *O. ovis* larvae counting. The larvae numbers were analyzed by completely randomized design by analysis of variance (ANOVA). IgG samples were evaluated with repeated measures using the General Linear Model (GLM). The chi-square test was used for categorical variable of nasal discharge. Values of $P < 0.05$ were considered statistically significant.

The associations between the quantity of larvae and nasal discharge score and quantity of larvae and OD were evaluated by linear regression and Spearman's Correlation. To evaluate the PCR as diagnostic method of oestrosis, the sensitivity, specificity, positive predictive value, negative predictive value, and Kappa index (k) of agreement (confidence interval 95%) were compared to the count of recovered larvae. All

statistical procedures were performed using the Statistical Analysis System, version 9.2 (SAS Institute, Inc., NC, USA).

Descriptive statistical analyses were used to summarize the data on the larval burden in agreement with Bush et al. (1997), using the following terms: prevalence, as 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 infested with that parasite.

Results

Clinical signs and larval counting

A total of 491 larvae were recovered, and all of them were alive. No third stage larvae were recovered regardless the group. Regarding the experimental groups, 24 lambs from the infested group (26) were parasitized by *O. ovis* and the mean of larvae per animal was 19 (±3.43), being 12 (±2.33) L1 and 6 (±1.31) L2. The maximum of larvae recovered in one lamb in this group were 54 specimens. In total, 483 larvae of *O. ovis* were recovered in animals from infested group, which were 66% (321) L1 and 34% (162) L2. Among the 321 L1 recovered, 90% (289) were in the nasal conchae (Figure 2A), 8% (27%) in the ethmoidal conchae, and 2% (5) in the frontal sinus. Out of 162 L2 recovered, 22% (36) were in the nasal conchae, 15% (24) in the ethmoidal conchae, and 63% (102) in the frontal sinus (Figure 2B). The biggest L1 found in this group was 3.61 mm long and the smallest was 1.69 mm long. From control group, three of the 13 animals were parasitized only by first stage *O. ovis* larvae recovered from nasal conchae: one animal with three larvae, another with four, and one animal with only one larva. The biggest L1 found in this group was 1.42 mm long and the smallest was 0.98 mm long.

Some of the recovered L1 were attached to the nasal mucosa by the oral hooks and others were loose in the mucus of the nasal cavity from both groups. Many larvae at this stage were recovered only by opening the nasal conchae (Figure 3). Prevalence data and mean intensity of infestation are showed in the Table 1.

During the study, each animal of infested and control treated group had their nasal secretions evaluated 17 times. The nasal secretion of lambs of the infested group was predominantly classified as mucus (51%) followed by serous (38%), mucopurulent (6%), and as thick mucus (5%) (Table 2), while in the control treated group the nasal secretion

was mostly classified as serous (61%) followed by mucus (31%), thick mucus (4%), and as mucopurulent (4%), with a statistically significant group effect on the frequency type of the nasal discharge (Table 2). Despite animals from infested group presenting higher number of larvae than control treated group, four animals showed no clinical signs of infestation even with a high number of *O. ovis* larvae. In contrast, six lambs with thick mucus or mucopurulent nasal secretions had few or no larvae, whereas two animals had mucopurulent nasal secretions and had numerous larvae.

In addition, there was no significant correlation between secretion score and the number of recovered larvae in the infested group ($R^2 = 0.012$; $P = 0.65$) (Figure 4), as observed in a lamb with the highest infestation intensity (54 larvae in total, 38 L1 and 16 L2) from infested group which at the time of larvae recovery did not show exacerbated presence of mucus. The same lamb presented thick mucus nasal secretion only on previous evaluations (February 9th and 16th), while in the other dates, the secretion was serous or mucus.

Sneezing and coughing were observed among the lambs, but the latter symptom was less frequent, regardless of the group, also blood streaks were rarely found in nasal secretions. The clinical manifestations of the control treated lambs were more discreet and they showed lower secretion scores in comparison to the infested group.

Over the experimental period, flies were frequently observed on the lambs and flying over the animals' muzzle. In their presence, the animals were annoyed, beating their paws on the ground and trying to hide their muzzle.

The lambs did not show any clinical signs of poisoning caused by the antiparasitic treatments administered.

