Instituto de Biociências

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

PG-BGA

# Interações entre as infecções por *Haemonchus contortus* e *Haemonchus placei* em ovinos

**Michelle Cardoso dos Santos**

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

*Prof. Titular Alessandro Francisco Talamini do Amarante*
*Orientador*

**BOTUCATU - SP**

**2017**

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# Dedico

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# Sumário

**Resumo**

Dois experimentos foram realizados no período de 2013 a 2015, em cordeiros da raça Suffolk, com objetivo de avaliar a dinâmica da infecção mista por *Haemonchus contortus* e *Haemonchus placei* em ovinos, bem como a competição e o cruzamento interespecífico com a formação de híbridos. Um cordeiro doador, livre de infecções helmínticas, recebeu infecção artificial oral única com 25.000 larvas infectantes (L3) de *H. contortus*, isolado de laboratório SpHco2, com múltipla resistência aos anti-helmínticos, enquanto um bezerro doador foi infectado via oral, em dose única, com 25.000 L3 de *H. placei*, isolado de laboratório SpHpl1, susceptível à ação do levamisol. No primeiro experimento, realizado no ano de 2013, cordeiros mantidos confinados e livres de infecções helmínticas foram inicialmente infectados com 2.000 L3 de *H. placei* no dia zero e 11 dias depois, os mesmos animais receberam 2.000 L3 de *H. contortus* (grupo parental, n=6). Coproculturas individuais dos animais do grupo parental foram utilizadas para a produção de L3, as quais foram destinadas à infecção dos animais dos grupos F1 (primeira geração filial), grupos F1-42 DPI (n=6) e F1-84 DPI (n=6), o que permitiu avaliar a produção de híbridos. Todos os animais pertencentes aos grupos: parental, F1-42 DPI e F1-84 DPI, foram eutanasiados aos 50, 42 e 84 dias pós-infecção (DPI), respectivamente, para a identificação e quantificação dos parasitas, a fim de determinar a competição interespecífica e a hibridização. Seis animais, não infectados, foram mantidos como grupo controle. No segundo experimento, desenvolvido no período de 2014 e 2015, cordeiros livres de infecções helmínticas foram infectados com 4.000 L3 de *H. placei* (n=16) ou com 4.000 L3 de *H. contortus* (n=16). Os animais foram inicialmente mantidos estabulados, para verificar o estabelecimento das infecções e depois foram alocados nas mesmas pastagens, as quais foram compartilhadas durante 357 dias. Para avaliar a evolução da infecção, dois cordeiros, de cada grupo experimental, foram eutanasiados em Fevereiro de 2014 e as eutanásias posteriores ocorreram a cada três meses, contendo três cordeiros por grupo experimental, com exceção da última eutanásia, em Fevereiro de 2015, na qual os cinco animais remanescentes de cada grupo foram eutanasiados. Animais traçadores, livres de infecções parasitárias, foram colocados na pastagem a cada três meses para avaliação da contaminação da pastagem e das espécies de nematódeos presentes. Em ambos os estudos, os animais eram pesados e nesse mesmo dia amostras de sangue e fezes eram coletadas para as análises laboratoriais. Nos dois estudos os animais foram eutanasiados para a recuperação e identificação dos parasitas. No primeiro experimento, em confinamento, houve estabelecimento da infecção por ambas as espécies, com elevadas contagens de ovos por grama de fezes (OPG). Os parasitas recuperados foram identificados morfologicamente e

molecularmente, comprovando o sucesso da infecção. As reações em cadeia da polimerase (PCR) com os oligonucleotídeos iniciadores (*primers*) espécies-específicos demonstraram a presença de *H. contortus* e *H. placei* em todos os grupos experimentais (grupo parental, F1-42 DPI e F1-84 DPI) e de oito híbridos (3,6% do total de parasitas avaliados), os quais foram recuperados dos grupos F1-42 DPI e F1-84 DPI. No segundo experimento também houve estabelecimento da infecção por ambas as espécies, com elevadas contagens de OPG e redução do volume globular nas coletas iniciais. As medidas das L3, bem como as análises morfológicas e moleculares de exemplares adultos, dos animais experimentais e traçadores demonstraram a eliminação da infecção por *H. placei* ao longo do tempo. Dois helmintos foram classificados como híbridos com a utilização dos mesmos *primers* espécies-específicos. No último estudo realizado, avaliou-se a mutação no gene da resistência ao levamisol por PCR convencional. Os primers utilizados propiciaram amplificação apenas das amostras oriundas de *H. contortus*. De 20 exemplares adultos de *H. contortus* provenientes do cordeiro doador, 85% (n=17) mostraram-se homozigotos resistentes e 15% (n=3), heterozigotos. Dos animais experimentais descritos no experimento 1, foram avaliados 126 exemplares de *H. contortus*, dos quais 97,6% (n=123) foram classificados como homozigotos resistentes e 2,4% (n=3) como heterozigotos. Em conclusão, demonstramos que ocorreu o estabelecimento de infecções mistas por *H. contortus* e *H. placei* em ovinos e, o acasalamento interespecífico pode resultar em descendentes híbridos. Contudo, quando ovinos infectados com *H. contortus* ou *H. placei* compartilharam as mesmas pastagens a competição interespecífica promoveu a extinção de *H. placei* (parasita oriundo de bovinos), com a permanência no rebanho apenas da população de *H. contortus*, espécie que possui os ovinos como hospedeiro preferencial. Em adição, os pares de primers de resistência ao levamisol não apresentaram bandas de amplificação com as amostras de DNA de exemplares de *H. placei*, contudo, o isolado de laboratório SpHco2 (*H. contortus*) apresentou resistência ao levamisol.

**Abstract**

Two trials were performed from 2013 until 2015 in Suffolk sheep breed, these studies aimed to evaluate the dynamics of mixed infection by *Haemonchus contortus* and *Haemonchus placei* in sheep, as well as interspecific competition and mating with the hybrid production. Initially, one worm-free donor lamb received orally, in a single dose, artificial infection with 25,000 infective larvae (L3) of *H. contortus*, laboratory isolate SpHco2, while the donor calf was infected orally, in a single dose, with 25,000 of *H. placei*, laboratory isolate SpHpl1. In the first trial, performed in 2013, worm-free lambs kept indoors received initially 2,000 L3 of *H. placei* on day zero and, 11 days later the same animals were infected with 2,000 L3 of *H. contortus* (parental group, n=6). Individual fecal cultures from parental animals were performed and were used to L3 production, which was used to the infection of the animals from F1 group (first filial generation), groups F1-42 DPI (n=6) and F1-84 DPI (n=6), which allowed the hybrid production. All animals from groups: parental, F1-42 DPI and F1-84 DPI were euthanized 50, 42 and 84 days post-infection (DPI), respectively, for the identification and quantification of the parasites in order to determine the interspecific competition and hybridization. Six animals remained as a non-infected control. On the second trial, developed in 2014 and 2015, worm-free lambs were infected with 4,000 L3 of *H. placei* (n=16) or 4,000 L3 of *H. contortus* (n=16). These animals were initially kept indoors to confirm the establishment of the infections and then the animals were allocated to the same pastures, which were shared for 357 days. To evaluate the progression of the infection, two animals for each experimental group were euthanized in February 2014 and the subsequent euthanasia occurred every three months, with three animals from each experimental group, except on the last euthanasia, in February 2015 in which the remaining five animals from each group were euthanized. Worm-free tracer animals were introduced in the pasture every three months to evaluate the pasture contamination and nematode species present. In both studies, the animals were weighed and blood and feces samples were collected on the same day to laboratory analysis. In both trials, the animals were euthanized for the parasites recovery and identification. In the first trial, in the feedlot, there was observed the establishment of the infection for both species, with high fecal egg counts (FEC). The parasites recovered were identified by morphological and molecular techniques, proving the success of the infection. Polymerase chain reaction (PCR) reactions with the oligonucleotide (primers) species-specific showed the presence of *H. contortus* and *H. placei* in all experimental groups (groups parental, F1-42 DPI and F1-84 DPI) and eight hybrids (3.6% of the total of parasites

evaluated), which were recovered from F1-42 DPI and F1-84 DPI groups. In the second trial, there was also the establishment of the infection for both species, with high FEC and low packed cell volume levels in the initial collections. Measurements of the L3 as well as morphological and molecular evaluation of the adult specimens, of the experimental and tracer sheep demonstrate the elimination of the *H. placei* over time. Two helminths were identified as hybrids using the same species-specific primers. On the last experiment, the mutation for levamisole resistance gene was evaluated by conventional PCR. These primers provided amplification only in *H. contortus* specimens. Of 20 adult specimens of *H. contortus* from the donor lamb 85% (n=17) showed homozygous resistant and 15% (n=3), heterozygous. From experimental animals described in trial 1 were evaluated 142 *H. contortus* specimens and 97.6% (n=123) were homozygous resistant and 2.4% (n=3) as heterozygous. In conclusion, the establishment of mixed infections by *H. contortus* and *H. placei* in sheep occurs, and the interspecific mating can produce F1 with hybrids offspring. Nonetheless, when sheep infected with *H. contortus* and *H. placei* shared the same pastures, interspecific competition promotes the extinction of *H. placei* (parasite from cattle) with the presence of only *H. contortus*, specie that has the sheep as preferred host. In addition, the primers pairs to levamisole resistance did not present amplification bands with DNA samples of *H. placei* - however, the SpHco2 laboratory isolate (*H. contortus*) showed molecular resistance to levamisole.

# Revisão de Literatura

## 1. Considerações iniciais

As infecções por nematódeos gastrintestinais são o mais grave problema na produção de ovinos, promovendo perda na produtividade do rebanho, acometendo principalmente as categorias jovens e as fêmeas no periparto (Rocha et al., 2004; 2005).

O controle desses helmintos baseia-se principalmente no tratamento com anti-helmínticos, porém o uso indiscriminado dessas drogas, ao longo dos anos, tornou essa estratégia ineficiente, tendo como consequência a seleção de populações de parasitas com resistência às drogas comerciais (revisado por Gilleard, 2006; Almeida et al., 2010; Salgado e Santos, 2016).

Visando favorecer sua sobrevivência, os parasitas resistentes aos anti-helmínticos apresentam mutações gênicas que podem, eventualmente, se tornar fixas nas gerações seguintes (Aguayo-Ortiz et al., 2013). Pode-se utilizar como exemplo o polimorfismo de nucleotídeo único (SNP) no códon F200Y, os genes que codificam a $\beta$-tubulina sofrem mutação em sua sequência e as proteínas codificadas pelos genes perdem a afinidade aos benzimidazóis (Silvestre e Humbert, 2002; Brasil et al., 2012; Chaudhry et al., 2015a; Redman et al., 2015).

Em virtude da ineficácia dos tratamentos, diferentes metodologias no manejo dos animais de interesse zootécnico podem ser empregadas, como por exemplo, o pastejo misto entre ovinos e bovinos (Amarante et al., 1997; Fernandes et al., 2004; Rocha et al., 2008). Essa metodologia pode ser utilizada para a descontaminação da pastagem, embora possa favorecer a ocorrência de infecções cruzadas (Fernandes et al., 2004; Rocha et al., 2008).

*Haemonchus contortus* têm os ovinos como hospedeiro preferencial, enquanto *Haemonchus placei* apresenta predileção por bovinos (Hoberg et al., 2004), no entanto, relatos sobre infecções mistas (*H. contortus* e *H. placei*) em ovinos e bovinos já foram reportados em diversos locais do mundo (Amarante et al., 1997; Jacquiet et al., 1998; Achi et al., 2003; Hoberg et al., 2004; Rocha et al., 2008; Gasbarre et al., 2009; Khalafalla et al., 2011). As infecções mistas interespecíficas podem promover a produção de híbridos, contudo, esses parasitas podem apresentar baixa eficiência reprodutiva (Le Jambre, 1979; 1981).

Desta maneira, no presente estudo, utilizaram-se as técnicas moleculares de reação em cadeia da polimerase (PCR) convencional com oligonucleotídeos iniciadores (*primers*) para: a identificação das espécies de *H. contortus* e *H. placei*, a detecção da resistência à levamisole nessas espécies, bem como o sequenciamento de marcadores microssatélites para a caracterização molecular de *Haemonchus* spp.

**2. Gênero *Haemonchus***

Infecções por helmintos do gênero *Haemonchus* causam grandes prejuízos econômicos, sendo os principais parasitas em ruminantes de interesse zootécnico (bovinos, ovinos, caprinos e búfalos) (Almeida, 1935; Achi et al., 2003; Hoberg et al., 2004). *Haemonchus placei* possui bovinos como hospedeiro preferencial enquanto *H. contortus* têm predileção por pequenos ruminantes. *Haemonchus contortus* promove prejuízos devido a sua elevada patogenicidade que pode resultar em taxas significativas de mortalidade em pequenos ruminantes (Besier et al., 2016).

*Haemonchus* spp. possuem ciclo direto, com uma fase de vida livre e outra parasitária. Durante o ciclo de vida livre, os ovos eliminados no ambiente junto às fezes do hospedeiro, sofrem eclosão e a larva se desenvolve até o estágio infectante (L3). O tempo entre a eclosão até as larvas de *H. contortus* atingirem o estágio infectante pode variar entre quatro e 16 dias, dependendo da temperatura e umidade relativa (revisado por O'Connor et al., 2006). As L3 são ativas e migram das fezes para a planta forrageira, desta maneira, as L3 são ingeridas junto com pastagem, iniciando a fase de vida parasitária (Silva et al., 2008; Santos et al., 2012). No trato gastrintestinal do hospedeiro, as L3 se alojam e sofrem mudas até atingirem o estádio adulto. Machos e fêmeas copulam e as fêmeas passam a produzir entre 5.000 a 15.000 ovos por dia (Le Jambre, 1995; Almeida, 1935; revisado por O'Connor et al., 2006; Emery et al., 2016). O ápice de fecundidade das fêmeas ocorre em média aos 52 dias após a infecção (Santos et al., 2014a).

Os parasitas adultos são grandes, as fêmeas podem medir de 15 a 33 mm de comprimento e os machos de 9 a 21 mm de comprimento, são delgados, de cor pardo-avermelhada, possuem cápsula bucal provida de lanceta e cutícula com estrias longitudinais e transversais. Os machos apresentam bolsa copuladora trilobada, gubernáculo e espículos providos de ganchos. As fêmeas possuem vulva, útero e ovários duplos. São ovíparas e liberam ovos elipsoides de casca delgada e segmentados (Almeida, 1935).

Durante a fase parasitária, os prejuízos na produção de ovinos são decorrentes do consumo de sangue que se inicia no quarto estádio (L4). Os parasitas adultos se alimentam de aproximadamente 30 µL de sangue por dia, assim, podem-se estimar perdas mensais de quase 1 litro de sangue em um animal albergando 1.000 vermes (Gennari et al., 1991; Nishi et al., 2002; Rocha et al., 2004; 2005; Emery et al., 2016).

Nos hospedeiros, a perda de sangue pode promover anemia, com redução no porcentual do volume globular do animal (Blackburn et al., 1991), com efeito deletério no potencial produtivo e, em casos extremos, ocasionando o óbito do hospedeiro (Dargie e

Allonby, 1975).

### 2.1. Produção de híbridos

Estratégias de manejo, como por exemplo, o pastejo misto e/ou alternado entre bovinos e ovinos têm como objetivo minimizar as perdas na ovinocultura devido às infecções por nematódeos gastrintestinais contribuindo com a descontaminação da pastagem e reduzindo o número de tratamentos com anti-helmínticos (Fernandes et al., 2004; Rocha et al., 2008).

Embora as espécies *H. contortus* e *H. placei* possuam hospedeiros preferenciais, elas podem infectar hospedeiros satélites, como por exemplo, ovinos podem albergar *H. placei* e bovinos *H. contortus* (Hoberg et al., 2004).

A coexistência de mais de uma espécie de *Haemonchus* no abomaso de ruminantes domésticos pode levar a três resultados: (1) as espécies são tão intimamente relacionadas que a produção de híbridos ocorre; (2) as espécies estão intimamente relacionadas, mas seus nichos se sobrepõem apenas ligeiramente (por exemplo: separação de hábitat); ou (3) a competição interespecífica ocorre levando à eliminação de uma espécie de acordo com o princípio de exclusão competitiva de Gause (Achi et al., 2003).

O cruzamento interespecífico é possível devido à poliandria em *Haemonchus* spp. Redman et al. (2008) utilizando marcadores de microssatélites ligados ao cromossomo X demonstraram que uma fêmea pode copular com múltiplos machos. A ocorrência da poliandria contribui para a variabilidade genética e está associada a uma maior oportunidade de recombinação dos genes transmitidos da geração parental para as futuras gerações (revisado por Gilleard e Redman, 2016).

A formação de indivíduos híbridos descendentes do cruzamento entre *H. contortus* e *H. placei* foi relatada em vários estudos, contudo, algumas barreiras podem impedir a introgressão de genes nos parasitas de gênero *Haemonchus,* produtos do cruzamento entre *H. contortus* e *H. placei*, como por exemplo, a esterilidade dos híbridos. A esterilidade pode levar a perdas na espécie com a menor proporção da população, uma vez que uma proporção correspondentemente maior de sua prole não conseguiria se reproduzir na geração subsequente (Le Jambre, 1979). Analisando-se os oito tipos de retrocruzamentos entre híbridos (F1 produtos do cruzamento entre *H. contortus* e *H. placei*) e as espécies parentais foi observado que nem todos os descentes eram inférteis e seis dos oito retrocruzamentos produziram ovos férteis e foram utilizadas na infecção de ovinos por quatro gerações (Le Jambre e Royal, 1980).

Recentemente, foram avaliados parasitas obtidos de bovinos, ovinos, caprinos e búfalos no Brasil e observou-se que apenas 1,28% (2 dos 156 exemplares avaliados) eram portadores de alelos na região do ITS-2 (espaçador interno transcrito tipo 2) de *H. placei* e de *H. contortus*, ou seja, apenas dois exemplares eram híbridos (Brasil et al., 2012).

A produção de híbridos consiste em uma abordagem alternativa para identificar genes associados à resistência aos anti-helmínticos. Ao cruzar uma cepa de *H. contortus* resistente com uma cepa de *H. placei* susceptível e realizar retrocruzamentos (a partir do cruzamento da F1 produzida com as cepas parentais), os híbridos produzidos podem ser rastreados para a identificação de genes de interesse. No entanto, os resultados devem ser interpretados com cautela, pois o gene que mostra uma frequência significativamente diferente da esperada não é necessariamente aquele que está envolvido na resistência. Ele pode estar próximo ao gene envolvido na resistência e, por conta disso, segrega-se juntamente com ele (Prichard, 2001).

No Sul da Índia e no Paquistão, parasitas adultos foram avaliados de 27 e 22 populações de ruminantes, respectivamente. Das populações analisadas, foram recuperados cinco exemplares heterozigotos de quatro caprinos e de um ovino (um exemplar por hospedeiro). Os heterozigotos apresentavam genótipo A/G na posição 24 (P24), G/A na P205 e A/G na P219 da região ITS-2 do DNA ribossomal (rDNA), indicando que eles eram provenientes do cruzamento entre *H. contortus* e *H. placei* (Chaudhry et al., 2015a). No mesmo estudo, ao analisarem a subunidade 4 da NADH desidrogenase mitocondrial (ND4). Nos cinco híbridos, observaram que quatro híbridos apresentaram na geração parental maternal de *H. placei* e um híbrido apresentou na geração parental maternal de *H. contortus*, demonstrando assim que a produção de híbridos poderia ocorrer em qualquer direção. Adicionalmente, os autores observaram que um exemplar híbrido apresentou o alelo de resistência à benzimidazóis (BZ) de *H. contortus* (isotipo-1 $\beta$-tubulina), sugerindo o potencial para a introgressão interespecífica do *loci* de resistência às drogas (Chaudhry et al., 2015a).

Em um estudo anterior, com a utilização de retrocruzamentos, Redman et al. (2012) relataram a introgressão de genes relacionados a resistência à ivermectina de duas cepas resistentes (MHco4 e MHco10) para uma cepa susceptível (MHco3).

## 2.2. Caracterização de *Haemonchus contortus* e *Haemonchus placei*

### 2.2.1. Morfologia

Registros antigos na literatura consideravam *H. placei* como sinonímia de *H. contortus* (Gibbons, 1979), contudo, estudos posteriores comprovaram que são espécies distintas (Lichtenfels et al., 1994; Amarante et al., 1997; Hoberg et al., 2004).

A diferenciação morfológica entre as espécies pode ser realizada nas L3 a partir da mensuração da distância entre a extremidade posterior da larva e o final da cauda da bainha (Santiago, 1968), que apresentam valores inferiores a 85 µm para *H. contortus* e superiores a 89 µm para *H. placei* (van Wyk e Mayhew, 2013; Santos et al., 2014b).

A distinção morfológica dos exemplares adultos pode ser realizada com a mensuração dos espículos e de seus ganchos em machos, que habitualmente apresentam maiores dimensões em *H. placei* (Jacquiet et al., 1997; 1998; Achi et al., 2003; Amarante, 2011; Santos et al., 2014b). A avaliação morfológica também pode ser realizada pela avaliação da sínlofe, que consiste na contagem das cristas cuticulares formadas pelas estrias longitudinais que recobrem o corpo do parasita, sendo que a espécie *H. contortus* possui 30 cristas, na metade posterior do esôfago, enquanto a espécie *H. placei* possui 34 cristas (Lichtenfels et al., 1994).

Na África ocidental, Jacquiet et al. (1998) observaram a partir de avaliações morfológicas que os pequenos ruminantes eram os hospedeiros preferenciais de *H. contortus*, e também albergavam 56% dos exemplares de *H. placei*. Posteriormente, na Costa do Marfim, Achi et al. (2003) com a utilização da função discriminante (combinação entre três medidas: o espículo e os ganchos direito e esquerdo dos machos adultos) observaram 96,1% de *H. contortus*, 3,5% *H. placei* e 0,4% de *H. similis* em 493 parasitas provenientes de ovinos. Na Etiópia foi avaliada a morfologia dos espículos de 1.159 machos adultos, provenientes de ovinos, e foi observado que 95,1% eram *H. contortus*, 3,4% *H. placei* e 1,5% *H. longistipes* (Kumsa et al., 2008).

No entanto, os exemplares de *H. contortus* podem apresentar medidas superiores ao padrão da espécie, sobrepondo com as medidas de *H. placei*, assim, estudos atuais relatam que esses exemplares podem ser erroneamente classificados como *H. placei*. Santos et al. (2014b) relataram que a identificação morfológica das L3 concordou com os resultados moleculares utilizando *primers* espécie-específicos (Zarlenga et al., 1994), sendo que apenas 0,71% das L3 apresentaram sobreposições de medidas, enquanto 6,09% dos machos adultos avaliados foram erroneamente identificados com base na morfologia. As sobreposições das medidas entre as espécies podem ocorrer, dificultando a sua identificação (Amarante, 2011; Santos et al, 2014b).

### 2.2.2. Diagnóstico molecular

Em virtude das dificuldades observadas nas avaliações morfológicas, devido à sobreposição de medidas (Amarante, 2011) as avaliações moleculares mais precisas, rápidas,

reprodutíveis e sensíveis podem ser empregadas. Assim sendo, acredita-se que os progressos em tecnologias moleculares continuarão a ocorrer neste campo de pesquisa, contribuindo para a identificação de espécies de nematódeos gastrintestinais (McKeand, 1999; von Samson-Himmelstjerna, 2006; Brasil et al., 2012; Zarlenga et al., 2016).

A implementação da técnica de PCR tem revolucionado a pesquisa parasitológica, pois sua sensibilidade permite a amplificação de genes, ou segmentos de genes, a partir de quantidades diminutas de material do parasita. Desse modo, a avaliação molecular contribuiu para avanços em várias áreas da parasitologia, relacionados à identificação, sistemática e epidemiologia, no diagnóstico de infecções, na análise da estrutura genética de populações, na expressão gênica e na resistência às drogas anti-helmínticas (Gasser, 1999).

### 2.2.3. Reação em cadeia da polimerase (PCR)

Zarlenga et al. (1994) desenvolveram o par de *primers* 75 e 86 (espécies-específicos) para a diferenciação de *H. contortus* e *H. placei*, a partir da clonagem e caracterização do rDNA dos helmintos. Em *H. placei* ocorreu amplificações de um único segmento com 1.630 pb e em *H. contortus*, de dois segmentos, um de 1.320 pb e outro de 1.630 pb de comprimento. Contudo, Santos et al. (2014b) observaram que nem todos os espécimes de *H. placei* e *H. contortus* apresentaram seu DNA amplificado com tais *primers*, embora as mesmas amostras, posteriormente tenham sido amplificadas pelos *primers* descritos por Dorris et al. (2002), comprovando a presença de DNA (Santos et al., 2014b).

A ausência de amplificação de DNA, a partir da utilização de *primers* descritos por Zarlenga et al. (1994), pode estar associada à variabilidade genética das espécies na região em que ocorre o anelamento. Nos Estados Unidos foi observada grande diversidade genética e intenso fluxo gênico dentro das populações de *H. placei* e também nas populações de *H. contortus* (Blouin et al.,1995). No entanto, no Brasil ao utilizar o sequenciamento da região ITS-2 foi observada baixa variabilidade genética em populações de *H. placei*, em isolados de campo em comparação com *H. contortus* (Brasil et al., 2012). Assim, a utilização de marcadores moleculares tem contribuído na avaliação do polimorfismo em diferentes populações.

A técnica de PCR também pode ser empregada em ovos e L3. Zarlenga et al. (2001) desenvolveram PCR multiplex, a partir do DNA genômico de parasitas adultos. Neste trabalho houve a discriminação de ovos das fezes de animais e diferenciação de cinco espécies de helmintos de bovinos (*Ostertagia ostertagi*, *H. placei*, *Oesophagostomum radiatum*, *Trichostrongylus colubriformis* e *Cooperia oncophora*).

Em conjunto com a PCR, técnicas de sequenciamento da região ITS-2 do rDNA para a identificação das espécies de *H. contortus* e *H. placei* foram descritas por Steverson et al. (1995) e mais recentemente por Brasil et al. (2012).

### 2.2.4. Marcadores moleculares

Marcadores moleculares constituem-se em *loci* (regiões do genoma onde se localizam sequências de DNA) que são polimórficas e que, portanto, apresentam diferenças entre indivíduos de uma população. Dentre as várias classes de marcadores moleculares podemos incluir os microssatélites, que podem ser denominados como repetições curtas (STRs = "short tandem repeats"), sequências simples repetidas (SSR = "simple sequence repeats" ou SRS = "simple repetitive sequence") (Kantartzi, 2013). Ou seja, os microssatélites consistem em repetições muito curtas (1 a 6 pares de bases) dispersas aleatoriamente ao longo do genoma. Esses marcadores costumam ser mais polimórficos que sequências transcritas, pois foram capazes de acumular mutações ao longo do tempo, por não sofrerem pressão de seleção (Hoekstra et al., 1997; Gasser, 1999; Kantartzi, 2013).

Portanto, microssatélites são adequados para utilização como marcadores genéticos em *H. contortus*, desde que apresentem polimorfismo em uma ou mais populações (Hoekstra et al., 1997). A utilização de 13 marcadores de microssatélites para *H. contortus*, de diferentes origens (Grã-Bretanha (SE e SW), Malásia (SM) e Zimbábue (SHS) que estão associados à expressão de genes relacionados à susceptibilidade à ação de drogas demonstrou haver diversidade dentro e entre populações. Além disso, a população africana SHS divergiu das populações SE, SW e SM (européias e asiáticas) o que pode ter sido resultante do isolamento geográfico e da criação intensiva (Hoekstra et al., 1997).

## 3. Drogas e resistência aos anti-helmínticos

Tratamentos com anti-helmínticos foram utilizados indiscriminadamente ao longo dos anos visando reduzir as perdas do rebanho, contudo, essa medida favoreceu o surgimento de populações resistentes às drogas comerciais (Almeida et al., 2010).

*Haemonchus contortus* tem demonstrado uma grande capacidade de desenvolver resistência às drogas e, em muitos casos, a resistência tem aparecido em menos de 10 anos após a introdução de uma nova classe de drogas (Kotze e Prichard, 2016). Assim, a resistência aos antiparasitários tornou-se um grave problema econômico na produção animal, ocorrendo de maneira generalizada em parasitas de ovinos, caprinos, equinos, bovinos e suínos (Prichard, 1994).

Denomina-se resistência quando há o declínio da eficiência de uma droga anti-helmíntica contra uma população de parasitas, que originalmente era sensível a sua ação (Sangster e Gill, 1999). O surgimento da resistência ocorre em três etapas: (1) estabelecimento, influenciado pelo tamanho e diversidade da população e taxa de mutação do gene envolvido, (2) desenvolvimento (depende da frequência de utilização da droga que seleciona os genes envolvidos) e (3) dispersão, que ocorre por migração e fluxo gênico (Sutherst e Comins, 1979; Humbert et al., 2001).

A resistência aos BZs, levamisol/morantel e ivermectinas tem sido extensivamente estudada em nematódeos de ovinos (Prichard, 19994; Sangster e Gill, 1999; Beech et al., 2011). Deve-se salientar relatos de resistência envolvendo a mais nova classe de droga, a aminoacetilnitrila, Zolvix®, recentemente disponível no mercado (van der Brom et al., 2015; Cintra et al., 2016, Kotze e Prichard, 2016).

Para a detecção da resistência às drogas *in vivo*, a técnica comumente empregada é o teste de redução de ovos por grama de fezes (FERCT, faecal egg count reduction test). Os testes mais empregados *in vitro* são o teste de eclodibilidade dos ovos (TEO) e o teste de desenvolvimento larval (TDL), contudo, essas avaliações são laboriosas, demoradas e muitas vezes apresentam baixa sensibilidade e reprodutibilidade (Roeber et al., 2013). Assim, técnicas moleculares têm sido propostas como novas alternativas (Coles et al., 2006; revisado por Roeber et al., 2013). Uma variedade de técnicas moleculares para a detecção de substituições específicas, que causam ou estão ligados à resistência anti-helmíntica, têm sido desenvolvidas. Pode-se citar algumas técnicas descritas por Beech et al. (2011):

(1) digestão com enzimas de restrição: as enzimas de restrição reconhecem sequências nucleotídicas específicas (geralmente de 4 ou 6 bases), clivam o DNA e geram fragmentos.

(2) Sequenciamento direto: o sequenciamento direto do DNA amplificado por PCR de regiões de interesse que pode revelar polimorfismo.

(3) Pirosequenciamento: com a utilização de *primers* desenhados para detectar polimorfismos específicos, podem ser utilizados para analisar um número relativamente grande de parasitas individualmente.

(4) Diagnóstico por PCR: Os *primers* podem ser desenhados para sequenciamento em larga escala (a partir de DNA).

Redman et al. (2008) utilizaram PCR e sequenciamento com um conjunto de oito microssatélites para estabelecer a frequência alélica entre isolados e espécimes de *H. contortus* (*genetic fingerprinting*). Porém, esses autores ressaltaram que, no caso de identificação individual, esta metodologia mostrou-se trabalhosa e apresentou custo elevado,

tornando-se impraticável para uso na rotina laboratorial.

Apesar da sua importância cada vez maior, a base molecular e genética de resistência ainda é pouco compreendida. Não está claro como as mutações contribuem para o fenótipo de resistência e como os alelos de resistência originam-se, são selecionados e espalham-se nas populações de parasitas (Gilleard, 2006).

### 3.1. Benzimidazóis

Os BZs são uma classe de compostos anti-helmínticos sintéticos, de amplo espectro, abundantemente utilizados no tratamento de infecções gastrintestinais de humanos e animais (von Samson-Himmelstjerna et al., 2009; Taman e Azab, 2014).

O modo de ação dos BZs consiste na ligação da droga às tubulinas, que compõem os microtúbulos, causando a sua despolimerização. Os microtúbulos são compostos de duas proteínas de 450 aminoácidos, conhecidas como *α*-tubulina e *β*-tubulina. São organelas intracelulares envolvidas em muitas funções essenciais ao longo do ciclo celular, como por exemplo: formação do fuso mitótico durante a divisão celular, formação do citoesqueleto celular e movimento de partículas intracelulares (Martin, 1997; Downing, 2000; von Samson-Himmelstjerna et al., 2007).

Os dímeros solúveis da tubulina contendo um peptídeo de *α*-tubulina e outro de *β*-tubulina se polimerizam e formam uma espiral oca com 13 membros. Cada volta da espiral permite que a *α*-/*β*-tubulina formem um feixe, dispostas alternadamente. Os microtúbulos resultantes são altamente dinâmicos e envolvem a polimerização da tubulina em uma extremidade (positiva) e a despolimerização na outra extremidade (negativa) (Martin, 1997) (Figura 1).

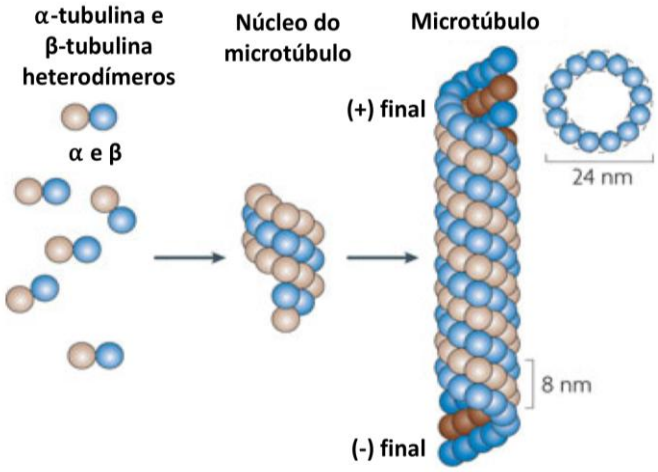


Figura 1. Dímeros de *α*-tubulina e outro de *β*-tubulina formando a estrutura do microtúbulo. Esquema adaptado de Dumontet e Jordan (2010).

### 3.1.1. Resistência aos benzimidazóis

O primeiro relato de resistência, no início da década de 1960, foi a um BZ em nematódeos, parasitas de animais de produção (Drudge et al., 1964 citado por Beech et al., 2011). No Brasil, o primeiro relato de resistência anti-helmíntica em ovinos foi descrito em 1967 no Rio Grande do Sul (Santos e Gonçalves, 1967 citado por Duarte et al., 2012).

O tratamento anti-helmíntico com BZs promove a inibição da formação dos microtúbulos ligando-se seletivamente à *β*-tubulina celular do helminto, todavia, em populações resistentes, essa ligação é impedida por uma mutação pontual no gene da *β*-tubulina (Kowal et al., 2016). Vários estudos apontam principalmente a presença de SNPs (single nucleotide polymorphism) nos códons 167, 198 e 200 do isotipo 1 da *β*-tubulina (Kowal et al., 2016).

A mutação comumente observada em diferentes locais do mundo acontece no sítio F200Y, quando ocorre a substituição de uma fenilalanina, Phe / F (T**T**C) para tirosina, Tyr / Y (T**A**C) na posição 200 do isótopo 1 do gene da *β*-tubulina (Lancey, 1988 citado por Le Jambre, 1993; Martin, 1997; Silvestre e Humbert, 2000; Coles et al., 2006; Gilleard, 2006; Čudeková et al., 2010; Niciura et al., 2012; Chagas et al., 2016; Kowal et al., 2016).

Um SNP no códon F167Y com a substituição de uma fenilalanina, Phe / F (T**T**C) para tirosina, Tyr / Y (T**A**C) no códon 167, também tem sido detectado em diversos países, embora menos comum do que a F200Y. A mutação F167Y apresentou baixa frequência na população de *H. placei* em bovinos no estado de Minas Gerais (2,5%) e alta frequência (32,5%) em ovinos no estado de São Paulo (Brasil et al., 2012). Mais recentemente, em fazendas de ovinos no Reino Unido foi observada a mutação F167Y em seis das sete fazendas avaliadas, com frequência similar a mutação no F200Y (Redman et al., 2015).

Um terceiro SNP, porém mais raro e pontual, já foi relatado no códon E198A, mutação de um ácido glutâmico, Glu / E (G**A**A) para uma alanina, Ala / A (G**C**A) (Chaudhry et al., 2015b). Em diferentes locais do Sul da Índia a mutação E198A foi relatada em oito das 23 populações de ruminantes avaliadas, com frequências entre 8% e 18% (Chaudhry et al., 2015b).

Embora estudos anteriores não relatassem a combinação da mutação F200Y e F167Y, no mesmo alelo, a combinação das mutações (F200Y, F167Y e E198A), no mesmo alelo, pode ser letal (Barrère et al. 2013; Kotze e Prichard, 2016).

### 3.2. Imidatiazóis

Os imidatiazóis (levamisol e pirantel) têm como alvo os receptores nicotínicos de acetilcolina (nAChR) (Martin, 1997; Sangster e Gill, 1999). Em mamíferos, os canais de acetilcolina são compostos por cinco subunidades do receptor que juntas formam um canal iônico transmembrana (Figura 2) (Sangster e Gill, 1999). Os imidatiazóis atuam seletivamente como agonistas colinérgicos nos nAChRs das células musculares do nematódeos, abrindo os canais iônicos, promovendo a contração e paralisia da musculatura (Martin, 1997; Sangster e Gill, 1999; Martin et al., 2012; Kotze et al., 2014).

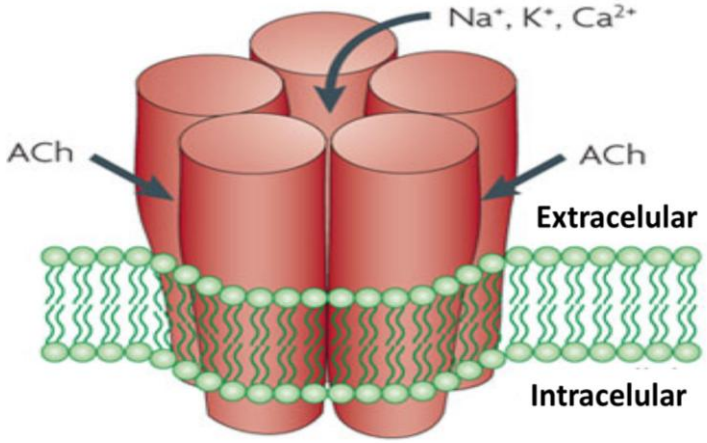


Figura 2. Receptores de acetilcolina nicotínicos (nAChR), esquema adaptado de Changeux (2010).

#### 3.2.1. Resistência aos imidatiazóis

Levamisol e pirantel são drogas antigas (1965) e muito utilizadas para tratar infecções helmínticas em humanos e animais (Martin et al., 2012).

A redução dos níveis de expressão dos genes codificantes das subunidades de nAChR, que compõem o receptor, têm implicado na resistência ao levamisol em *H. contortus* (Sarai et al., 2015). A resistência também tem sido associada com a presença de formas truncadas de duas subunidades do receptor (acr-8b, como forma truncada de acr-8a, e unc-63b, como forma truncada de unc-63a) em isolados de *H. contortus*, *T. colubriformis* e *Teladorsagia circumcincta* (Barrère et al., 2014; Sarai et al., 2015).

Estudos demonstraram também que as subpopulações resistentes de um isolado de *H. contortus* apresentaram redução na expressão de genes que codificam proteínas auxiliares UNC-74, RIC-3 e UNC-50 (Sarai et al., 2015).

### 3.3. Lactonas macrocíclicas (ivermectina)

A ivermectina foi introduzida comercialmente em 1981 para o controle de endo e ectoparasitas (Campbell, 2016).

A ivermectina tem forte afinidade pelo ácido gama-amino butírico (GABA "gamma aminobutyric acid") ou aos canais de cloreto iônicos dependente de glutamato (GluCls), causando, seletivamente, paralisia da musculatura do parasita (Ashraf et al., 2015).

#### 3.3.1. Resistência as lactonas macrocíclicas (ivermectina)

A resistência a ivermectina é generalizada e crescente em endoparasitas de ovinos, caprinos e bovinos. Na África Ocidental, Eng et al. (2006) utilizaram a resistência à ivermectina em *H. contortus* como um parâmetro para avaliação da mutação em *Onchocerca volvulus*, cuja infecção em humanos, naquela região, é tratada com ivermectina, a qual tem apresentado eficácia reduzida. Foi observado que a ivermectina pode selecionar para a mutação F200Y da *β*-tubulina (T**A**C) em ambas as espécies, podendo predispor os nematódeos também a resistência aos BZs (Eng et al., 2006). Recentemente, Asharaf et al. (2015) avaliaram um isolado de *H. contortus* e observaram que a ivermectina pode ligar-se e estabilizar os microtúbulos (alterando o equilíbrio de polimerização das tubulinas), apresentando um potencial como agente anti-mitótico.

Ao avaliar *H. contortus* provenientes do cruzamento de uma cepa parental susceptível à ivermectina (MHco3 (ISE)) e duas cepas parentais resistentes à ação da ivermectina (MHco4 (WRS) e MHco10 (CAVR)), as F1 produzidas foram nomeadas como MHco3/4.BC e MHco3/10.BC, respectivamente (Redman et al., 2012). As F1 foram utilizadas para infectar cordeiros doadores, os quais receberam tratamento com ivermectina, e os parasitas F1 sobreviventes foram retrocruzados por quatro gerações consecutivas com a cepa MHco3 (ISE). A quarta geração (MHco3/4.$BC_4$ e MHco3/10.$BC_4$) expressavam fenótipo de resistência, embora apresentassem um fundo genético semelhante à cepa de referência susceptível (MHco3 (ISE)), com base na genotipagem em massa com 18 *loci* de microssatélites e genotipagem individual com nove *loci* de microssatélites (Redman et al., 2012). Os autores relataram que apenas um marcador de microssatélite (Hcms8a20) mostrou evidência de ligação genética a um *locus* que confere resistência à ivermectina, detectando a sua introgressão (Redman et al., 2012).