Plasma IgG anti-*O. ovis* levels

The animals in the infested group had means of IgG anti-*O. ovis* L2 higher than the control treated animals during almost the entire experiment, except for the first two evaluations in which the percentage of OD was similar between groups ($P \geq 0.05$) (Figure 5).

There was a time effect ($P<0.001$) on IgG levels. The time and treatment interaction was significant ($P<0.001$). The OD did not show significant correlation with the number of recovered larvae considering each animal of the infested group ($R^2 = 0.091$, $P = 0.608$) (Figure 6).

Molecular diagnosis

Among animals with *O. ovis* larvae (27/39) only 7 (7/27) had positive PCR test results. All animals that did not have recovered larvae tested negative in the PCR, as well as none of the animals in the control treated group showed a positive result in this test. This test showed low sensitivity (26%) and high specificity (100%). The PCR results showed a slight agreement with the results of the larvae counting after slaughter (k = 0.177) and the confidence interval was 0.034 – 0.320. The false negative was 74%, the false positive was 0, the positive predictive value was 100% and the negative predictive value was 35%

PCR amplification (Figure 7) was used to identify the presence of *O. ovis* in mucus samples collected from the caudal regions of both the ventral nasal and common nasal conchae. One amplification band of 406 bp is observed in the line 3 that corresponds to L2 larvae sample and was employed as a positive control. Lines 5 and 8 also show the same amplification band which denotes the presence of *O. ovis* DNA. No amplification band is observed in the negative control (line 2, host) and also in lines 4, 6 and 7 which means there was no evidence of *O. ovis* DNA in those mucus samples.

It is important to explain that there were no larvae in the mucus samples during the process of DNA extraction. Then, the diagnosis using PCR was based on the detection of DNA traces eliminated by the larvae in the nasal cavity.

Climatic conditions

The average temperature from December 22nd to April 14th was 22.6 °C (18.7 and 28.5 °C, average minimum and maximum), the average relative humidity was 76.5% and the accumulated rainfall of 701.5 mm.

Discussion

The control treated animals remained practically asymptomatic throughout the experimental period demonstrating that the treatment with closantel prevented the evolution of clinical signs of oestrosis as observed in a previous study (Bello et al., 2022). Thirty days after the last treatment only a few control lambs had larvae in the initial stage of development with lengths of 0.98 and 1.42 mm. According to Gianetto et al. (1999) the first stage larvae are about 1 mm long at the time they are deposited by the fly, therefore, L1 recovered from the control treated were possibly deposited in the nostrils of the lambs a few days before slaughter, also they were smaller than the others L1 recovered

from the infested group which were between 1.69 and 3.61 mm. Presumably, larvae in this early stage of development have limited feeding, so they are less susceptible to systemic parasiticides (Rugg et al., 2012). When the *O. ovis* larvae start to develop they increase their food intake, consequently ingest a higher dose of the drug and die (Martínez-Valladares et al., 2013).

The nasal discharge and sneezing represent the main clinical signs of *O. ovis* infestation in sheep that may characterize the presence of larva and the subsequent sinusitis. However, in the present study, some lambs did not present frequent clinical signs of oestrosis even with large numbers of larvae recovered from the nasal cavity and frontal sinus at the necropsy. In contrast, some sheep presented strong evidences of infestation, even with few or none *O. ovis* larva, phenomenon also pointed out by Angulo-Valadez et al. (2011) in a review. During larval development, the migration of *O. ovis* larvae in the nasal cavities of the sheep induces an inflammatory response in the host due to the parasite hooks and spines, also the serine proteases secreted and excreted on host mucosae by the larvae (Tabouret et al., 2003a). A specific immune response develops with a local (IgG and IgA) and systemic (IgG) response along with the recruitment of eosinophils and mast cells in the upper airways' mucosae (Nguyen et al., 1999; Tabouret et al., 2003a). The respective roles of these two types of responses are not yet known. The development of *O. ovis* larvae after artificial infections was higher in immunosuppressed animals compared to the controls (Marchenko and Marchenko, 1989; Jacquiet et al., 2005) implying that at least a partial immune regulation of *O. ovis* larval establishment could occur. The lambs in the infested group showed increasing levels of IgG over the trial, which did not prevent the establishment and development of the larvae. It indicates that the young sheep did not develop an efficient protective immune response as a result of exposure to the parasite. Besides age influence, there are also individual variations in genetic resistance in sheep (Angulo-Valadez et al., 2008). Thus, possibly, the most susceptible animals were the ones with less intense clinical signs, despite the presence of many larvae, while the most resistant animals were those with more severe clinical signs presenting fewer larvae with more efficient response to the parasite presence.