## 4. Considerações

A avaliação morfológica para a diferenciação das espécies de *Haemonchus* consiste na técnica mais acessível, contudo, a identificação dos espécimes pode ser dificultada pela sobreposição das medidas entre *H. contortus* e *H. placei*, impossibilitando a distinção das espécies e, consequentemente, de indivíduos híbridos. Mediante o exposto, o presente estudo utilizou as técnicas moleculares como o sequenciamento de microssatélites para a identificação das espécies de *H. contortus* e *H. placei*, a PCR convencional para a identificação das espécies de, bem como de híbridos, e ainda para a caracterização molecular da resistência ao levamisole.

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# Capítulo 1

*Short communication redigida de acordo com as normas do periódico Molecular & Biochemical Parasitology

A panel of microsatellite markers to discriminate and investigate *Haemonchus contortus* and *Haemonchus placei*

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**Highlights**

- Description of five loci to characterize *H. contortus* and *H. placei*;
- Development and validate of two new microsatellite markers;

**Abstract**

The characterization of *Haemonchus contortus* and *Haemonchus placei* in mixed infections is important, thus, molecular techniques as microsatellite markers were developed and validated in this trial. Sheep and calf received artificial oral infection with 25,000 infective larvae (L3) of *H. contortus* (SpHco2 laboratory isolate) or *H. placei* (SpHpl1 laboratory isolate). The parasites were recovered and analyzed with five microsatellites (Hcms3561, Hp53, Hp52, Hcms53265, and Hcms36) to compare the homozygous and heterozygous profile. Higher gene diversity over loci was observed for SpHco2 isolate (0.711751 ± 0.406429) than for SpHpl1 isolate (0.529082 ± 0.318804) and variation among populations of 30.76%. In summary, the loci characterization of SpHco2 and SpHpl1 laboratory isolates showed differences between the populations using five loci, the characterization of the species was better observed with the microsatellite Hp52, showing that this microsatellite could be applied to distinguish both species in mixed infections.

**Keywords:** Gastrointestinal nematode, molecular biology, ruminant.

*Haemonchus contortus* and *Haemonchus placei* species are important gastrointestinal nematodes affecting the animal productivity. Although sheep and cattle are preferential hosts in extensive production systems these parasites can contribute to parasitic cross-infections when sheep and cattle share the same pastures. In mixed infections these parasites can crosses and hybrid production occurs [1, 2, 3, 4, 5].

Morphological evaluations are used to identify *Haemonchus* species, however, the overlap between measures of *H. contortus* and *H. placei* induce to misidentified the specimens [6]. Thus, molecular techniques present advantage for tackling diagnostic problems identification of parasites, their genetic characterization, the isolation and characterization of expressed genes, and the detection of drug resistance genes and mutations [7, 8]. Microsatellites markers enable the analysis of the polymorphism, evaluating alleles in different populations and individuals within a population [9, 10].

This study aimed the development, characterization, and validation of microsatellite markers to identify *H. contortus* and *H. placei* specimens from a laboratory isolates.

The trial was approved and conducted in accordance with the experimental protocol approved by the local Ethics Committee (protocol number 449-CEEA) of São Paulo State University (Unesp), Institute of Biosciences, Botucatu, Brazil.

Sheep and calf were maintained indoors and, before being infected, received monepantel (2.5 mg/kg, Zolvix®, Novartis Animal Health) in a single oral dose to eliminate any natural nematode infection. Their worm-free status was confirmed before the infection by a series of faecal examinations using a modified McMaster technique [11] and if no eggs were detected a more sensitive flotation method (modified Willis) was performed [11].

Calf and sheep were kept in separate pens to avoid cross-contamination and, during the trial, they had free access to tap water and grass hay (*Cynodon dactylon* cv. Tifton 85 *ad libitum*) purchased from a farm with no ruminants, avoiding risks of food contamination by

nematode infective larvae (L3).

Infective larvae of *H. placei* and *H. contortus* had been preserved in liquid nitrogen. *Haemonchus contortus* (laboratory isolate SpHco2) was used to infect a Suffolk male sheep exclusively with 25,000 L3 while *H. placei* (laboratory isolate SpHpl1) was used to infect a calf (25,000 L3). SpHco2 larvae were isolated in 2006 from two male lambs raised on a farm in Sao Paulo state, Brazil naturally infected with gastrointestinal nematodes, while SpHpl1 larvae were isolated from a bovine naturally infected with gastrointestinal nematode in Sao Paulo state in 2005. Both isolates were passed only once to infect donor sheep and calf, respectively. Previously, SpHpl1 adult specimens were evaluated to rDNA ITS-2 sequence in other study confirming the *H. placei* identity, based on SNPs differences between the *Haemonchus* species [5].

Faecal cultures were performed separately for the production of L3. The species were confirmed based on the length of the sheath tail extension using an ocular micrometer (Zeiss®), *H. placei* larvae usually have a sheath tail longer than 89 μm and *H. contortus* a sheath tail shorter than 85 μm [6].

Sheep and calf were euthanized 106 and 161 days post-infection (DPI), respectively. The abomasum was opened along the greater curvature, and its content was placed in a graduated beaker. The mucosa surface was washed with saline solution (0.9%) so that the parasites adhering to the mucosa could be detached from the organ. The volume was brought up to 1 L with saline solution; the content was homogenized and divided into two containers of 500 mL each. The containers were immediately frozen (-20°C) for the preservation of parasites. Worm identification and counting procedures were performed and the recovered worms were stored in ethanol 70%.

Adult male *Haemonchus* worms were chosen at random for analysis. Individual adult males (n=30 for SpHco2 and n=30 for SpHpl1) were randomly chosen for analysis, the

specimens were placed on a glass slide and with the needle of an insulin syringe the worms were cut in the middle and divided into two tubes. The half worm was placed in a tube with ethanol 70% and the other half was used for DNA extraction. The lysates were prepared as previously described by Redman [12].

Five microsatellite markers (Hcms3561, Hcms36, Hcms53265, Hp52, and Hp53) were tested to compare the allele size in 30 samples of SpHco2 and SpHpl1 lysates.

The microsatellites Hcms3561, Hcms36, and Hcms53265 were previously described by Chaudhry [5] and Redman [12, 16]. Details about the unpublished markers follow: microsatellite Hp52 was amplified using primers 5'-GAGTGAGACATCGTAATCCA-3' and 5'-GGTATCAAACTACTACTTGTC-3' allele size range (340-470 bp). The microsatellite Hp53 was amplified using primers 5'-TCGACTCTCACCCGAATACA-3' and 5'-TTTGTGGCAGTTGGCCGTT-3'allele size range of 170-220 bp. A total of five microsatellite markers (Hcms3561, Hcms36, Hcms53265, Hp52, and Hp53) were used in this study.

The PCR was performed in 20 μL using Qiagen® multiplex PCR kit (QIAGEN, Hilden, Germany) and 0.1 μM forward and reverse primers and 2μL of lysate DNA. Thermocycling conditions evaluated were 95°C for 15 minutes followed by 40 cycles of 94°C for 30 seconds, 54°C for 90 seconds and 72°C for 30 seconds with a single final extension cycle of 72 °C for 15 minutes.

The forward primer of each microsatellite primer pair was 5'end labelled with 6-FAM and HEX fluorescent dyes (MWG) and electrophoresed. Microsatellite PCR products by capillary electrophoresis were performed using GeneScan ROX 400 internal size standard was used on the ABI Prism 3100 genetic analyser (Applied Biosystems, USA®). Individual chromatograms were analysed using Peak Scanner™ software to determine the genotypes of

each sample and each of these five molecular markers produced distinct genotypes with a single or double peak (homozygous or heterozygous) as predictable in a diploid organism.

The number of alleles (Na), the average gene diversity over loci, expected heterozygosity ($H_e$), and observed heterozygosity ($H_o$) were evaluated. Hardy–Weinberg equilibrium (HWE) was tested in AMOVA (Analysis of Molecular Variance) test (1000 permutations) for Arlequin software (version 3.5.2.2) [17] considering genotypic differences between isolates, at significance level (0.05). Fixation indices were evaluated and population pairwise $F_{ST}$ statistics were calculated for all samples.

To characterize *H. contortus* and *H. placei* populations five loci were evaluated using SpHco2 and SpHpl1 laboratory isolates (Figure 1, A to E). Although *H. contortus* and *H. placei* were considered synonymy during many years [13], recent studies showed morphologically and molecular differences between both species [3, 4, 12, 14].

Thirty specimens of each isolate (SpHco2 and SpHpl1) were evaluated, however, seven specimens from SpHpl1 laboratory isolate did not amplify and only n=23 specimens were included in the study. AMOVA analysis showed pairwise differences with variation among populations of 30.76% (global $F_{ST}$ value of 0.30763) and higher gene diversity over loci was observed for SpHco2 (0.711751 ± 0.406429) than for SpHpl1 (0.529082 ± 0.318804), Table 1. Our results previous studies conducted in U.S. and in Brazil. In U.S. was observed a lower genetic diversity among *H. placei* populations compared to *H. contortus* using the sequence of mitochondrial DNA(mtDNA) ND4 gene [15]. Similarly, in Brazil, the genetic diversity among populations was lower in *H. placei* compared to *H. contortus* from sheep, goat, cattle, and buffalo from different regions of Brazil, [14].

Molecular methods are important advances to the comprehension of population genetics [10]. In our study, the microsatellite technique contributed to the distinction between allele range in both species with the microsatellite Hp52, showing different patterns for

SpHco2 (*H. contortus*) and SpHpl1 (*H. placei*). The SpHco2 isolate showed allele range between 358bp to 374bp and SpHpl1 isolate between 379bp to 484 bp, with this microsatellite did not was observed overlap.

Differences on allele range for each locus and species were observed between SpHco2 and SpHpl1 populations (Fig. 1, A to E). Microsatellites markers are commonly used in population studies and are important in characterizing the polymorphism among and within species and the hybrids products (F1 populations) of their mating [10].

The number of alleles was similar in both isolates (SpHpl1Na=30 and SpHco2 Na=31) to the five loci evaluated (Figure 1, A to E and Table 1). In addition, the SpHpl1 isolate showed higher Na on loci Hcms3561 (Na=9), while SpHco2 laboratory isolate showed higher Na on loci Hcms53265 (Na=9), Table 1.

In the evaluation of the Hardy-Weinberg equilibrium of the SpHpl1 laboratory isolate, differences between $H_o$ and $H_e$ was observed on loci Hcms3561 (0.47826 and 0.79903, respectively), and on loci Hcms53265 (0.27778 and 0.38889, respectively), while to SpHco2, there was no statistical difference ($p>0.05$) in the five loci evaluated (Table 1).

The loci characterization of *H. contortus* and *H. placei* populations using the microsatellite marker Hp52 could be applied to distinguish both species in mixed infections.

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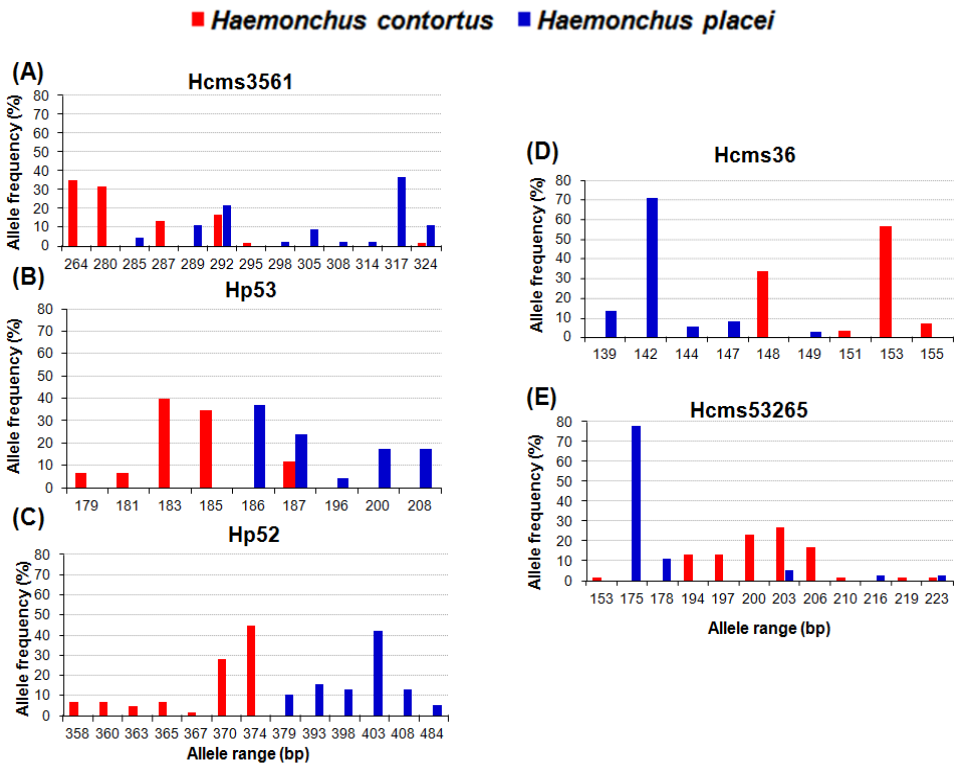


Figure 1. Allele frequencies (%) of five microsatellites markers (Hcms3561 (A), Hp53(B), Hp52 (C), Hcms36 (D) and Hcms53265 (E)) based on populations of each laboratory isolate SpHco2 (*Haemonchus contortus*) and SpHpl1 (*Haemonchus placei*).

Table 1. Hardy-Weinberg equilibrium for *Haemonchus contortus* (SpHco2) and *Haemonchus placei* (SpHpl1) populations to the five microsatellites (Hcms3561, Hp53, Hp52, Hcms36 and Hcms53265) and AMOVA results for polymorphic loci.

| Loci | Hcms3561 | Hp53 | Hp52 | Hcms36 | Hcms53265 |
|---|---|---|---|---|---|
| *Haemonchus contortus* | | | | | |
| Genotypes | 30 | 30 | 30 | 30 | 30 |
| Na | 6 | 5 | 7 | 4 | 9 |
| $H_o$ | 0.76667 | 0.66667 | 0.63333 | 0.53333 | 0.73333 |
| $H_e$ | 0.74350 | 0.70678 | 0.71299 | 0.57175 | 0.82373 |
| p-value | 0.14048 | 0.84859 | 0.16947 | 0.43332 | 0.31930 |
| s.d. | 0.00025 | 0.00036 | 0.00037 | 0.00045 | 0.00047 |
| *Haemonchus placei* | | | | | |
| Genotypes | 23 | 23 | 19 | 19 | 18 |
| Na | 9 | 5 | 6 | 5 | 5 |
| $H_o$ | 0.47826 | 0.56522 | 0.63158 | 0.52632 | 0.27778 |
| $H_e$ | 0.79903 | 0.76039 | 0.76956 | 0.48080 | 0.38889 |
| p-value | 0.00003* | 0.06515 | 0.22626 | 0.47369 | 0.02102* |
| s.d. | 0.00001 | 0.00024 | 0.00045 | 0.00052 | 0.00011 |
| AMOVA results for polymorphic loci: | | | | | |
| % Variation among populations | 19.65 | 24.42 | 25.83 | 46.85 | 36.01 |
| % Variation among individuals within populations | 13.41 | 11.30 | 10.50 | 0.62 | 9.78 |
| % Variation within individuals | 66.94 | 64.28 | 63.67 | 52.54 | 54.21 |
| Fixation indices: | | | | | |
| $F_{IS}$ | 0.17 | 0.15 | 0.14 | 0.01 | 0.15 |
| p-value | 0.01271 | 0.05181 | 0.05376 | 0.51320 | 0.03226 |
| $F_{ST}$ | 0.20 | 0.24 | 0.26 | 0.47 | 0.36 |
| p-value | 0 | 0 | 0 | 0 | 0 |
| $F_{IT}$ | 0.33 | 0.36 | 0.36 | 0.47 | 0.46 |
| p-value | 0 | 0 | 0 | 0 | 0 |

Na: number of alleles, $H_o$: observed heterozygosity and $H_e$: expected heterozygosity.

*p-value < 0.05 indicate a significant deviation from Hardy–Weinberg equilibrium.

Global AMOVA results: average F-Statistics over all loci for fixation indices: inbreeding coefficient ($F_{IS}$): 0.12080, fixation index ($F_{ST}$): 0.30763 and overall fixation index ($F_{IT}$): 0.39819.

# Capítulo 2

*Manuscrito redigido de acordo com as normas do periódico Veterinary Parasitology.

**Establishment of coinfection and hybridization of *Haemonchus contortus* and *Haemonchus placei* laboratory isolate in sheep**

**Abstract**

This study aimed to evaluate the simultaneous infections with *Haemonchus contortus* and *Haemonchus placei* in sheep, as well as the production of hybrids. Lambs from the parental group (n=6) were infected with 2,000 infective larvae (L3) from *H. placei* (SpHpl1 isolate) on day 0 and 11 days later the same animals received 2,000 L3 of *H. contortus* (SpHco2 isolate). Fecal cultures were performed individually and the first generation (F1) of L3 were designed to infect the animals from groups F1-42 DPI (n=6) and F1-84 DPI (n=6). All animals from groups parental, F1-42 DPI and F1-84 DPI were euthanized at 50, 42 and 84 days post-infection (DPI), respectively, to evaluate the establishment and patency of each species and also the production of hybrids. Uninfected animals (n=6) were kept as a control group. Fecal egg counts and identification of L3 demonstrated that the artificial infection with *H. contortus* and *H. placei* established successfully in all lambs of the parental group. The higher worm burden mean was recorded at 42 days post-infection (Group F1-42 DPI) in comparison with 84 days post-infection (Group F1-84 DPI). Adult worms of both species were also detected in similar proportions by PCR in animals of the F1-42 DPI and F1-84 DPI groups. However, larvae with *H. contortus* morphology predominated in fecal cultures. Species-specific primer pairs were employed to analyze individual *Haemonchus* adult specimens (n=219), then111 *H. contortus*, 100 *H. placei* and eight hybrids (3.65%) were recovered from groups F1-42 DPI and F1-84 DPI. In summary, artificial mixed infections by *H. contortus* and *H. placei* may promotes the establishment of both species in sheep with a small number of hybrids found among their offspring.

Keywords: cattle, gastrointestinal nematode, host-specificity, sheep, mixed infections.

## 1. Introduction

*Haemonchus contortus* and *Haemonchus placei* are two valid distinct species supported by morphological, biological and molecular evaluations (Zarlenga et al., 1994; Achi et al., 2003; Hoberg et al., 2004; Santos et al., 2014a; Chaudhry et al., 2015), these species have, respectively, small ruminants and cattle as preferential hosts. Nevertheless, mixed infections can occur when cattle and small ruminants share the same pasture in livestock production systems of Brazil (Amarante et al., 1997; Rocha et al., 2008), West Africa (Jacquiet et al., 1998), North Côte d'Ivoire (Achi et al., 2003), Egypt (Khalafalla et al., 2011), Western Australia (Jabbar et al., 2014) and United States (Chaudhry et al., 2014).

In mixed infection, the hybridization through interspecific crossing can have major evolutionary consequences on species and populations by either promoting or preventing divergence, depending on the viability and reproductive abilities of the hybrids. In addition, adaptive traits can also be acquired through hybridization, resulting in an increase in fitness of hybrids (reviewed by Gilabert and Wasmuth, 2013). Nevertheless, in the case of *Haemonchus*, the offspring of hybrids backcrossing with *H. contortus* or *H. placei* can be sterile or show reduced fertility (Le Jambre, 1979; 1981).

*Haemonchus contortus* and *H. placei* differentiation could be performed based on the morphology of the infective larvae (L3) (van Wyk and Mayhew, 2013) or adult males (Jacquiet et al., 1998; Achi et al., 2003), however, overlapping in the values of morphometry can interfere with the precise identification (reviewed in Amarante, 2011; Santos et al, 2014b). Therefore, molecular techniques offer some advantages, because these methods are more objective, more scalable and easier to implement (Chaudhry et al., 2014).

Thus, this study aimed to evaluate the hybrid production in artificial mixed infection with *H. contortus* and *H. placei* in sheep as well as the establishment rate of both species in lambs.

## 2. Material and Methods

This trial was approved and conducted in accordance with the experimental protocol approved by the local Ethics Committee (protocol number 449-CEEA) of Sao Paulo State University (Unesp), Institute of Biosciences, Botucatu, Brazil.

*Haemonchus placei* and *H. contortus* had been preserved in liquid nitrogen. *Haemonchus contortus* (laboratory isolate SpHco2) was used to infect a Suffolk male sheep exclusively with 25,000 L3 while *H. placei* (laboratory isolate SpHpl1) was used to infect a calf (25,000 L3). The larvae SpHco2 were isolated in 2006 from two male lambs, naturally infected with gastrointestinal nematode, these lambs were raised on a farm in Sao Paulo state, Brazil. While the SpHpl1 larvae were isolated from a bovine in 2005 with natural infections with gastrointestinal nematode in Sao Paulo state. Both isolates were passed only once to infect donor sheep and calf, respectively.

Donor sheep and cattle were maintained indoors and, before being infected, received monepantel (2.5 mg/kg, Zolvix®, Novartis Animal Health) in a single oral dose to eliminate any natural nematode infection. These animals were maintained indoor, in separate pens to avoid cross-contamination and fecal cultures were performed separately for the production of L3 and, during the trial, they had free access to tap water and grass hay (*Cynodon dactylon* cv. Tifton 85 *ad libitum*) purchased from a farm with no ruminants, avoiding risks of food contamination by nematode L3. All L3 were stored in tubes with 20 and 25 mL of distilled water and used to infect the experimental animals (parental group). The species identification was confirmed based on the length of the sheath tail extension, the measurement was made using an ocular micrometer (Zeiss®) according to the descriptions by Santos et al. (2014b).

*Management and diet*

Initially, all animals were vaccinated against clostridiosis (Sintoxan T Polivalente®,

Merial S.A.) and during the trial they received decoquinate (Deccox®, Alpharma), according to the manufacturer's recommendations.

The animals had free access to tap water; mineral salt (Presensefós®, Presence) and ground hay (Tifton 85, *Cynodon dactilon*. cv. Tifton 85) and received a commercial concentrate (Suplementa Ovino Campo®, Presence, 18% of crude protein) in an amount corresponding to 1% of their mean body weight.

*Experimental design*

Twenty-four Suffolk male lambs of approximately five months old (mean of 23.6 Kg) were acquired from a commercial farm located in Paranapanema-Sao Paulo, and maintained in pens with a concrete floor in the facilities for small ruminants of the Sao Paulo State University (Unesp). All animals were treated with monepantel (2.5 mg/kg, Zolvix®, Novartis Animal Health) orally, in a single dose. Then, a series of fecal examinations was performed to confirm the elimination of infection by nematodes (worm-free status).

Six lambs were used as a parental group. Each of these animals received orally artificial infection with 2,000 L3 of SpHpl1 on day zero and eleven days later the same animals received 2,000 L3 of SpHco2 (Fig. 1). This difference in the time range of the infections was due to the fact that the prepatent periods is approximately 11 days shorter in *H. contortus* in comparison with *H. placei* (Riggs, 2001; Santos et al., 2014a). Worm-free lambs (control group, n=6) were kept to verify any risk of environmental contamination with L3.

Feces were recovered from lambs of the parental group between 25 and 50 days post SpHco2 infection and fecal cultures were performed individually for L3 production. This first filial generation (F1) of L3 were assigned for infection of animals of F1 group (n=12). Larvae from each animal of the parental group were used to infect two animals of the F1 group (Fig. 1). The lambs of the F1 group were subdivided in F1-42 DPI (n=6), euthanized at

42 days post infection (DPI) and F1-84 DPI (n=6), euthanized at 84 DPI.

Animals from the parental group were euthanized at 50 DPI with SpHpl1 (39 DPI with SpHco2). All infective larvae produced on fecal cultures were quantified and identified.

### 2.1. Laboratory analyses

*Fecal examination*

Fecal samples were collected once a week directly from the rectum of animals for egg counting. Fecal cultures were prepared separately with samples of each animal. Measurement of sheath tail (distance between the tip of the larval tail and the end of the sheath tail) of L3 obtained from each experimental animal was performed according to Santos et al. (2014b).

*Worm burden*

The animals were fasted for 12 hours before being euthanized. The abomasum was opened along the greater curvature, and its content was placed in a graduated beaker. The mucosa surface was washed with saline solution so that the parasites adhering to the mucosa could be detached from the organ. The volume was brought up to 1 L with saline solution, the content was homogenized and divided into two containers of 500 mL each. The containers were immediately frozen for the preservation of parasites. The mucosal layers of all abomasums were soaked in saline solution at 38°C for 4 hours. All content of the digested material was collected and frozen (-20°C).

Worm identification and counting procedures were performed on 50% of the content of the frozen material. If no worms were found in that sample, all the remaining content was also carefully examined for worms.

The morphometry of the spicules of 10 male adult worms, randomly chosen, was determined from each animal. These specimens were placed on a glass slide and the body was

cut near the copulatory bursa, the anterior region of the worm body was transferred to a 1.5 mL tube for DNA extraction, while its posterior portion was left on the slide to be measured. The discriminate function (DF) using morphometry of spicules was calculated according to the formula described by Jacquiet et al. (1997;1998) and Achi et al. (2003).

*Molecular evaluation*

These gold standard analyses aimed to identify the species and evaluate the hybrid production. From each lamb, 20 males were evaluated, including those 10 with morphological assessment (morphometry of spicules) plus other 10 without morphological evaluations.

Genomic DNA was extracted from each *Haemonchus* specimen using a QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. DNA from the cattle and sheep blood samples was also extracted for use as controls in PCR analysis.

The identification of the *Haemonchus* species was performed by PCR using species-specific primer pairs HcBotuF1/R2 and HpBotuF/R (Amarante et al., 2017). Each primer pair produced one single and distinct amplification band for each species. The primer pair HcBotuF1/R2 presented band with approximately 260 base pair (260 bp) on *H. contortus* samples and the HpBotuF/R one presented a PCR product of approximately 459 bp on *H. placei* samples. The PCR conditions have been described by Amarante et al. (2017).

The PCR products were electrophoresed on 2% agarose gel in 1% TAE buffer containing ethidium bromide and photographed under UV light using a Sony Cyber-shot DSC-HX1 camera (Sony Electronics, San Diego, California, USA). All PCR reactions were performed at least in duplicate.

### 2.2. Statistical analyses

The Dot plots of the sheath tail length measures were performed using the Minitab® software, version 17 (2013). Agreement between the results of the morphological and molecular analysis was evaluated by McNemar test ($p<0.05$), and the Kappa statistics (coefficient of agreement) using the Statistical Analysis System, version 9.2 (SAS® Institute, Inc., Cary, NC, USA).

## 3. Results

### 3.1. Fecal egg counts (FEC)

The mean FEC from the parental group was 2,733 (±664 EPG, eggs per gram of feces) at 49 DPI (Fig. 2A). High mean FECs were observed on groups F1-42 DPI and F1-84 DPI, with high FEC at 42 DPI (5,700 ± 379 EPG) and at 63 DPI (7,800 ± 1,699 EPG), respectively (Fig. 2B and 2C). On group F1-84 DPI a progressive decrease in FEC was observed starting 63 DPI. On the last sampling day (83 DPI), lambs presented 2,633 (± 1,026) EPG (Fig. 2C). The control group never shed eggs in feces, i.e., there was no evidence of undesired infections at experimental facility.

### 3.2. Sheath tail length of infective larvae

*Haemonchus contortus* L3 used to infect parental animals were produced from the donor lamb. The sheath tail lengths of a total of 500 L3 measured on five occasions (days 39, 43, 71, 81 and 114 post-infection) were in the range between 60.64 µm and 86.17 µm (Fig. 3A) and a mean of 69.41 (±0.23) µm. The *H. placei* L3 were obtained from the donor calf in seven sampling days (days 36, 41, 45, 56, 105, 114 and 117 post-infection). The sheath tail lengths of 700 L3 measured between 86.17 µm and 121.28 µm (Fig. 3B), mean of 102.61 (±0.27).

These measures were used to infer the *H. contortus* and *H. placei* establishment in the experimental animals. We assumed that L3 with measures < 85µm were *H. contortus*, and those with measures >89 µm were *H. placei* (Figs. 3A and 3B), while intermediate values, between >85 µm and <89 µm, were considered inconsistent to species classification due to overlap between *H. contortus* and *H. placei* measures.

The experimental animals showed measures of the sheath tail length of the L3 produced with fecal samples of the parental lambs (numbers 1 to 6). Infective larvae from the parental group (Fig. 4, A to F) showed measures of *H. contortus* (57.45 to 85 µm) and *H. placei* (89 to 124.47 µm). Infective larvae produced by parental group were used to infect the lambs of group F1. From group F1, L3 were measured at the last sampling (group F1-42 DPI euthanized 42 DPI and group F1-84 DPI 84 DPI). In the group F1-42 DPI (Fig. 4, H to L), lambs 14, 15, 16, 17 and 18 showed L3 with *H. contortus* and *H. placei* measures. The animal number 13 (Fig. 4, G) showed on last sampling (42 DPI) only L3 with *H. contortus* measures (63.83 µm to 79.79 µm). For the group F1-84 DPI (Fig. 4, M to R) all lambs showed L3 with *H. contortus* and *H. placei* measures. L3 with *H. contortus* morphology predominated in animals of the F1 group.

### 3.3. Worm burden

Worm recoveries were higher on group F1-42 DPI euthanized 42 DPI (average of 2,395 adults specimens) than in group F1-84 DPI euthanized 84 DPI (average of 1020 adults) indicating that a considerable number of worms had been naturally eliminated at the end of the study (Table 1). The establishment of the parasites and the dynamics of the infection were not uniform within groups. A few juvenile parasites were found in most of the lambs, however in small numbers with a maximum of 36 specimens.

3.4. Morphological and molecular analysis of the adult worms

*Donors*

Twenty adult males from each donor were evaluated by PCR confirming that the pure infection with each isolate established in each donor (Table 2, Fig. 6).

*Experimental animals*

The animals from parental group received a mix infection with L3 of *H. contortus* (donor sheep) and *H. placei* (donor cattle). The establishment rate of both species was similar (51.7% *H. contortus* and 48.3% *H. placei*) based on PCR analysis of 60 adult males.

Both species were detected until the last sampling at 42 DPI on F1-42 DPI group and 83 DPI on the F1-84 DPI group by morphological evaluation. The analysis of 219 adult males specimens by PCR showed a similar proportion of *H. contortus* (52.2%) and *H. placei* (47.8%) in F1-42 DPI and in F1-84 DPI groups (53.1% *H. contortus* and 46.9% *H. placei*).

For the F1-42 DPI group, 60 specimens were identified as *H. contortus*, 55 as *H. placei* and five hybrids and the group F1-84 DPI showed 51 *H. contortus*, 45 *H. placei* and three hybrids. Hybrid specimens showed amplification with both species-specific primers (Fig. 7). According to Hardy-Weinberg equilibrium ($p^2+2pq+q^2$), the infection with 2,000 L3 of *H. contortus* and 2,000 L3 of *H. placei* should result in the production of 50% specimens hybrids, however, only 3.65% of hybrids were recovered (8 in 219 worms)

The agreement between PCR using species-specific primers and morphological analysis by discriminate function (DF) was assessed to all groups. For the parental group, one specimen was identified as *H. contortus* using DF, but identified as *H. placei* by species-specific PCR, and four specimens identified as *H. placei* by DF were in fact *H. contortus* by species-specific PCR (Table 2). On groups F1 one specimen was identified as *H. contortus* by

morphology and identified as *H. placei* by species-specific PCR, and 12 *H. placei* specimens by DF were confirmed as *H. contortus* by species-specific PCR (Table 3).

Eight hybrids were recovered, nonetheless, just four of them had been previously evaluated by morphology, three specimens from the group F1-42 DPI and one from the group F1-84 DPI. Three were identified as *H. contortus* and one as *H. placei* based on morphometrics of spicules, using the discriminate function (Table 4).

## 4. Discussion

The establishment of the artificial infection by *H. contortus* and *H. placei* was confirmed by morphological evaluation of the L3 and afterwards by the molecular analysis of adult males with the use of species-specific primers. The PCR results showed a similar establishment percentage for both species indicating a favorable environment for interspecific mating.

Brasil et al. (2012) evaluated a total of 156 *Haemonchus* spp. from cattle, buffalo, goat and sheep in seven different herds raised in three states in Brazil, these animals showed natural infections by gastrointestinal nematodes and only two (1.3%) heterozygous specimens were detected carrying the ITS-2 alleles from *H. placei* and *H. contortus*. In Asia, Chaudhry et al. (2015) observed, among 644 worms from sheep and goats, five specimens *H. contortus/H. placei* F1 hybrids (0.078% of the total), which displayed heterozygosis for species-specific alleles of five independent genetic markers from the nuclear genome. Therefore, due the experimental infections in the present study the number of hybrids recovered differed from previously study 3.65% (n=8).

In the present trial, lambs were initially infected with *H. placei* and 11 days later with *H. contortus,* due the longer prepatent period in *H. placei* (Santos et al., 2014a), probably, both species reached maturity at the same time allowing the occurrence of interspecific

mating. Polyandry is observed in *Haemonchus* specimens and females could mate with three or more males, which in theory increase the probability of mating between both species (Redman et al., 2008) and the hybridization occurrence.

Strict host specificity can allow two closely related species of parasites to have a sympatric geographic distribution and still never come together for mating (Le Jambre, 1979). This was not the case of the present trial, where the artificial infections with *H. placei* and *H. contortus* resulted in the establishment of both species in lambs, creating an opportunity for intra- and interspecific mating. Animals from the parental group presented mixed infections with similar establishment rate (51.7% *H. contortus* and 48.3% *H. placei*) on the basis of PCR evaluation. Our results indicated that these parasites apparently avoid mating with individuals of other species or if the interspecific mating occurs the progeny is not viable to complete its development until the adult stage, consequently, the production of hybrids was very limited, only 3.65% in the present study. In Australia, Bremner (1955) evaluated the sheep type (*H. contortus*), cattle type (*H. placei*) and hybrids on the basis of giant X chromosome in a F1 sheep infected with L3 from a parental sheep that received 7,000 L3 of *Haemonchus* isolate from sheep and 7,000 L3 of *Haemonchus* isolate from cattle. These has been observed that a small number of hybrids were produced (3.9%: 3 of a total of 77 worms examined), value similar to that record in the present trial. In the same study, Bremner (1955) found 7% hybrids among 100 specimens in one calf, with high FEC (8,200 EPG), that had been mix grazing with goats.

Individuals hosting mixed infections with *Haemonchus* species are not rare, particularly in Africa, showing the complexity in evolutionary history (reviewed Hoberg and Zarlenga, 2016). It is important to highlight that *H. contortus* and *H. placei* populations may have come into contact numerous times over the centuries allowing the interspecific crossing,

even though these species have maintained their morphological and their distinct genetic identities (Bremner, 1955).

Thus, assuming that the mating between *H. contortus* and *H. placei* in mixed infections are common and the hybridization could occur, why is the recovery of hybrids so low? It is possible that hybrids present some metabolic and/or genetic modification, which unable them to develop from egg until the adult stage. Our results corroborate with the findings of Le Jambre (1979, 1981) who reported that crossing between *H. contortus* and *H. placei* is not beneficial for neither species.

After the adult recovery, male adults were subjected to morphological and molecular evaluations and Kappa coefficients obtained (> 0.76) indicated a strong agreement between techniques. Santos et al. (2014b) observed that 6.09% of the male adult specimens would be misidentified based on the discriminate function described by Jacquiet et al. (1997) while only 0.71% of the L3 were misidentified. In the present study, 166 samples were used to compare the morphological and molecular techniques and 10.8% (n=18) were misidentified.

Low FEC was observed at group parental when compared to F1-42 DPI and F1-84 DPI groups (Fig. 2A, 2B and 2C), this parental group was infected in two time points (day 0 with *H. placei* and day 11 with *H. contortus*). The artificial mixed infections with *H. contortus* and *H. placei* were established in sheep with a small number of hybrids found among their offspring. Further studies are needed to evaluate the possibility of gene introgression in field populations of both *Haemonchus* species.

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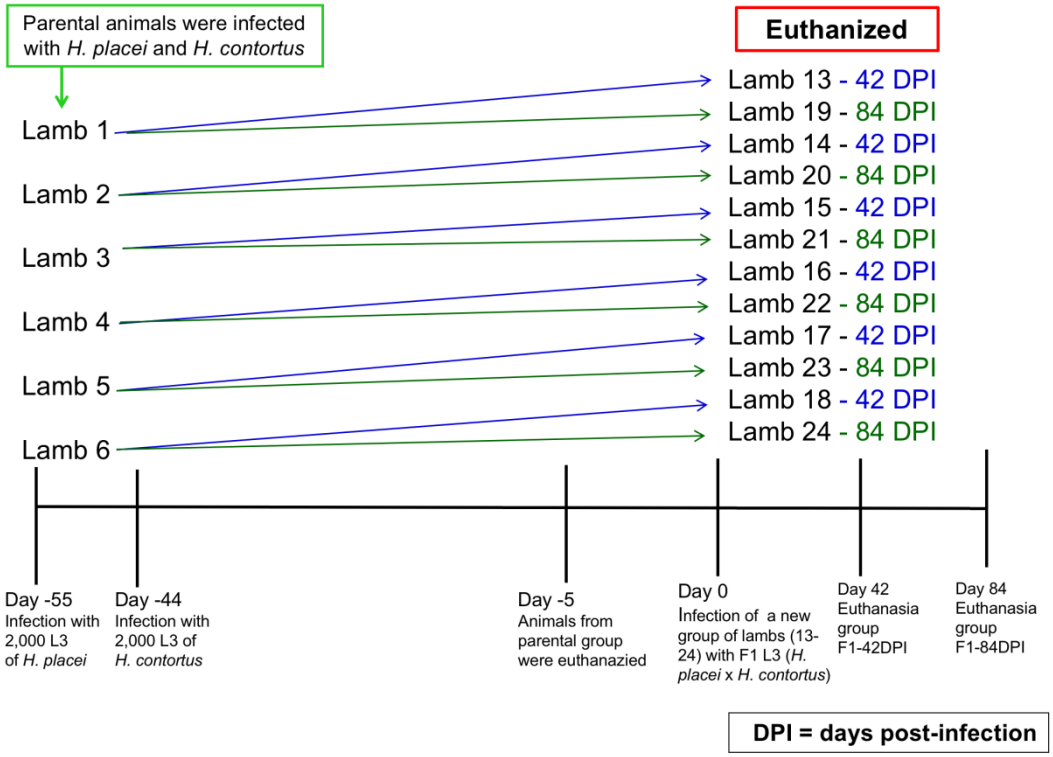



Figure 1. Experimental design: lambs from the parental group (1 to 6) received artificial infection with 2,000 infective larvae (L3) of *Haemonchus placei* (day 0), eleven days post-infection (DPI) the same animals were infected with 2,000 *Haemonchus contortus* L3. First filial generation (F1) of L3 produced from parental animals were used to infect lambs from F1 group (n=12, lambs 13 to 24). These lambs were subdivided into two groups and euthanized at 42 DPI (F1-42 DPI) or 84 DPI (F1-84 DPI). Animals 7 to 12 remained as a control group (uninfected).

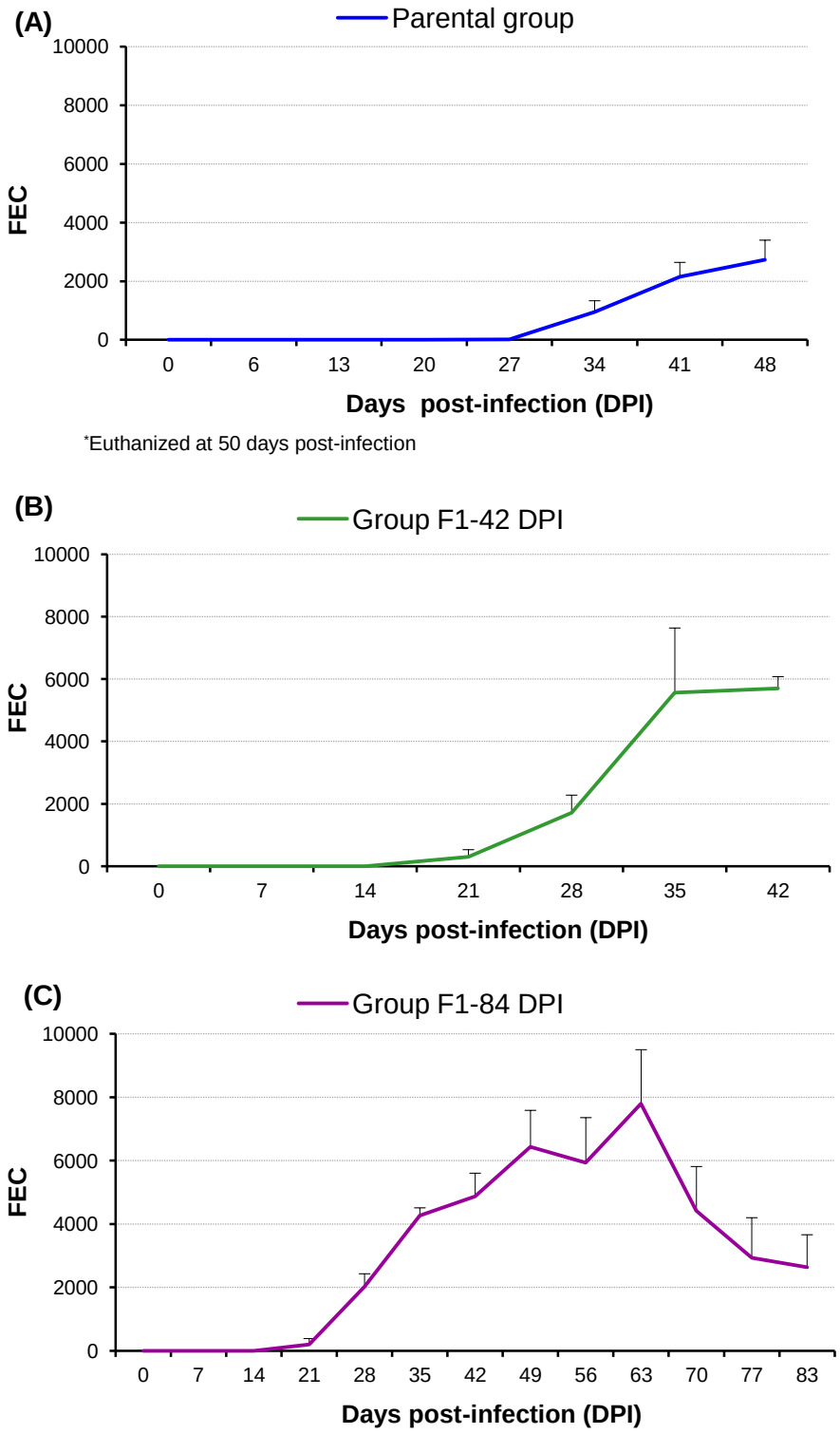



Figure 2. Fecal egg counts (FEC) from animals exclusively infected with *H. contortus* or *H. placei*. The parental group (A) was infected with 2,000 infective larvae (L3) of *Haemonchus placei* (day 0) and 11 days later received 2,000 L3 of *Haemonchus contortus*. The first filial generation (F1) groups were infected with L3 produced by parental lambs, the group F1-42 DPI (B) was euthanized 42 days post-infection (DPI) and group F1-84 DPI (C) was euthanized 84 DPI. Bars: standard error. Animals 7 to 12 remained as a control group (uninfected).