No L3 were recovered from any animal at necropsy, which may indicate that the first generation of larvae may have had time to complete the development, leave the host, and pupate in the soil. Possibly, at necropsy, we detected the beginning of the second generation of larvae at the study site. During all study period, climatic conditions were

favorable for the development and survival of the fly and the larvae of *O. ovis*. Therefore, the animals were constantly exposed to natural infestation.

Throughout an *O. ovis* infestation, the systemic humoral IgG response usually reaches seroconversion within 2 to 4 weeks of first infestation and the highest levels are observed during L2 and L3 larval development (Alcaide et al., 2005; Ângulo-Valadez et al., 2011). In an endemic environment, lambs got infested with *O. ovis* at an early age and start to produce detectable levels of anti-parasitic IgG in plasma samples at three months of age (Silva et al., 2018). In the course of the present trial, the immune response against *O. ovis* was clearly demonstrated since January 5th by the presence of IgG values with significant group differences. Therefore, the ELISA using L2 antigen to detect anti-parasitic IgG proved to be suitable to identify *O. ovis* infestation in young lambs and may be considered a valid method for the diagnosis of oestrosis.

Only a few animals of the treated group presented larvae that were probably newly deposited by the fly. Thus, in this group there was not enough stimulus for IgG production. Experimental studies have suggested that IgG can be detected as early as 2 to 4 weeks after repeated infestations, however, animals infested only with L1 larvae or with a very small number of mixed larvae reach minimal IgG thresholds after 6 to 9 weeks. Furthermore, specific IgG disappears within 160 days after effective antiparasitic treatment (Angulo-Valadez et al., 2009; Angulo-Valadez et al., 2011; Terefe et al., 2005). The PCR technique showed low sensitivity (26%) but high specificity (100%) in our study. In a similar study, Ípek and Altan, (2017) observed 100% sensitivity and specificity of PCR when they evaluated 158 sheep, which 133 were positive in both larval recovery and PCR. High diagnostic sensitivity (88.5%) and specificity (100%) by semi-nested PCR assay were obtained in mucus samples collected from the pharynxes of 26 slaughtered horses infested with 1–10 *Rhinoestrus* nasal botfly third stage larvae (Traversa and Otranto, 2006). In the same study, false-negative results were associated with low parasite loads. The animals with negative test were infested with ≤3 larvae and therefore had insufficient amount of DNA in the mucus (Traversa and Otranto, 2006). The false negative results observed in the present study were likely due to the insufficient amount of DNA in the mucus, which did not have a relationship with the parasite burdens.

The diagnosis of gastrointestinal nematodes by molecular methods is well advanced (Harmon et al., 2007; Roeber et al., 2013; McNally et al., 2013; Hoglund et al., 2019). In this case, the parasite's DNA is easily obtained from eggs presented in fecal samples or in larvae produced in coprocultures. The obstacle in diagnosing oestrosis by PCR is

related to the difficulty to reach the parasite itself at the time of material collected with the swab. Thus, we had to detect DNA excreted or secreted by the parasite mixed with the sheep's nasal secretion. Our results indicate that the presence of DNA molecules eliminated by the larvae and present in the mucus was very scarce in the animals of the current study and also most animals had serous nasal discharge during most of the study, which explains the high number of negative PCR results. In order to improve PCR as a diagnostic test for oestrosis, further studies are needed to improve collection of samples to allow the proper amplification of parasitic DNA.

Conclusion

The PCR protocol used in the present study presented low sensitivity for the individual diagnosis. However, it can be used to confirm whether the parasite is present or absent in a sheep flock. The severity of the clinical signs of oestrosis in lambs had no relationship with the intensity of infestation and ELISA showed to be a valuable tool for identifying animals that are carrying *O. ovis*.