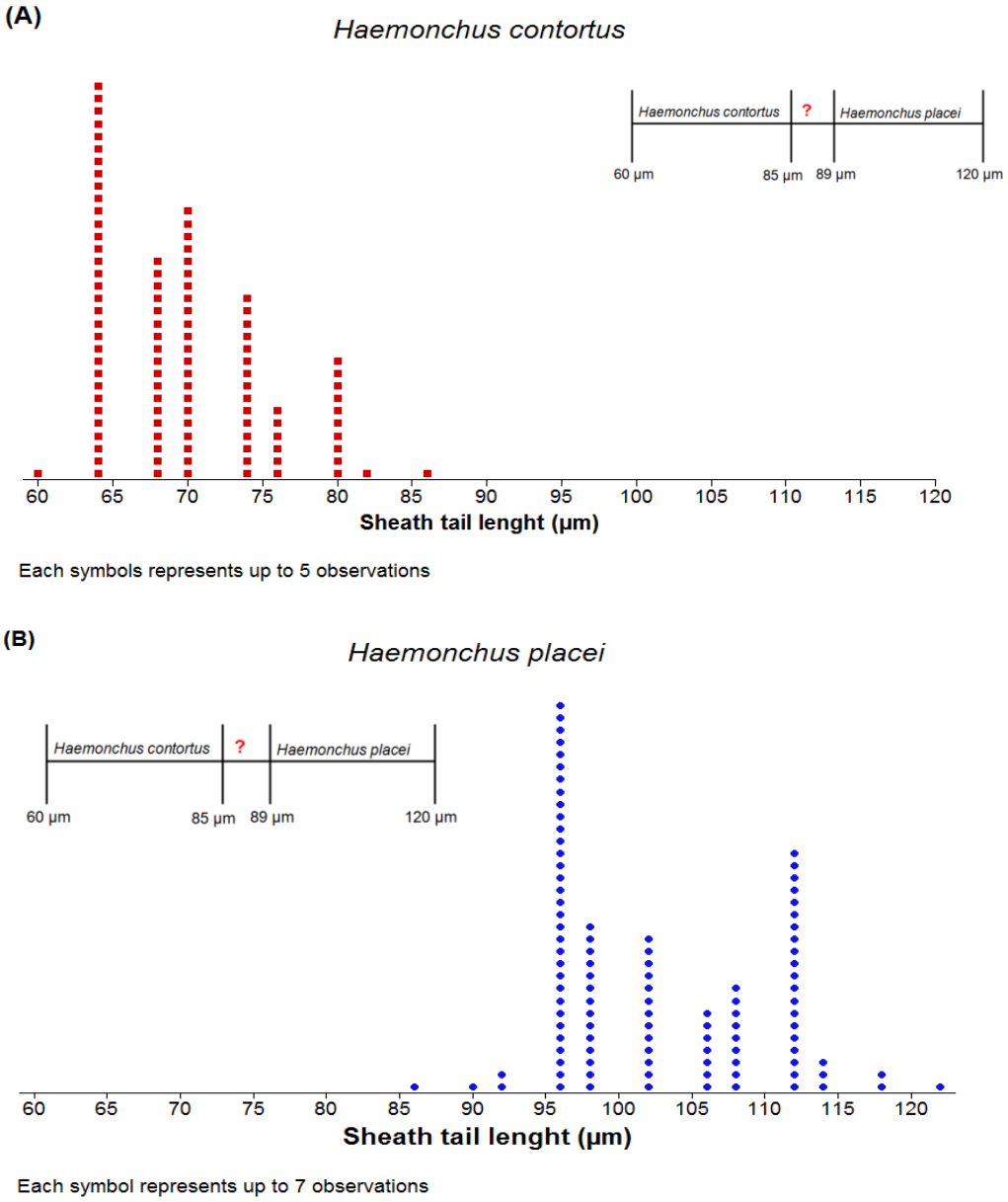
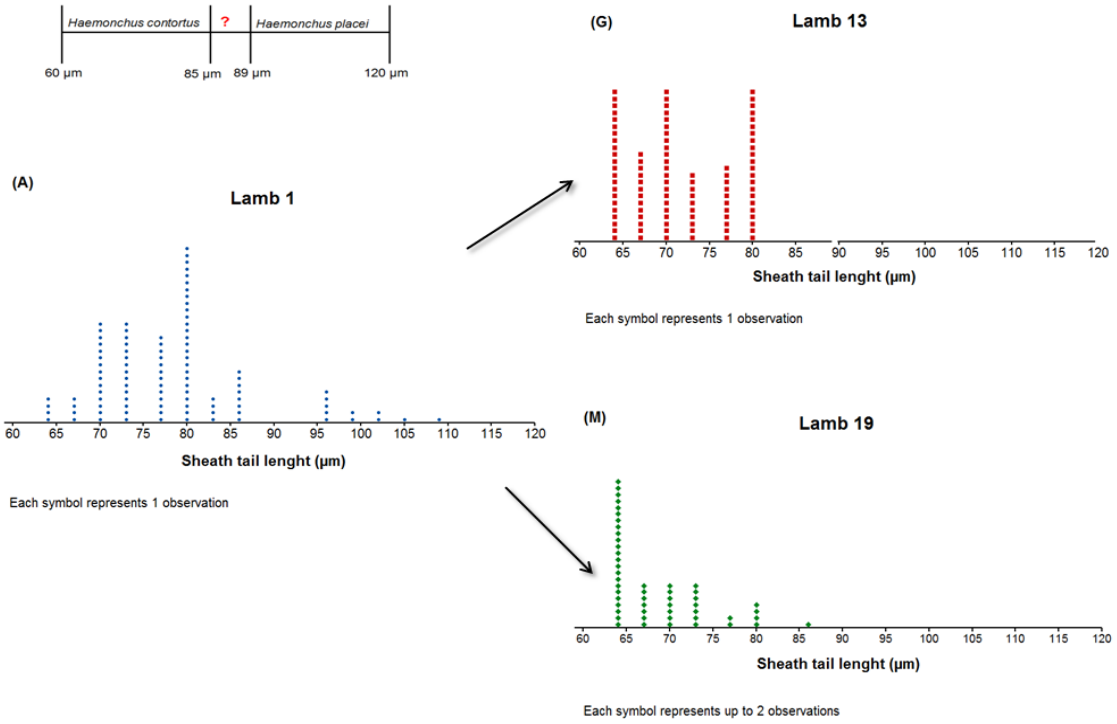
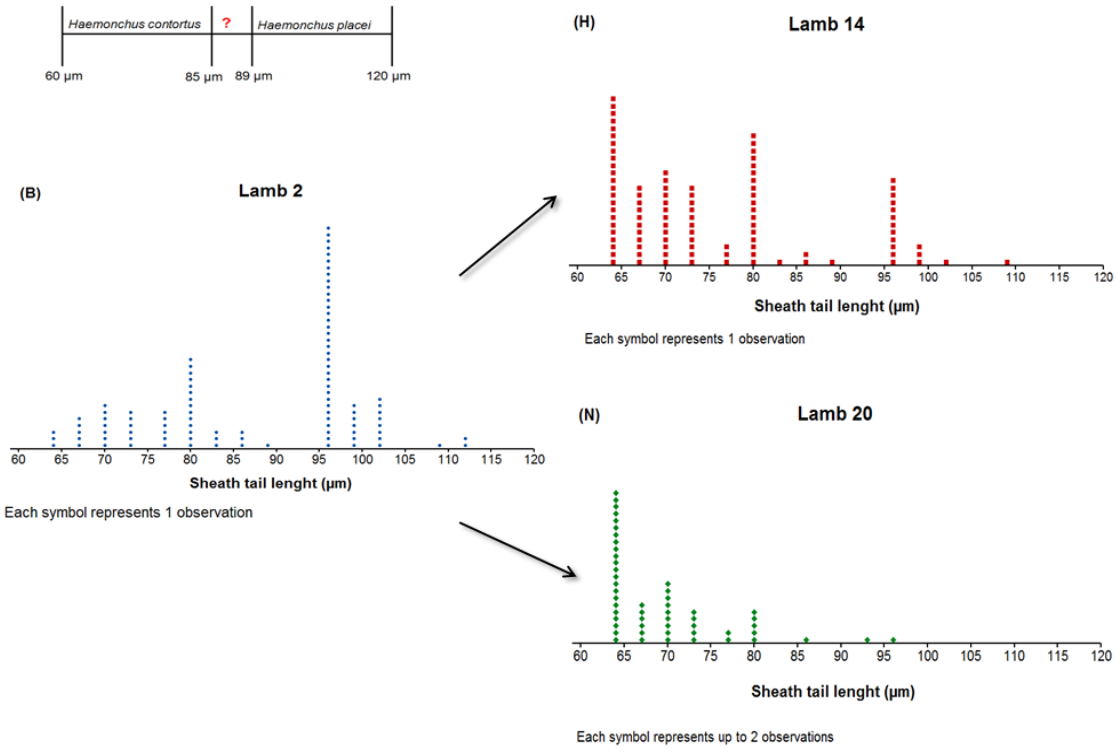
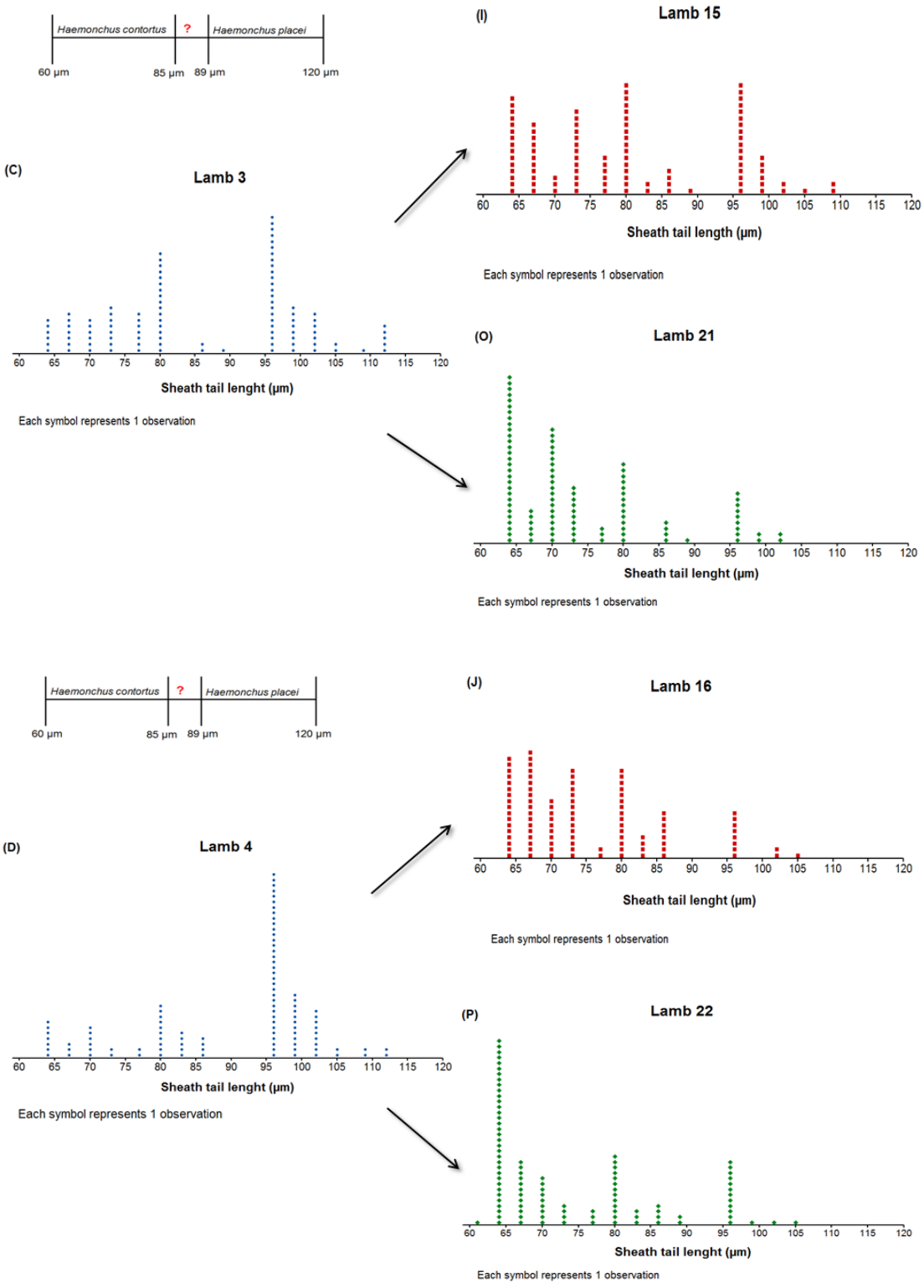



Figure 3. Dot plot of sheath tail length (µm) of third stage larvae (L3) of *Haemonchus contortus* from the donor lamb (A) and *Haemonchus placei* from the donor calf (B). The sheath tail length comprises the distance between the tip of the larval tail and the end of the sheath tail of the L3.


Haemonchus contortus
?
Haemonchus placei
60 µm
85 µm
89 µm
120 µm
(A)
Lamb 1
Sheath tail lenght (µm)
Each symbol represents 1 observation
(G)
Lamb 13
Sheath tail lenght (µm)
Each symbol represents 1 observation
(M)
Lamb 19
Sheath tail lenght (µm)
Each symbol represents up to 2 observations


Haemonchus contortus
?
Haemonchus placei
60 µm
85 µm
89 µm
120 µm
(B)
Lamb 2
Sheath tail lenght (µm)
Each symbol represents 1 observation
(H)
Lamb 14
Sheath tail lenght (µm)
Each symbol represents 1 observation
(N)
Lamb 20
Sheath tail lenght (µm)
Each symbol represents up to 2 observations


Haemonchus contortus
?
Haemonchus placei
60 µm
85 µm
89 µm
120 µm
(C)
Lamb 3
Sheath tail lenght (µm)
Each symbol represents 1 observation
(I)
Lamb 15
Sheath tail length (µm)
Each symbol represents 1 observation
(O)
Lamb 21
Sheath tail lenght (µm)
Each symbol represents 1 observation
(D)
Lamb 4
Sheath tail lenght (µm)
Each symbol represents 1 observation
(J)
Lamb 16
Sheath tail lenght (µm)
Each symbol represents 1 observation
(P)
Lamb 22
Sheath tail lenght (µm)
Each symbol represents 1 observation

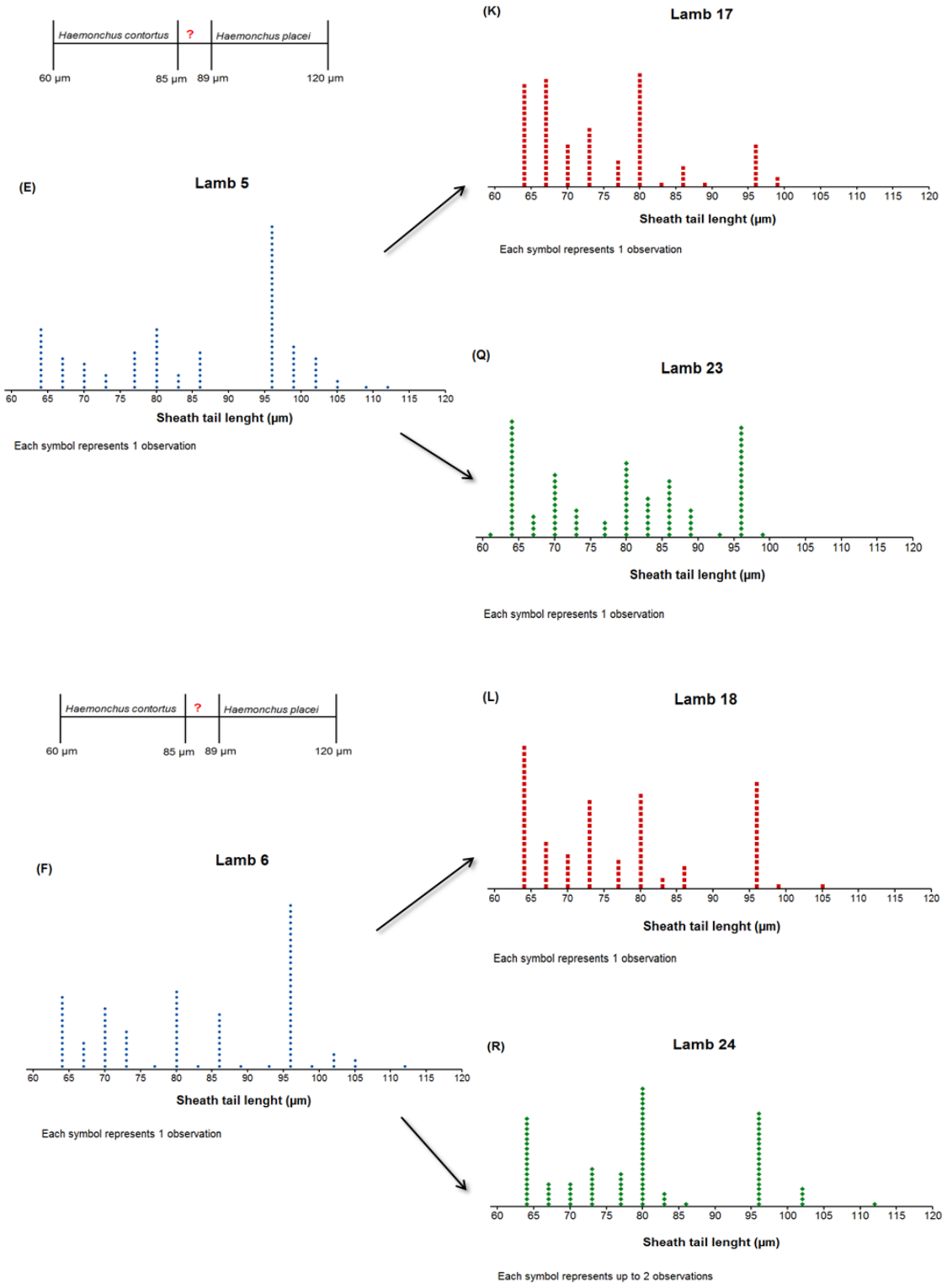



Figure 4. Dot plot of sheath tail length (µm) of third stage larvae (L3) of the parental group (A to F) infected with 2,000 infective larvae (L3) of *Haemonchus placei* (day 0) and 11 days later received 2,000 L3 of *Haemonchus contortus*. The first filial generation (F1) of L3 produced by parental lambs has been used to infect the animals from group F1-42 DPI (G to L) euthanized 42 days post-infection (DPI) and group F1-84 DPI (M to R) euthanized 84 DPI.

Table 1. Worm burden and identification of *Haemonchus* specimens by PCR. The parental lambs (1-6) were infected with 2,000 infective larvae (L3) of *Haemonchus placei* (Hp), on day zero and 11 days later received 2,000 L3 of *Haemonchus contortus* (Hc). The first filial generation (F1) of L3 produced by parental lambs has been used to infect the animals from group F1-42 DPI (lambs 13 to 18) euthanized 42 days post-infection (DPI) and group F1-84 DPI (lambs 19 to 24) euthanized 84 DPI. The parental animals were euthanized 50 days post *H. placei* infection.

| Group | Animal number | Infected with | Total number of adults | | Specimens identification by PCR | | |
|---|---|---|---|---|---|---|---|
| | | | Males | Females | Hc | Hp | Hybrid |
| Parental | 1 | 2,000 L3 Hc + 2,000 L3 Hp | 208 | 217 | 9 | 1 | 0 |
| | 2 | 2,000 L3 Hc + 2,000 L3 Hp | 1850 | 2047 | 3 | 7 | 0 |
| | 3 | 2,000 L3 Hc + 2,000 L3 Hp | 452 | 652 | 7 | 3 | 0 |
| | 4 | 2,000 L3 Hc + 2,000 L3 Hp | 778 | 792 | 3 | 7 | 0 |
| | 5 | 2,000 L3 Hc + 2,000 L3 Hp | 118 | 400 | 4 | 6 | 0 |
| | 6 | 2,000 L3 Hc + 2,000 L3 Hp | 164 | 140 | 5 | 5 | 0 |
| F1-42 DPI | 13 | 4,000 F1 L3 from lamb 1 | 952 | 1027 | 19 | 1 | 0 |
| | 14 | 4,000 F1 L3 from lamb 2 | 1142 | 1171 | 10 | 10 | 0 |
| | 15 | 4,000 F1 L3 from lamb 3 | 991 | 1063 | 9 | 10 | 1 |
| | 16 | 4,000 F1 L3 from lamb 4 | 1745 | 1479 | 6 | 14 | 0 |
| | 17 | 4,000 F1 L3 from lamb 5 | 1222 | 1223 | 4 | 13 | 3 |
| | 18 | 4,000 F1 L3 from lamb 6 | 1223 | 1132 | 12 | 7 | 1 |
| F1-84 DPI | 19 | 4,000 F1 L3 from lamb 1 | 582 | 601 | 20 | 0 | 0 |
| | 20 | 4,000 F1 L3 from lamb 2 | 60 | 11 | 12 | 5 | 0 |
| | 21 | 4,000 F1 L3 from lamb 3 | 526 | 572 | 10 | 8 | 2 |
| | 22 | 4,000 F1 L3 from lamb 4 | 1261 | 1182 | 3 | 17 | 0 |
| | 23 | 4,000 F1 L3 from lamb 5 | 691 | 633 | 4 | 15 | 1 |
| | 24 | 4,000 F1 L3 from lamb 6 | 1 | 1 | 2 | 0 | 0 |
| Donor | Calf | Hp | 465 | 610 | 0 | 20 | 0 |
| Donor | Lamb | Hc | 904 | 930 | 20 | 0 | 0 |

The donor calf and the donor lamb were euthanized, respectively, 157 and 188 days post-infection.

Table 2. Agreement between PCR and discriminate function analysis for the diagnosis of *Haemonchus contortus* and *Haemonchus placei* male worms obtained from the parental group.

| | | PCR | | |
|---|---|---|---|---|
| | | *H. contortus* | *H. placei* | Total |
| *Discriminate function | *H. contortus* | 26 | 1 | 27 |
| | *H. placei* | 4 | 28 | 32 |
| | total | 30 | 29 | 59 |

Kappa coefficient: 0.8308 (95% lower confidence limit = 0.6896 and 95% upper confidence limit = 0.9719)

*Discriminate function calculated based on descriptions by Achi et al. (2003).

Table 3. Agreement between PCR and discriminate function analysis for the diagnosis of *Haemonchus contortus* and *Haemonchus placei* male worms obtained from the first filial generation (F1) groups.

| | | PCR | | |
|---|---|---|---|---|
| | | *H. contortus* | *H. placei* | Total |
| *Discriminate function | *H. contortus* | 45 | 1 | 46 |
| | *H. placei* | 12 | 49 | 61 |
| | total | 57 | 50 | 107 |

Kappa coefficient: 0.7592 (95% lower confidence limit=0.6391 and 95% upper confidence limit=0.8793)

*Discriminate function calculated based on descriptions by Achi et al. (2003).

Table 4. Morphometry of four *Haemonchus* hybrids recovered from animals from groups F1-42 DPI (euthanized 42 days post-infection, DPI) or F1-84 DPI (84 DPI). These animals were infected with 4,000 first filial generation (F1) of infective larvae (L3) from the parental group. After the morphological analyses, the PCR were performed (primers HcBotuF1 and HcBotuR2and HpBotuF and HpBotuR).

| Lamb number | 15 | 17 | 18 | 21 |
|---|---|---|---|---|
| Group | F1-42 DPI | F1-42 DPI | F1-42 DPI | F1-84 DPI |
| Infected from parental lamb number | 3 | 5 | 6 | 3 |
| Parasite number | 1 | 10 | 8 | 4 |
| Body length (mm) | 17 | 16 | 16 | 17 |
| Spicule length (µm) | 442.1 | 454.7 | 454.7 | 442.1 |
| Right spicule barb length* (µm) | 47.9 | 44.7 | 47.9 | 41.5 |
| Left spicule barb length* (µm) | 19.1 | 22.3 | 25.5 | 22.3 |
| Discriminate function (Achi et al., 2003) | -0.224 | -0.128 | 0.766 | -0.556 |
| Morphological classification | *H. contortus* | *H. contortus* | *H. placei* | *H. contortus* |

*Distances from spicules barbs to distal tips of spicules.

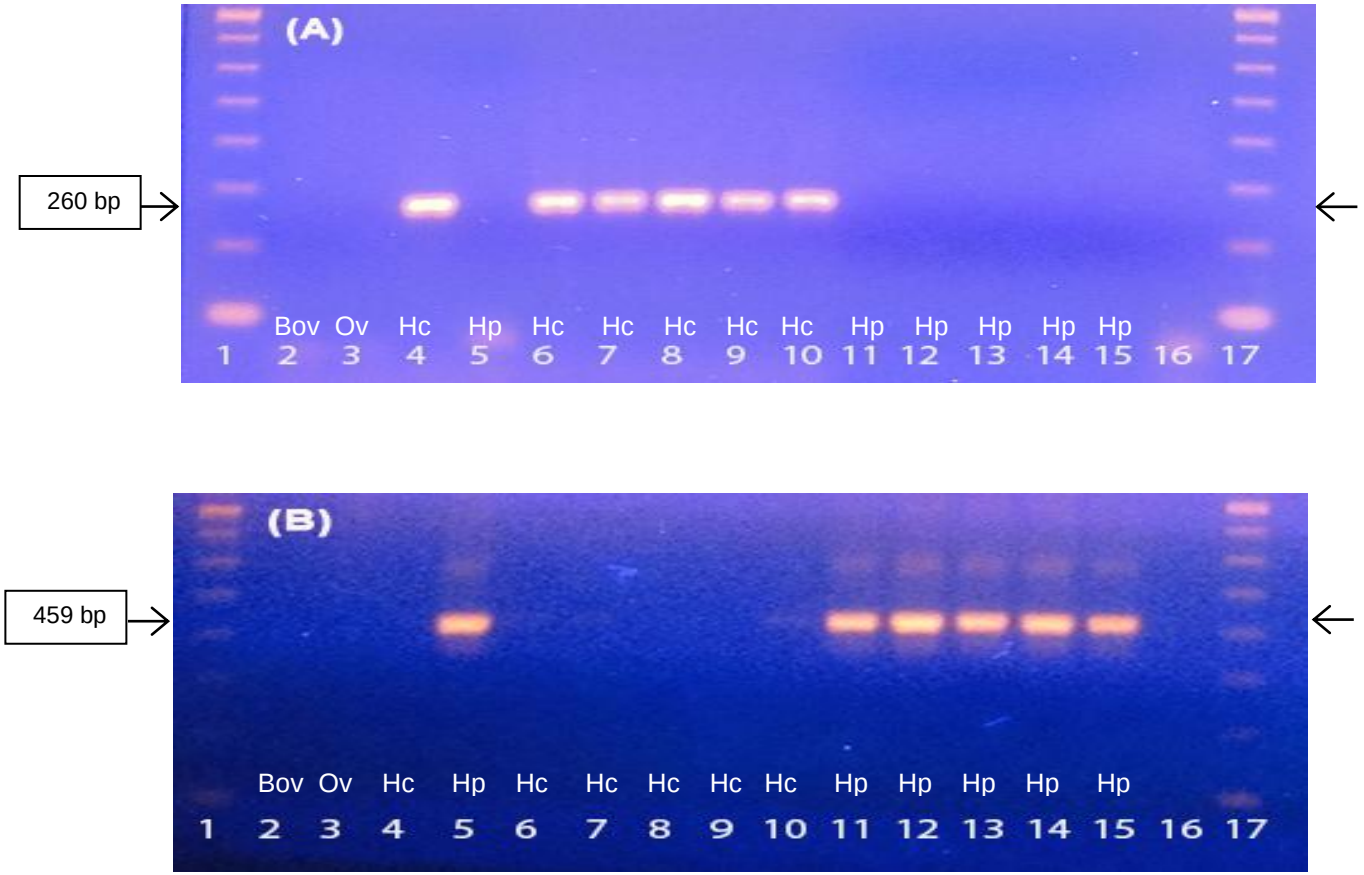



Figure 6. PCR reactions with the primers HcBotuF1/R2 for *Haemonchus contortus* (A), and HpBotuF/R for *Haemonchus placei* (B). Lines 1 and 17 show 100 bp molecular markers (GE Healthcare); 2 and 3 – bovine and ovine DNA, respectively (hosts); 4 – *H. contortus* (control); 5 – *H. placei* (control*);* 6 to 10 – *H. contortus* samples and 11 to 15 *H. placei* samples and 16 - reagents without DNA.

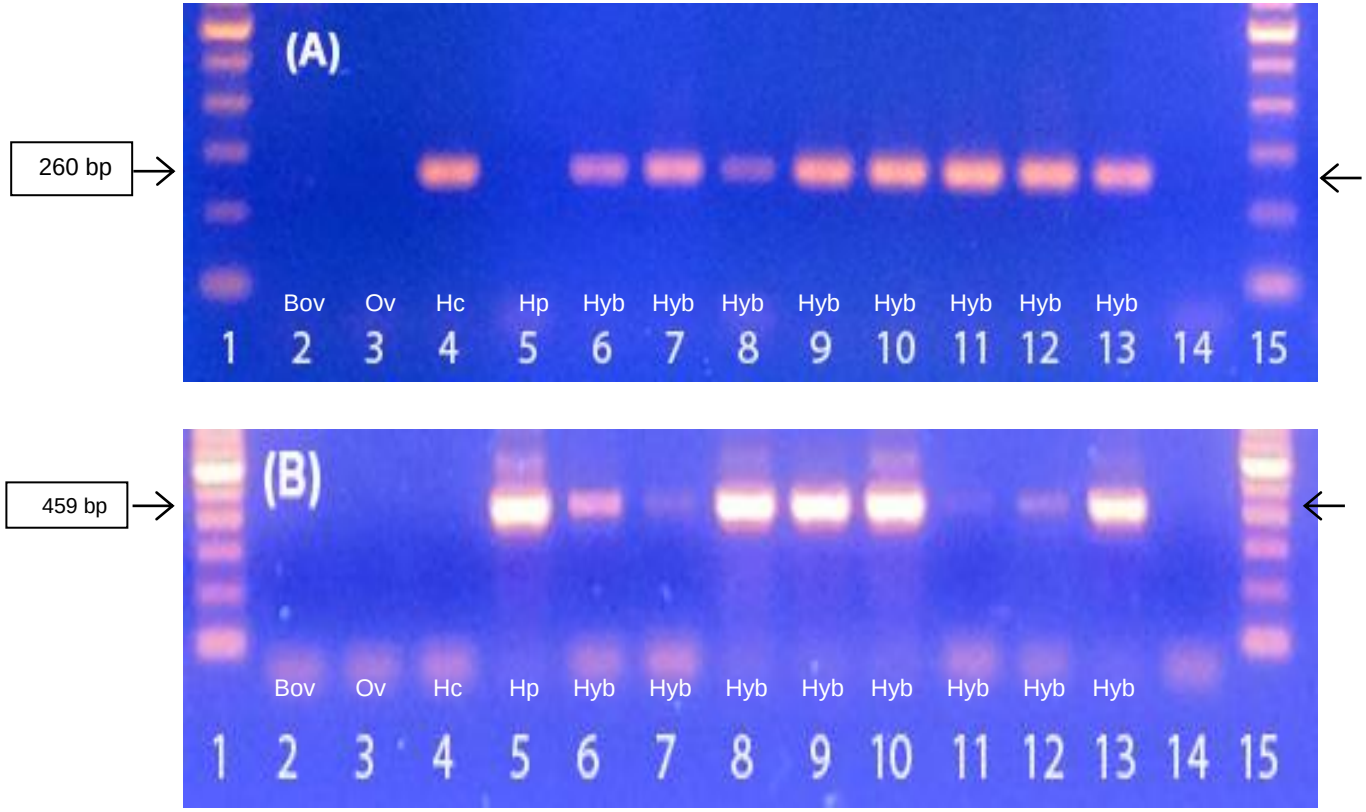



Figure 7. PCR reactions with the primers HcBotuF1/R2 for *Haemonchus contortus* (A), HpBotuF/R for *Haemonchus placei* (B) and DNA samples. Lines 1 and 15 show 100 bp molecular markers (GE Healthcare); 2 and 3-bovine and ovine DNA, respectively (hosts); 4 – *H. contortus* (control); 5 - *H. placei* (control*);* 6 – 13 hybrids from groups F1-42 DPI and F1-84 DPI and 14 - reagents without DNA.

# Capítulo 3

*Manuscrito redigido de acordo com as normas do periódico Veterinary Parasitology.

**Hybrids production from sheep artificially infected with *Haemonchus placei* and *Haemonchus contortus* with later disappearance of *Haemonchus placei***

**Abstract**

This study aimed to evaluate the dynamics of *Haemonchus contortus* and *Haemonchus placei* infections and crossing between these species in sheep grazing without contact with cattle. On January 14, 2014 (day zero), sixteen sheep were infected with 4,000 L3 of *H. placei;* 11 days later another group (n=16) was infected with 4,000 L3 of *H. contortus*. The animals were kept indoors and 35 days post-infection (DPI) with *H. placei* all sheep started to share the same clean pasture. In order to better evaluate the L3 population on pasture, worm-free tracer sheep were introduced on the pasture every three months. The initial establishment of the infection was confirmed by worm counting from group euthanized on February 24, 2014 and subsequent euthanasias were performed every three months. The last five animals of each group were euthanized a year later (February 2015). The parasites were recovered and identified using morphological and molecular techniques. The L3 morphological evaluation showed that the *H. placei* infection was progressively replaced by *H. contortus* populations starting in March, 2014. By September, 2014 until February (2015), no L3 with *H. placei* measures were found. These results were confirmed by molecular evaluation of the adult specimens. In addition, two hybrids specimens were recovered during this trial, one was recovered from the experimental animal euthanized in August (2014) and the other from the tracer animal that grazed in May 2014. In conclusion, the hybridization between *H. contortus* and *H. placei* can occur on the field and hybrids may be produced during co-infection by *H. contortus* and *H. placei*. Although *H. placei* species can establish in sheep, the natural reinfection with *H. contortus* provides the *H. placei* elimination in less than 12 months.

Keywords: cross-infection, gastrointestinal nematodes, specificity, sheep.

## 1. Introduction

Although *Haemonchus placei* had bovine as a preferential host, in artificial infections *Haemonchus contortus* (preferential parasite from ovine) can establish in cattle in sheep (Bassetto et al., 2011) and the opposite can occur in sheep (Santos et al., 2014a). When small ruminants share pasture with cattle, mixed infections can occur (Le Jambre and Royal, 1980; Amarante et al., 1997; Jacquiet et al., 1998; Achi et al, 2003; Hoberg et al., 2004), although over time the host apparently eliminate the parasite species that is not well adapted to them (Amarante et al., 1997). Sheep repeatedly exposed to *H. placei* artificial infections acquires strong resistance against this parasite, showing an intense immune response that leads to a low rate of parasite establishment (Santos et al., 2014a).

The morphological analyses are easily low-cost applied technique, however, the overlap between measurements of *H. placei* and *H. contortus* can occur, casting doubt upon the correct identification (Amarante, 2011).

Molecular techniques used for differentiation of *H. contortus* and *H. placei* may be based on the analysis of mitochondrial DNA (mtDNA) NADH dehydrogenase subunit 4 (ND4) gene (Blouin et al., 1995; 1997), small subunit (SSU) of ribossomal DNA (rDNA) external-spacer region (Zarlenga et al., 1994), second internal transcribed spacer (ITS-2) of rDNA (Stevenson et al., 1995; Brasil et al., 2012), and genotyping using microsatellites markers (Redman et al., 2008; Chaudhry et al., 2015).

Interspecific crossing with the production of hybrids have been reported in mixed infections (natural or artificial) with *H. contortus* and *H. placei* (Bremner, 1955; Le Jambre, 1979; 1981; Brasil et al., 2012, Chaudhry et al., 2015; Santos et al., unpublished). According to Le Jambre (1981) in artificial backcrossing males and females hybrids can be semi-sterile, possibly perpetuating a low level of reproductive efficiency within the population.

Thus, this study aimed to evaluate the survival of a *H. placei* population competing

with *H. contortus* in grazing sheep without contact with cattle and the production of hybrids under natural conditions of infection.

## 2. Material and Methods

The trial was approved and conducted in accordance with the experimental protocol approved by the local Ethical Committee (protocol number 449-CEEA-UNESP, Institute of Biosciences, Brazil).

Production of *Haemonchus placei* and *Haemonchus contortus* infective larvae

Infective larvae production of *H. placei* (SpHpl1 laboratory isolate) and *H. contortus* (SpHco2 laboratory isolate) described by Santos et al. (*unpublished*). *Haemonchus contortus* was used to infect a donor sheep while *H. placei* was used to infect a donor calf. Fecal cultures were performed separately for the production of L3, which were stored in tubes with 20 and 25 mL with distilled water Santos et al. (*unpublished*). Then, these isolates were maintained in Suffolk male sheep, with fifteen months old. These animals were kept indoors and before being infected, they received monepantel (2.5 mg/kg, Zolvix®, Novartis Animal Health) in a single dose, in order to eliminate any natural strongyle infection. After the confirmation of the worm-free status two animals received 4,000 L3 of *H. contortus* (SpHco2) and the other two were infected with 4,000 L3 of *H. placei* (SpHpl1). Their worm-free status was confirmed before the infection by a series of fecal examinations. They were kept in separate pens to avoid cross-contamination and, during the trial they had free access to tap water and hay grass (*Cynodon dactylon* cv. Tifton 85), purchased from a farm with no ruminants, avoiding risks of food contamination with nematode infective larvae. Fecal cultures from each of these sheep were performed separately for the production of L3, used to infect the experimental sheep.

2.1. Experimental design

The trial design is summarized in Fig. 1. The trial was carried out with 32 male Suffolk lambs. All of the lambs were acquired from a commercial farm located in Paranapanema, Sao Paulo state, Brazil just after weaning (2-3 months of age) with the average body weight of 19 Kg.

The animals were placed in pens with a concrete floor in the facilities for small ruminants of the University of Sao Paulo State and all animals were vaccinated against clostridiosis (Sintoxan Polivalente T®, Merial, Brasil) and received treatment with monepantel (2.5 mg/kg, Zolvix®, Novartis Animal Health), in a single dose. After treatment, the determination of their worm-free status was based on a series of fecal examinations.

Thirty-two animals were randomly distributed into two groups and were artificially infected orally in a single dose with 4,000 L3 of either *H. contortus* or *H. placei*. The group *H. placei* (n=16) was infected on January 14, 2014 (day zero) and the group *H. contortus* (n=16) was infected 11 days later on January 25, 2014. This period between the infections was planned to allow both species getting the patency at the same time (Riggs, 2001; Santos et al., 2014a; Santos et al., unpublished).

All animals were kept indoors and 35 days after the *H. placei* infection (February 18, 2014), they were allocated together in the same pasture of *Brachiaria decumbens* and *Cynodon* spp. (UNESP, Botucatu - Sao Paulo) where they remained until the end of the trial. The pasture was considered worm-free because it had not been grazed by ruminants for a long period of time. Therefore, the environmental contamination and subsequent re-infections were exclusively derived from *Haemonchus* eggs shed by the experimental animals.

The animals had free access to water and mineral salt, the diet consisted of the grass and daily supplementation with concentrate (Suplementa Ovino Campo® - Presence, with 17% of crude protein (CP)). Initially, each animal received 100 g of concentrate/day. Because

the sheep showed a decrease in packed cell volume (PCV) percentual associated with high nematode fecal egg counts (FEC), in order to improve their resilience, it was decided to increase the amount of commercial concentrate to an amount corresponding to 1.5% of their body weight until the day 126 post-infection. This allowed a recovery in the PCV percentual, which was associated, however, with a decrease in FEC. For this reason and to avoid the elimination of the parasites, the animals return to receive 100 g/animal per day. During the dry season, from May until November, hay grass (*Cynodon dactylon* cv. Tifton 85 with 8% CP) was added to the diet (1-2 Kg/animal per day) and after this period, with the start of the rainy season, the animals were fed exclusively with the pasture available in the paddock.

*Necropsy of the experimental sheep*

To evaluate the establishment of the infection, two animals of each experimental group were euthanized on February 24, 2014: 30 DPI with *H. contortus* and 41 DPI with *H. placei*. Subsequently, three animals of each group were euthanized every three months, to recovery, identification and quantification of the worms (Fig. 1). In the last sampling day of the following year (February 2015), the five remaining animals of each group were euthanized.