Data Availability Statement

The original contributions presented in the study were included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

CRediT authorship contribution statement

Bello, Hornblenda Joaquina Silva: Conceptualization, Methodology, Formal analysis, Investigation, Writing - Original Draft. **Lins, José Gabriel Gonçalves**, **Albuquerque, Ana Cláudia Alexandre**, **Kozlowski Neto, Vitoldo Antonio**, and **Silva, Naiara Mirelly Marinho:** Investigation and Writing - Original Draft. **Amarante, Mônica Regina Vendrame:** Conceptualization, Methodology, Investigation, Writing - Original Draft, Funding acquisition. **Amarante, Alessandro F. T.:** Conceptualization, Methodology, Supervision, Writing - Original Draft, Funding acquisition.

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Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Table 1. Prevalence and mean intensity of infestation of *Oestrus ovis* in the infested and treated control groups 30 days after the last closantel treatment of the naturally infested lambs.

Group	Larval stage	Prevalence (%)	Mean intensity of infestation
Infested (n=26)	L1	88	13.96
	L2	73	8.53
	Total (L1+L2)	92	20.13
Treated Control (n=13)	L1	23	2.67
	L2	0	0
	Total (L1+L2)	23	2.67

Table 2. Frequency of nasal discharge score observed in the 39 lambs naturally infested by *Oestrus ovis*, infested and control treated group, totaling 17 evaluations from December 2020 to April 2021.

Group	Serous	Mucus	Mucus thick	Mucopurulent
Infested (n=26)	166 (38%)	227 (51%)	24 (5%)	25 (6%)
Control treated (n=13)	135 (61%)	69 (31%)	8 (4%)	9 (4%)
Total	301 (45%)	296 (45%)	32 (5%)	34 (5%)

There was a significant difference between group percentages ($P < 0.001$; Chi-square test).

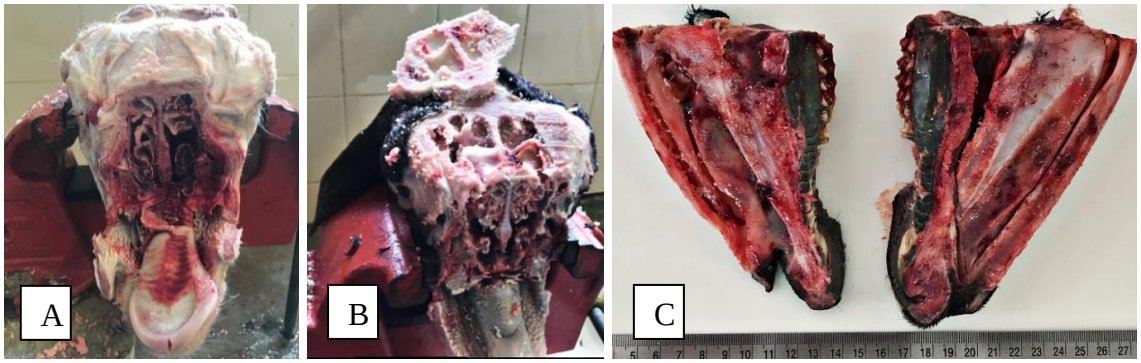

Figure 1. Frontal (A), transversal (B), and sagittal cut (C) were made on each lambs' head for oestrosis diagnosis through the recovery of *Oestrus ovis* larvae.

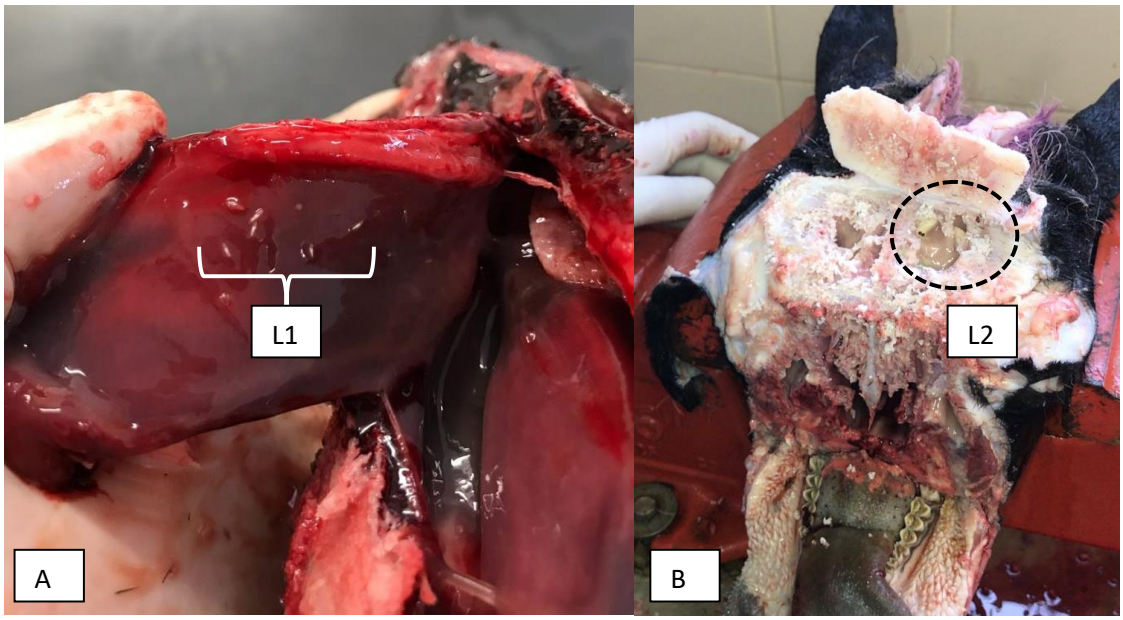

Figure 2. Opening the nasal cavities of lambs for *Oestrus ovis* larvae recovery. (A) Opening and exposure of nasal septum to recovery of larvae attached to the mucosa. (B) Transversal cut of the frontal sinus.

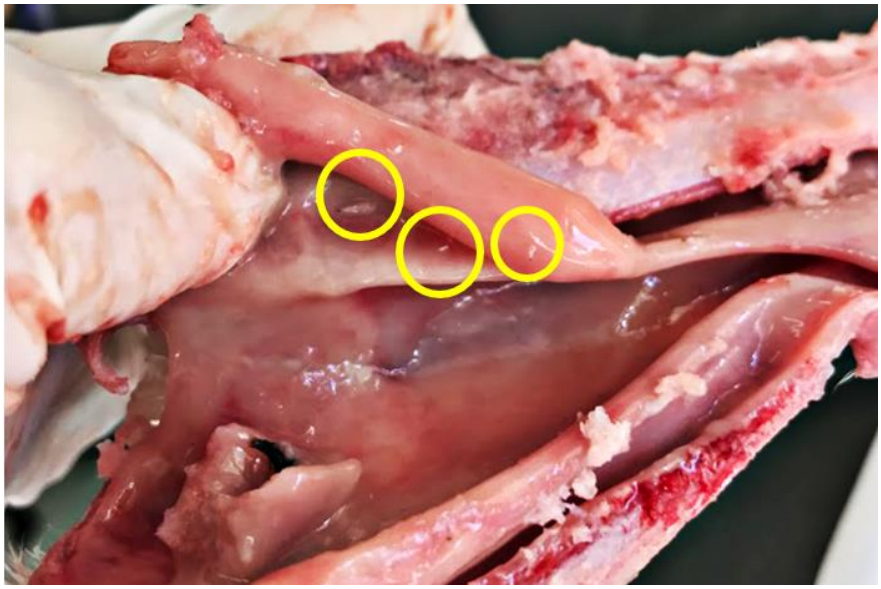

Figure 3. Opening of nasal conchae to recovery of *Oestrus ovis* larvae of naturally infested lambs.