*Tracer animals*

The experimental animals re-infected constantly with the L3 during grazing, which might induce the development of the immune response, with a consequent reduction on establishment of the parasites (Santos et al., 2014a). In order to better evaluate the L3 population on pasture, worm-free tracer sheep were also used. Two tracer animals were introduced on the pasture, together with the experimental animals where they grazed for two weeks (Fig. 1). Then, the tracer animals were housed for 20 days and at the end of this period,

they were euthanized for worm recovery, counts, and identification. The tracer lambs were placed on pasture 14 days before the date of the necropsy of the three experimental animals of each group (a total of seven tracer animals were used in the present study).

2.2. Laboratory analyses

Every 14 days blood and fecal samples were obtained to evaluate FEC. The feces were collected directly from the rectum of animals and the L3 production was prepared separately for each group. The larvae identification was based on the L3 sheath tail length, which comprises the measurement between the tip of the larval tail and the end of the sheath tail (Santos et al., 2014b).

*Worm burden*

The animals were fasted for 12 hours before being euthanized. The abomasum was opened along the greater curvature, and its content was placed in a graduated beaker and washed with saline solution so the parasites adhering to the mucosa could be detached from the organ. The volume was brought up to 1 L with saline solution, the content was homogenized and divided into two containers of 500 mL each. The containers were immediately frozen for the preservation of parasites. The mucosal layers of all abomasum were soaked in saline solution at 38°C for 4 hours. All content of the digested material was collected and frozen.

Worm counting and identification procedures were performed on 50% of the content of the frozen material. If no worms were found in that sample, all the remaining content was also carefully examined for worms. The juvenile forms of the parasites were morphologically identified and the distinction between adults (males and females) was carried out observing the presence or absence of the copulatory bursa (Ueno and Gonçalves, 1998).

For *Haemonchus* species morphological evaluation, 10 adult males were randomly chosen from each experimental and tracer animals. The morphometry of the spicules was used to calculate the discriminate function (DF) (Jacquiet et al., 1998), previously described by Santos et al. (2014b). Briefly, the specimens were placed on a glass slide and with an insulin syringe needle, the worms were cut near the copulatory bursa. After the measurement of spicules, the anterior region of the body of the parasite was individually stored in 1.5 mL microtubes, previously identified. Each Eppendorf tube had 150 µL of ultrapure water and immediately frozen to be used for the DNA extraction. When the animal had less than 10 adult males, females and/or juveniles were used in order to complete 10 samples per sheep.

*Molecular evaluation*

These analyses aimed to identify the *Haemonchus* species and evaluate the hybrid production. When possible, 10 adult males from each experimental animal were collected for measurement of spicules and DNA extraction. From each tracer sheep, 20 adult males were used (10 with morphometry plus 10 males submitted only to molecular analysis).

Genomic DNA was extracted from each *Haemonchus* specimen using a QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. DNA from the cattle and sheep blood samples was also extracted to be used as controls in PCR analysis.

The species-specific primer pair for *H. contortus* (HcBotuF1/R2) showing an amplification band with approximately 260 base pair (~260 bp) and a species-specific primer pair to *H. placei* (HpBotuF/R) that resulted in an amplification band with approximately 459 base pair (~459bp) (Amarante et al., 2017) were employed for species identification.

The PCR products were electrophoresed on 2% agarose gel in 1% TAE buffer containing ethidium bromide and photographed under UV light using a Sony Cyber-shot DSC-HX1 camera (Sony Electronics, San Diego, California, USA).

2.3. Statistical analyses

The Dot plots of the measures of the sheath tail length (distance between the tip of the larval tail and the end of the sheath tail) were performed using the Minitab® software, version 17 (2013). The results of molecular analyses were presented descriptively. The results of morphologic and molecular diagnostics were compared by McNemar's test ($p<0.05$), and the Kappa statistics (coefficient of agreement) was calculated using the Statistical Analysis System, version 9.2 (SAS® Institute, Inc., Cary, NC, USA).

## 3. Results

3.1. Fecal evaluations

A peak in the FEC occurred at 35 DPI (February 18, 2014) in both groups 7,063 eggs per gram (EPG) (±1,250) on the group initially infected with *H. contortus* and 6,394 EPG (±847) at group initially infected with *H. placei*. It was also observed a reduction on PCV levels from 36% (±0.79) on day 0 to 24% (±0.76) at 70 DPI on *H. contortus* group and from 35% (±0.73) on day 0 to 23% (±0.91) at 70 DPI on *H. placei* group, for this reason the sheep received concentrate (17% CP) at 1.5% of their body weight per animal daily from day 70 until day 126 post-infection (March 25, 2014 until May 20, 2014). Consequently, on 140 DPI (June 03, 2014), it was observed an increase in PCV levels on both groups 1,855 cells/µL (±431) and 31% (±1.8) for *H. contortus* and 2,836 cells/µL (±569) and 34% (±0.73) for *H. placei* (data not shown).

3.2. Sheath tail length of infective larvae

*Donor animals*

One hundred L3 sheath tail length were measured from each *H. contortus* and *H. placei* donor sheep (Fig. 2). The *H. contortus* donor animals presented L3 with the maximum sheath tail length of 79.79 µm and the minimum of 60.64 µm, with an average of 66.32 µm (±0.46). The *H. placei* larvae from the donor sheep showed maximum and minimum measures of 105.32 µm and 86.17 µm, respectively, and the mean of the sheath tail was 95.55 µm (±0.36).

*Experimental animals*

Throughout the trial, measurements were taken from 200 L3 of each group on each sampling day. In some fecal cultures that produced less than 200 *Haemonchus* L3, all larvae present was measured.

The establishment of the artificial infection with *H. contortus* was confirmed by the presence of L3 with *H. contortus* measures in February 2014 (Fig. 3A). This group presented some L3 with *H. placei* measures (above 86 µm) from March to September (2014). Then, L3 with *H. placei* measures were no longer recovered, i.e., they were absent from October 2014 until the end of the trial, in February 2015 (Fig. 3B to M).

As expected, the group initially infected with *H. placei* started the trial in February (2014) with larvae displaying *H. placei* measures. However, in March (2014), it was possible to observe L3 with *H. contortus* measures (60 to 85 µm), although the *H. placei* larvae percentage was still high (Fig. 3O). In the following months, *H. contortus* larvae predominated and from September (2014) until February (2015), L3 with *H. placei* measures were no longer found (Fig. 3P to Z).

3.3. Worm burden, morphological, and molecular evaluation of *Haemonchus* specimens

*Experimental animals*

The highest worm burdens were observed on the first euthanasia: means of 2,466 ± 262.50 and 3,235 ± 634.50 in the groups initially infected with *H. contortus* and *H. placei*, respectively (Table 1). On the second euthanasia in May, two animals showed high total worm burden: one animal firstly infected with *H. contortus* (11,282 worms) and another of the group firstly infected with *H. placei* (2,503 worms). In contrast, one of the sheep did not present worms (Table 1). Over the experimental period, the worm burden from both experimental groups decreased, except on the last euthanasia (February 11, 2015) when one animal from the group *H. contortus* showed high total worm burden (18,755).

Considering 272 worms analysed by PCR with bands of identification, only 21 (7.72%) were *H. placei* specimens and showed amplification bands of ~459 bp. These specimens were recovered exclusively on February and March (2014) from sheep that received initially artificial infection with *H. placei* (Table 3, Fig. 4A and B). The remaining, 250 (90.28%) were identified as *H. contortus* and presented PCR products of ~260bp. Only one specimen was identified as hybrid (0.37%) and showed both ~260 and ~459 bp amplification bands. That specimen was recovered from one sheep initially infected with *H. contortus* and euthanized on August 2014 (Table 3). In addition, three specimens showed no amplification bands with both primers.

*Tracer animals*

Worm-free sheep (tracer animals) were introduced together with the flock every three months from May 2014 until the end of the trial in February 2015. They always acquired

*Haemonchus* infection, proving the presence of *Haemonchus* L3 on the pasture throughout the trial (Table 2).

Considering 160 specimens recovered from the tracer sheep and evaluated by PCR analyses, 140 (87.5%) were identified as *H. contortus* and showed ~260 bp amplification bands; 19, (11.88%) as *H. placei*, and presented PCR products of 459 bp; and only one specimen (0.63%) was identified as hybrid, presenting both ~260 and ~459 bp amplification bands (Table 4, Fig. 5A and B). Only tracer animals that grazed in May acquired *H. placei* infection.

The agreement between morphometry of adult males and PCR techniques for species identification was 95.1% (293 of a total of 308 specimens, Kappa coefficient = 0.7503) (Table 5).

## 4. Discussion

In artificial infections, *H. placei* established very well in young sheep and *H. contortus* in young cattle (Santos et al., 2014a; Fávero et al., 2016). However, in the present study, under natural re-infection over time in the grazing system without cattle, it was observed a fast decrease in *H. placei* population. *Haemonchus placei* L3 were not recovered from the pasture from June (2014) until the end of the trial (February 2015), while *H. placei* L3 were not observed in fecal cultures from the experimental animals since September (2014) until the last sampling (February 2015). Thus, in the group initially infected with *H. placei*, this worm population was gradually replaced by *H. contortus* becoming apparently extinct from the experimental site by the end of the trial. Several reasons may have caused the disappearance of *H. placei*, which include a role of the immune response in the host-parasite relationship. In comparison with *H. contortus*, a strong immune response was observed in sheep repeatedly infected with *H. placei* limiting the establishment of this species (Santos et al., 2014a).

Similarly, in experimental infections in sheep, Le Jambre (1983) observed 80% loss of *H. placei* when infection with this parasite was followed by dosing with *H. contortus*, showing the ability of *H. contortus* to exclude and dislodge *H. placei* from sheep. In sheep and cattle that share pastures in São Paulo state, it has been also demonstrated *Haemonchus* host-specificity (Amarante et al., 1997; Silva et al., 2015).

Possibly the development of the free-living stages is influenced by the moisture content of the host's dung. We observed, under controlled conditions (incubator) or on the pasture, during dry and rainy periods, that *H. placei* presented a lower rate of development with lower production of L3 in comparison with *H. contortus* (*unpublished data*). This fact may be associated with the adaptation of *H. placei* to develop in cattle dung that presents a higher content of moisture in comparison with the sheep feces pellets. This aspect of the biology of the free-living stages needs further investigation.

A total of 435 adult males (from experimental and tracer animals) were analysed by PCR using species-specific primers pairs. From these specimens, only two (0.46%) worms were identified as hybrids. These results corroborate the findings of the low percentage of hybrids in field studies reported by Brasil et al. (2012) and Chaudhry et al. (2015) and in artificial infections on Santos et al. (unpublished).

To identify the *Haemonchus* species, parasites were recovered and evaluated by morphology and PCR. In this trial, 4.9% adult male worms have been misidentified using only the morphological technique. These results were similar to the overlap between the measures as previously described by Amarante (2011), Santos et al (2014b) and Santos et al (unpublished).

In Botucatu (Brazil), the environmental conditions are highly favorable for the year-round transmission of *H. contortus* (Wilmsen et al., 2014). In our study tracer animals showed positive FEC and *Haemonchus* specimens were recovered in all periods. This result shows

that the employment of tracer animals is essential to accurately assess environmental contamination with L3. The introduction of worm-free (tracer) animals, when used together, allow obtaining consistent results in the evaluation of the seasonal variation of the L3 numbers on the pasture (Amarante and Barbosa, 1998).

Due to host-specificity adaptation (Amarante et al., 1997; Achi et al., 2003; Hoberg et al., 2004; Santos et al., 2014a; Zarlenga et al., 2016), *H. placei* population from the sheep flock has declined in a relatively short time period while *H. contortus* increased and dominated the parasitic fauna of experimental animals, demonstrating that the management systems using cattle and sheep could be an useful tool for the prevention of haemonchosis, corroborating with previous studies (Amarante et al., 1997; Fernandes et al, 2004; Torres et al., 2009; d'Alexis et al., 2012; Marshall et al., 2012; Brito et al., 2013).

*Haemonchus placei* established successfully in worm free sheep, however, under concomitant natural reinfection with *H. contortus*, the *H. placei* population is eliminated in less than 12 months from an environment without cattle. In addition, a few hybrids was produced during co-infection with *H. contortus* and *H. placei*, however further studies are necessary to evaluate a possible role of such parasites in the introgression of genes from *H. placei* to the *H. contortus* population.

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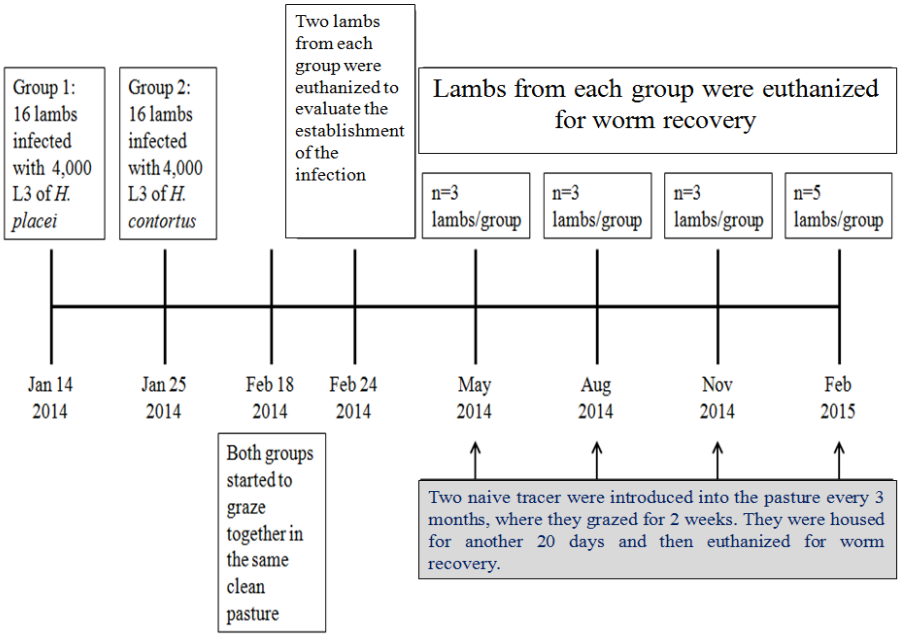



Figure 1. Experimental design: Sixteen worm-free sheep were artificially infected with *Haemonchus placei* (day 0, January 14, 2014) and another group (n=16) received *Haemonchus contortus* 11 days later (January 25, 2014). Animals shared the same pasture from February 18, 2014 until February 10, 2015. Every three months three animals were euthanized. Tracer animals were introduced together with the flock in May, August, and November (2014) and in February (2015).

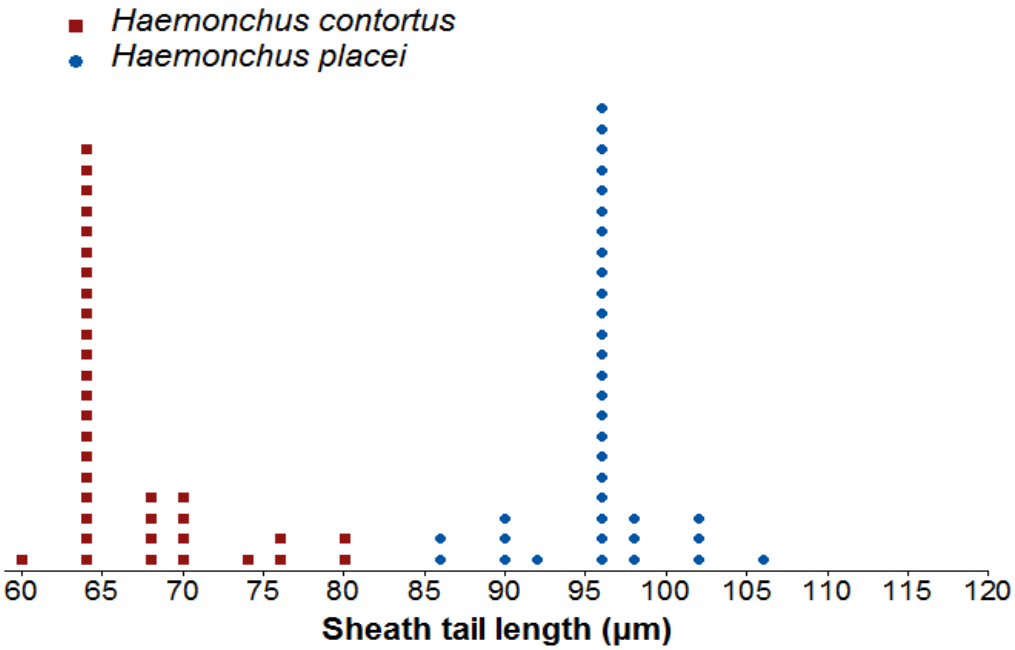
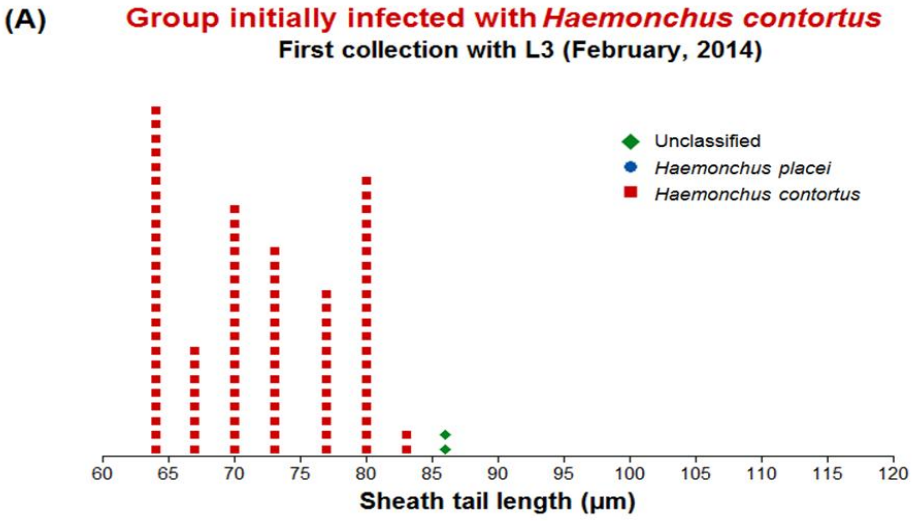
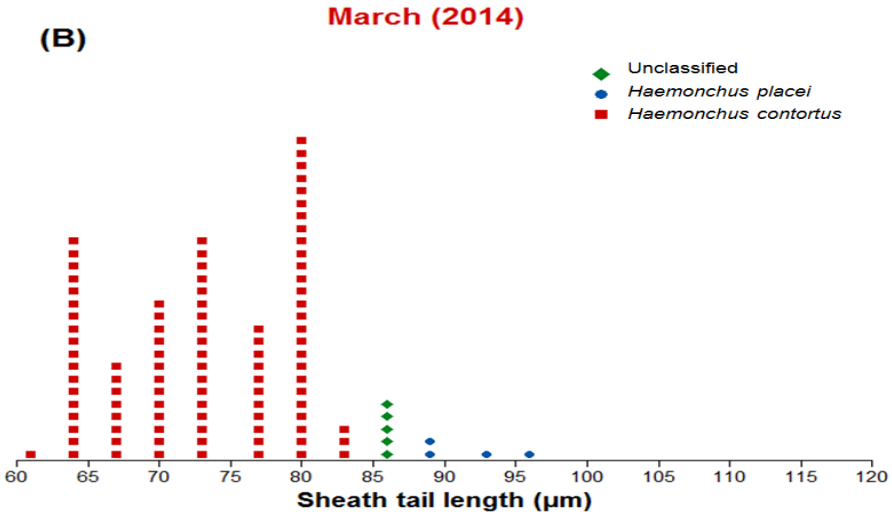


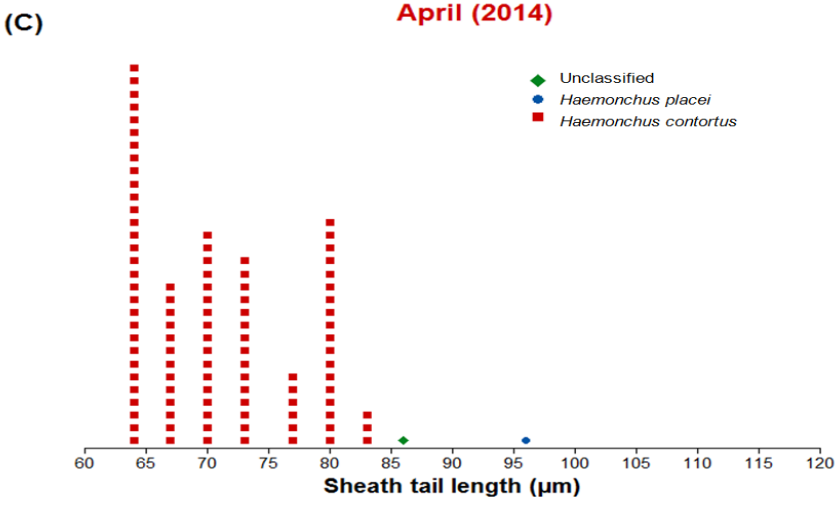

Figure 2. Dot plot of sheath tail length (µm) of third stage larvae (L3) of *Haemonchus contortus* sheep donor and *Haemonchus placei* sheep donor. The Sheath tail length comprises the distance between the tip of the larval tail and the end of the sheath tail of the L3. The L3 come from one donor sheep exclusively infected with *H. contortus* or *H. placei*. Each symbol represents up to three observations. Infective larvae with measures between 60µm to 85µm were identified as *H. contortus*, between 85.1µm to 88.9µm was not confirmed and between 89µm to 120µm as *H. placei*.