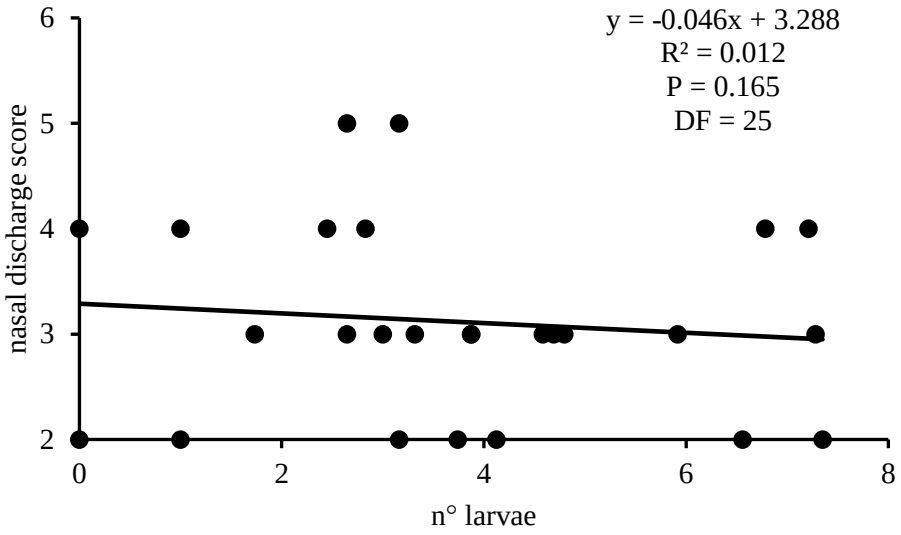

Figure 4. Correlation between the nasal discharge score evaluated before slaughter and the number of larvae recovered from the heads of lambs in the infested group after slaughter. Numbers of larvae were transformed into $\sqrt{(x+0.5)}$. Equation, R^2: Spearman's correlation coefficient, P: probability and GL= degrees of freedom. There is correlation between the variables when $P < 0.05$.

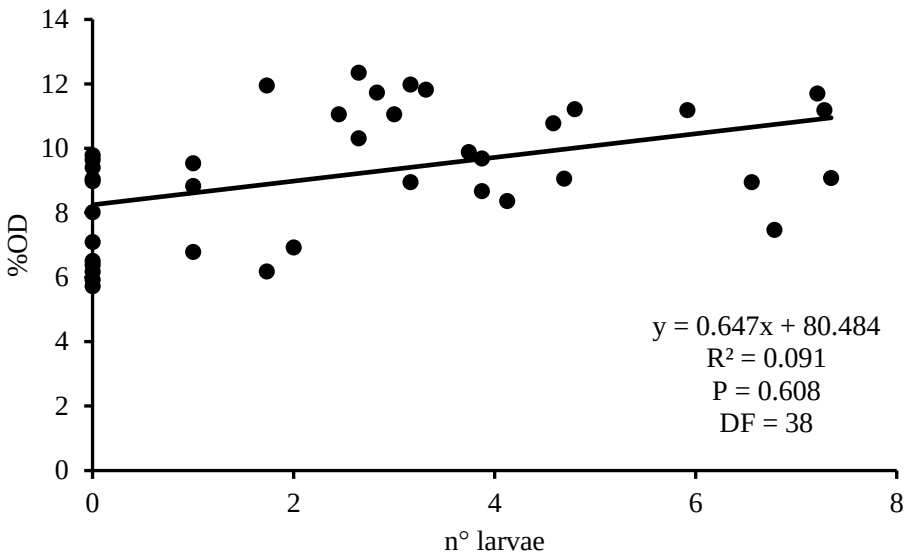

Figure 5. Correlation between the optical density (% OD) evaluated before slaughter and the number of larvae recovered from the heads of lambs in the infested group after slaughter. In this figure the data were transformed into $\sqrt{(x+0.5)}$. Equation, R^2: Spearman's correlation coefficient, P: probability and GL= degrees of freedom. There is correlation between the variables when $P < 0.05$.

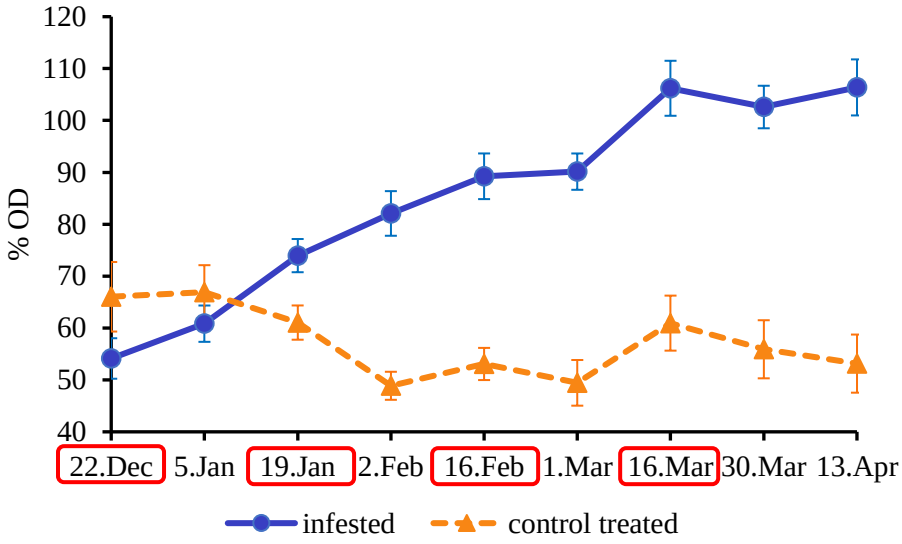

Figure 6. Means levels (±S.E.) of anti-*Oestrus ovis* L2 IgG (% OD) in plasma of the infested and control treated group, totaling 9 evaluations from December 2020 to April 2021. The highlighted dates correspond to the days of treatments of the animals from control treated group.

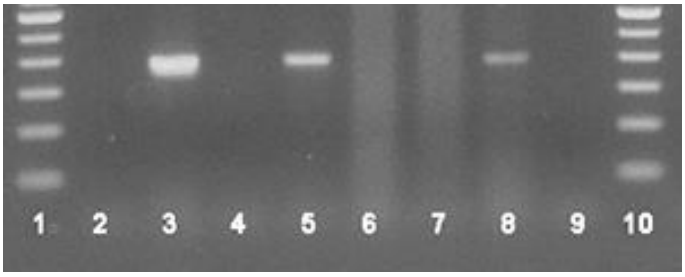

Figure 7. PCR reactions using DNA samples from nasal mucus of lambs with the designed forward primer OoBotu_F and the reverse primer UEA10 (Otranto et. al., 2003) in order to identify the presence of *Oestrus ovis*. Lines 1 and 10 show 100 bp molecular markers (Invitrogen, Vilnius, Lithuania); 2. ovine DNA (host and negative control); 3: *Oestrus ovis* L2 larvae DNA (positive control). 4-8 DNA extracted from mucus samples. 9: reagents without DNA.

CAPÍTULO IV

Discussão geral

A profilaxia e o tratamento da oestrose têm como base o uso de antiparasitários, sendo os mais utilizados para este fim o closantel e as lactonas macrocíclicas, principalmente a ivermectina (DORCHIES et al., 1997; LUCIENTES et al., 1998). Apesar de virem sendo utilizados há várias décadas, no presente estudo foi demonstrado que os dois antiparasitários mantém elevada eficácia contra *O. ovis*. A situação é bem diferente da observada com os nematódeos gastrintestinais, que ao serem expostos a mesma pressão de seleção, demonstraram grande capacidade de desenvolver resistência anti-helmíntica, problema que se tornou generalizado (ECHEVARRIA et al., 1996; ALMEIDA et al., 2010). No que diz respeito a ectoparasitas de bovinos a situação também tem gerado preocupação com registros de resistência envolvendo *Dermatobia hominis* (NEVES et al., 2015; CONDE et al., 2020), *Rhipicephalus microplus* (RECK et al., 2014) e *Cochliomyia hominivorax* (LOPES et al., 2013).

Os distúrbios causados por *O. ovis* começam no momento da larviposição, pois a mosca irrita os animais, que tentam se proteger dos ataques, escondendo o focinho próximo ao solo ou se aglomerando, balançando a cabeça e espirrando. Esses sinais foram comumente observados durante o experimento e são característicos da presença da mosca no rebanho (ZUMPT, 1965). Por sua vez, as larvas, com seus ganchos e espinhos, irritam a mucosa nasal, provocando inflamação acompanhada de produção de exsudato mucoso que pode até dificultar a respiração (DORCHIES et al., 1998). O parasitismo pelas larvas de *O. ovis* causou em alguns animais do presente estudo a produção exacerbada de secreção nasal, assim como espirros frequentes, sinais clínicos típicos da oestrose em pequenos ruminantes (ANGULO-VALADEZ et al., 2011). Vale ressaltar, que nem todos os cordeiros parasitados por larvas de *O. ovis*, demonstraram sinais clínicos da infestação, indicando que há variações individuais na intensidade da resposta inflamatória nos seios nasais e frontais, a qual independe do número de larvas presente.

No presente estudo não foram encontradas evidencias de que a infestação por larvas de *O. ovis* tenha causado prejuízo no ganho de peso dos cordeiros. Porém um fato a ser considerado é que os animais tratados com produtos oestricidas e aqueles do grupo sem tratamento foram mantidos juntos na mesma área e foram expostos, portanto, a mesma ação irritativa da mosca. Com isso, não foi possível determinar neste trabalho, se o estresse causado pela presença das moscas causou alguma interferência no desempenho

dos animais. De qualquer forma, a oestrose afeta o bem-estar dos ovinos, o que justificaria a aplicação de antiparasitários, independentemente da redução na produtividade do rebanho. Ao contrário do observado neste estudo, prejuízos econômicos devido a oestrose, como redução no ganho de peso (HORAK e SNYDERS, 1974) e queda na produção de leite de até 9% (DORCHIES et al., 2003) foram descritas em outros experimentos.

O diagnóstico da grande maioria das ectoparasitoses é realizado pela detecção visual de parasitas no corpo do animal como, por exemplo, de *Haemotobia irritans*, miíase por *Cochliomyia hominivorax*, infestação por carrapatos ou em amostras biológicas (ácaros da sarna detectáveis em crostas). Na maioria dos parasitas causadores de miíases internas, como oestrose em pequenos ruminantes e gasterofilose em equinos, o diagnóstico preciso depende do exame *post mortem* de órgãos internos (TRAVERSA e OTRANTO, 2006). No entanto, a necessidade da necropsia para diagnosticar e determinar a intensidade do parasitismo limita as possibilidades de investigação (ARIAS, 2014) e é economicamente inviável na rotina de uma criação de ovinos.

Neste estudo o teste sorológico por ELISA mostrou-se eficiente para indicar a ocorrência de oestrose no rebanho. Esses dados corroboram com os obtidos por outros autores (OTRANTO, 2001; ALCAIDE et al., 2005; ANGULO-VALADEZ et al., 2008; SILVA et al., 2012; 2018; ARIAS et al., 2014). Em associação com a sintomatologia, o teste de ELISA pode ser também empregado a fim de avaliar a eficácia de tratamentos com antiparasitários, pois na ausência de infestação larval, há queda na produção de anticorpos e redução acentuada dos sinais clínicos respiratórios (diminuição de espirros e secreção nasal).

Em contrapartida a PCR apresentou baixa sensibilidade para o diagnóstico individual, porém alta especificidade, o que habilita este método para ser utilizada com o objetivo de confirmar se o parasita está presente ou ausente em um rebanho ovino. O método PCR poderia ser útil para avaliar a eficácia dos medicamentos contra *O. ovis* através da monitorização da presença ou ausência de DNA específico de parasitas no muco nasal após tratamento de ovinos com oestrose, evitando assim sacrificá-los para confirmar a presença de larvas. Contudo este método deve ser aprimorado e avaliado em novos ensaios. Uma alternativa para tentar otimizar a técnica seria realizar a extração do DNA do parasita de um *pool* de amostras de muco, o que também diminuiria os gastos com reagentes e o trabalho envolvido com as análises laboratoriais.

Em relação às estratégias de tratamento e profilaxia da oestrose, em áreas com baixa infestação e pouco incômodo causado nos animais pela presença da mosca, talvez seja mais indicado tratar apenas os ovinos com sinais clínicos. Porém, em áreas com infestação intensa, o tratamento de todos os animais do rebanho pode ser a medida mais conveniente, pois teria o intuito de reduzir a população do parasita, tanto da fase de vida parasitária como das fases de vida livre. Consequentemente haveria redução na incidência de miíases e da ação irritante provocada pelas moscas durante a larviposição. Logo, é de grande importância o diagnóstico correto da oestrose para que seja planejado o uso adequado dos antiparasitários.

Conclusão geral

Closantel e ivermectina apresentaram elevada eficácia e período longo de proteção contra o estabelecimento de miíase por *O. ovis.* Na área em que os experimentos foram realizados não houve evidência da presença de *O. ovis* com resistência aos antiparasitários avaliados.

A miíase causada por *O. ovis* não causa prejuízo no desempenho de ovinos em crescimento, porém a presença da mosca causa desconforto e afeta o bem-estar dos animais.

A quantificação de larvas na necropsia se constitui no "padrão-ouro" para o diagnóstico da oestrose. Porém, a sorologia se mostrou muito útil, possibilitando discriminar grupos de cordeiros parasitados de grupos mantidos livres de infestação.

A PCR apresenta alta especificidade, porém sua limitação é a baixa sensibilidade por apresentar alto índice de resultados falso-negativos, não sendo indicada para diagnóstico individual de oestrose. Porém, pode ser uma ferramenta útil para confirmar a presença de *O. ovis* no rebanho.

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