Each symbols represents up to 2 observations.

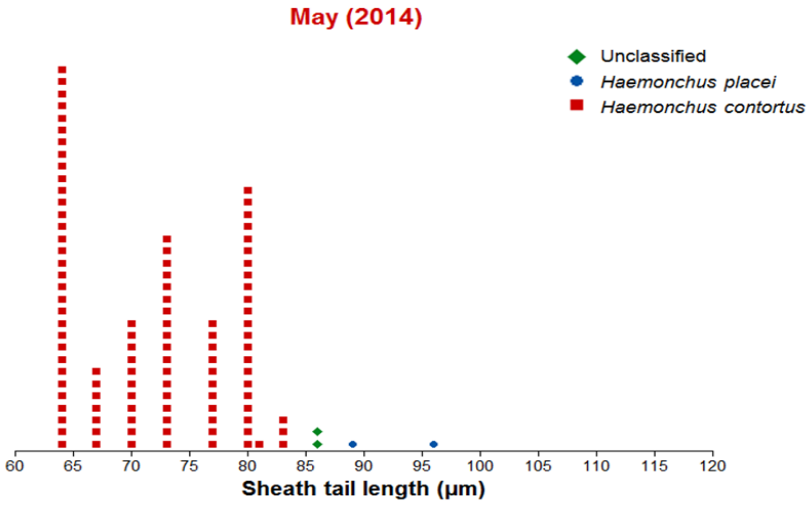



Each symbols represents up to 4 observations



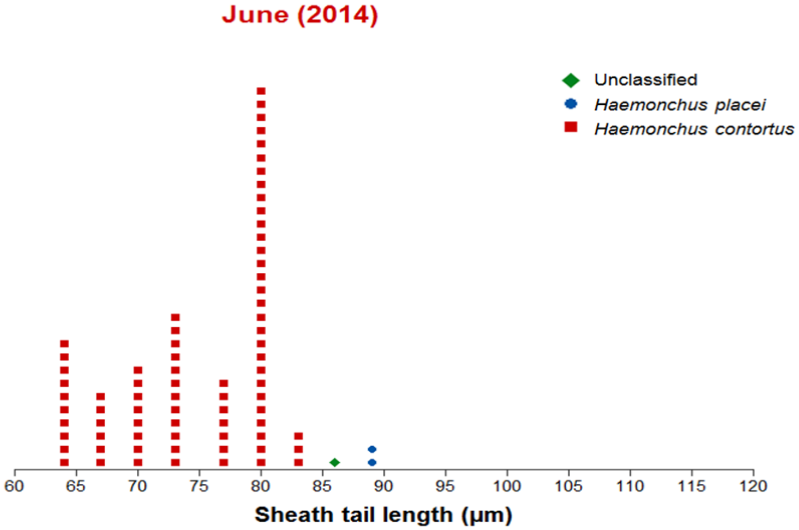

Each symbols represents up to 4 observations

(D)



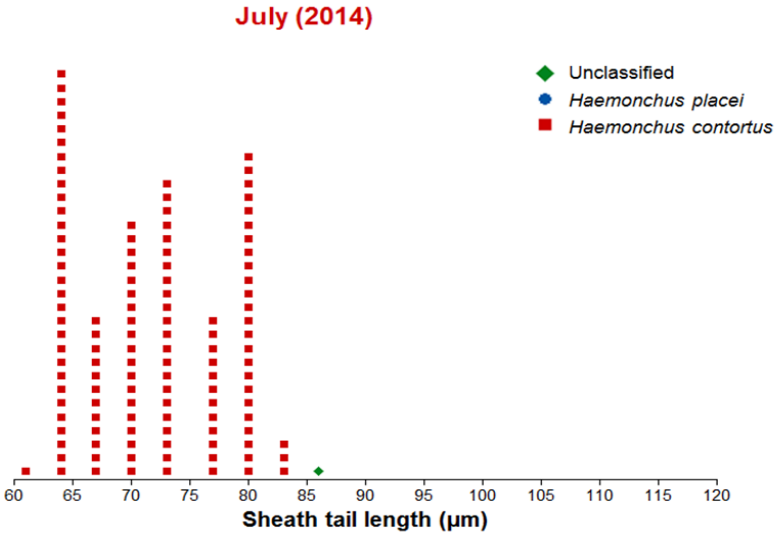

Each symbols represents up to 2 observations.

(E)



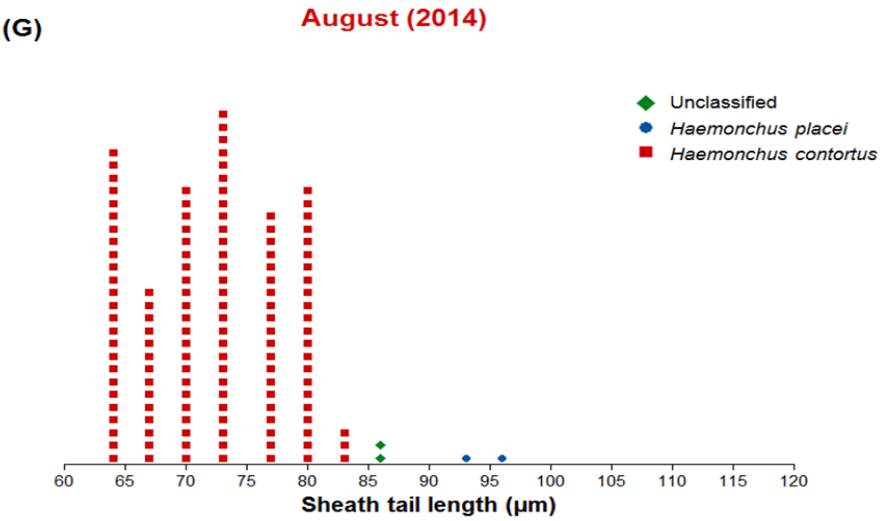

Each symbols represents 1 observation.

(F)



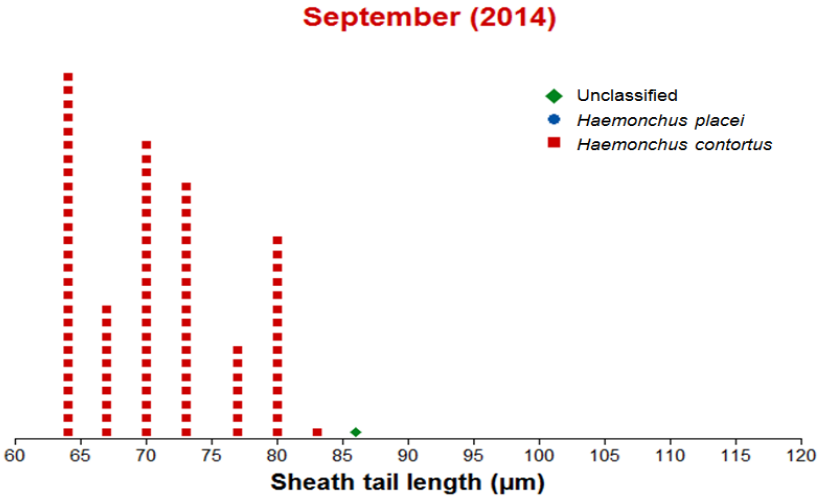

Each symbols represents up to 4 observations.

(G)



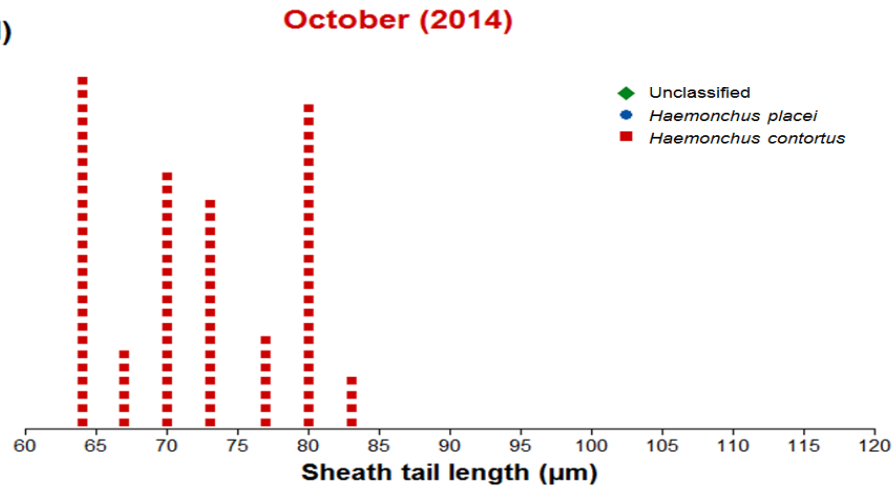

Each symbols represents up to 3 observations.

(H)



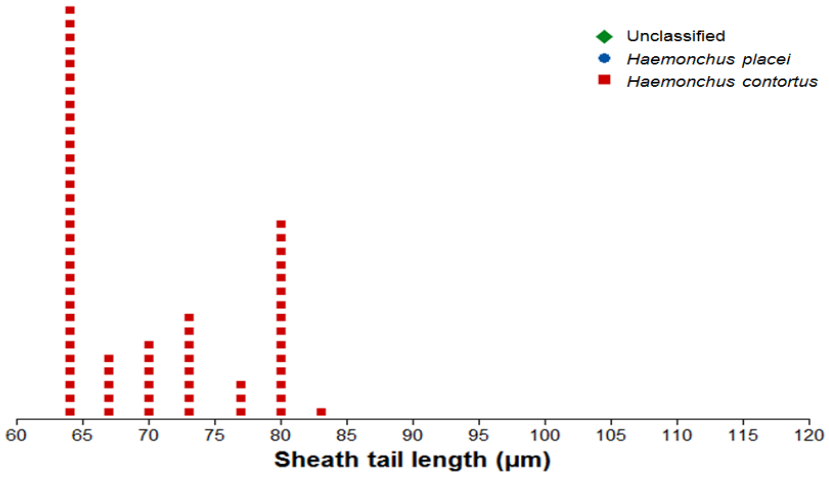

Each symbols represents up to 3 observations.

(I)



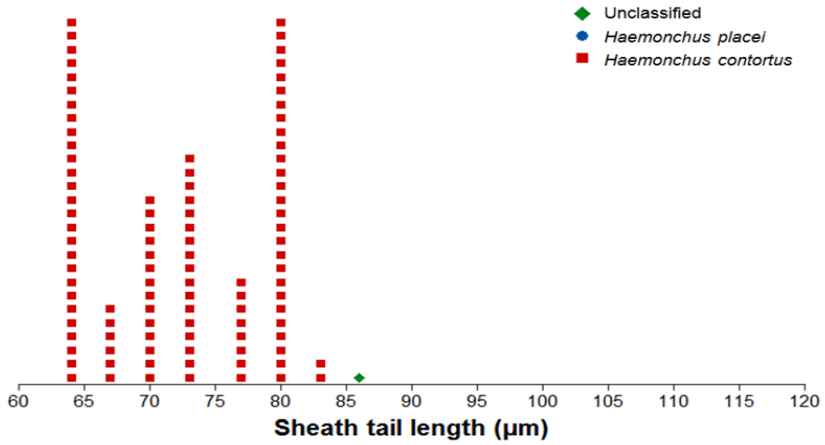

Each symbols represents up tp 3 observations.

(J) **November (2014)**



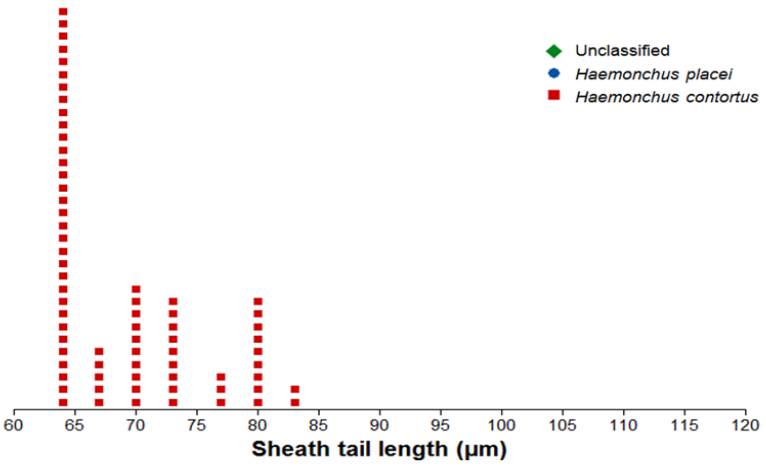

Each symbols represents up to 5 observations.

(K) **December (2014)**



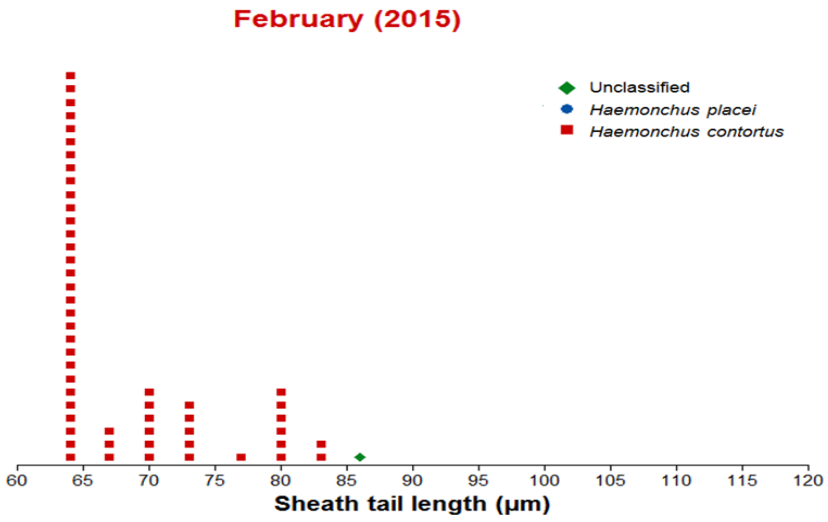

Each symbols represents up to 3 observations.

(L) **January (2015)**

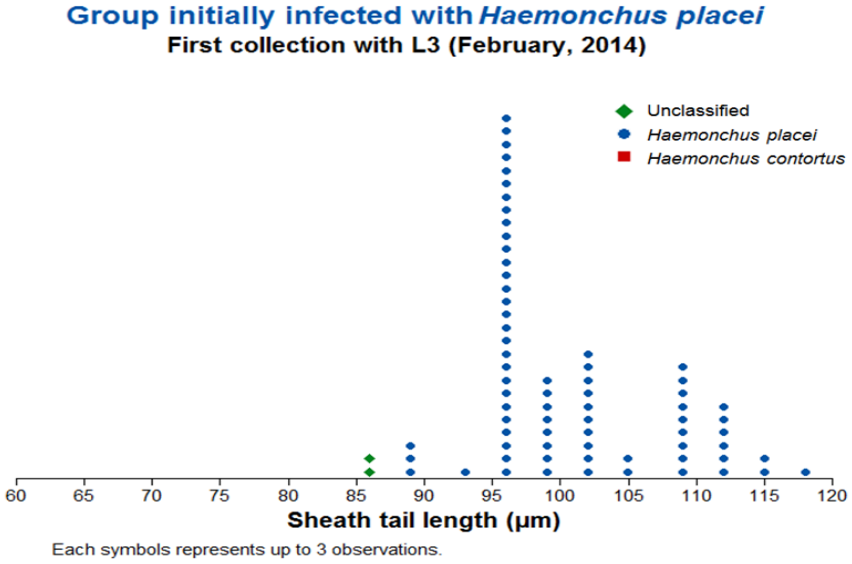



Each symbols represents up to 3 observations.

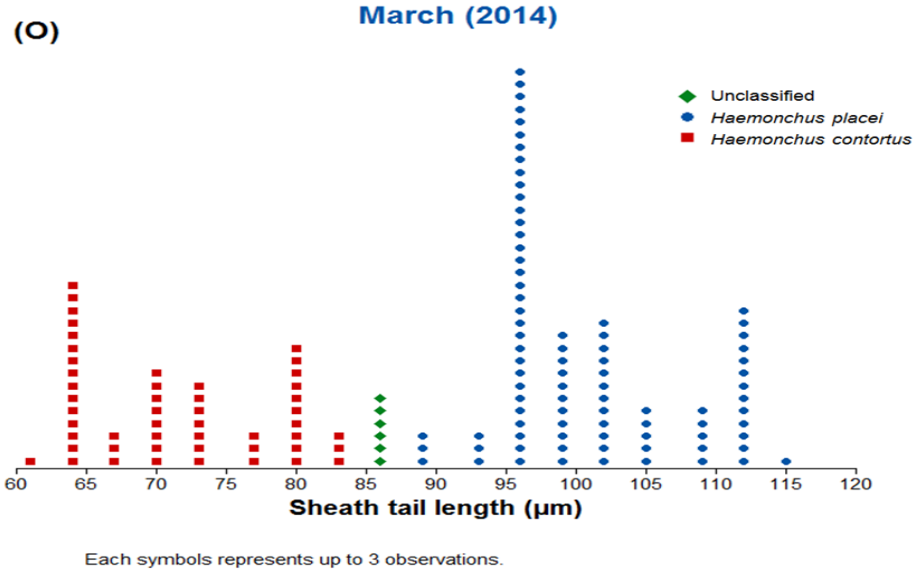
(M)


February (2015)
Unclassified
Haemonchus placei
Haemonchus contortus
60 65 70 75 80 85 90 95 100 105 110 115 120
Sheath tail length (µm)
Each symbols represents up to 3 observations.

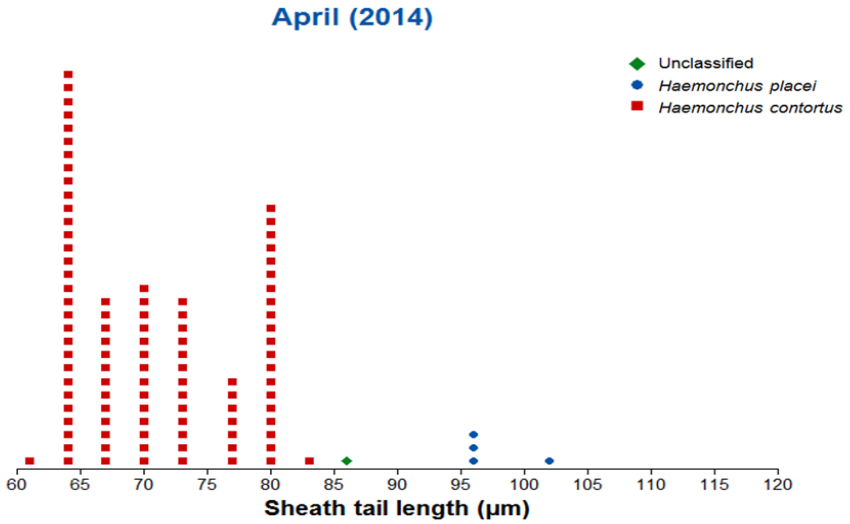
(N)


Group initially infected with Haemonchus placei
First collection with L3 (February, 2014)
Unclassified
Haemonchus placei
Haemonchus contortus
60 65 70 75 80 85 90 95 100 105 110 115 120
Sheath tail length (µm)
Each symbols represents up to 3 observations.

(O)

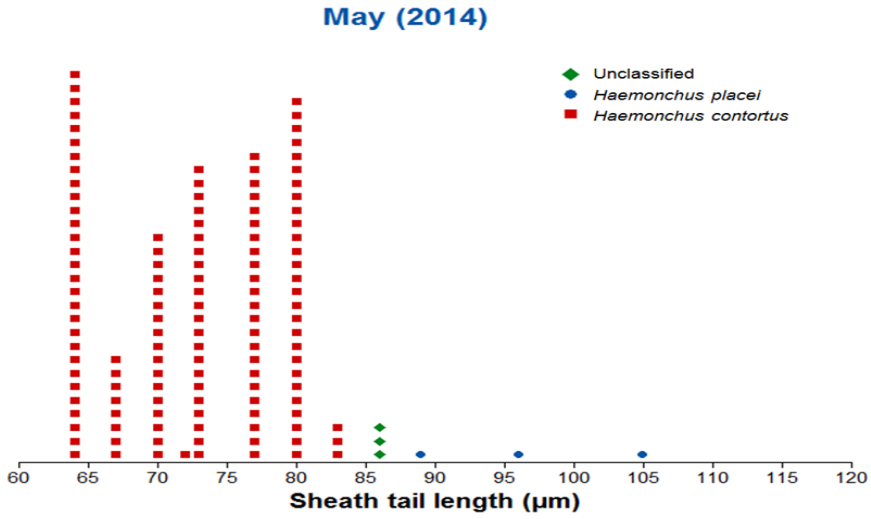

March (2014)
Unclassified
Haemonchus placei
Haemonchus contortus
60 65 70 75 80 85 90 95 100 105 110 115 120
Sheath tail length (µm)
Each symbols represents up to 3 observations.

(P)



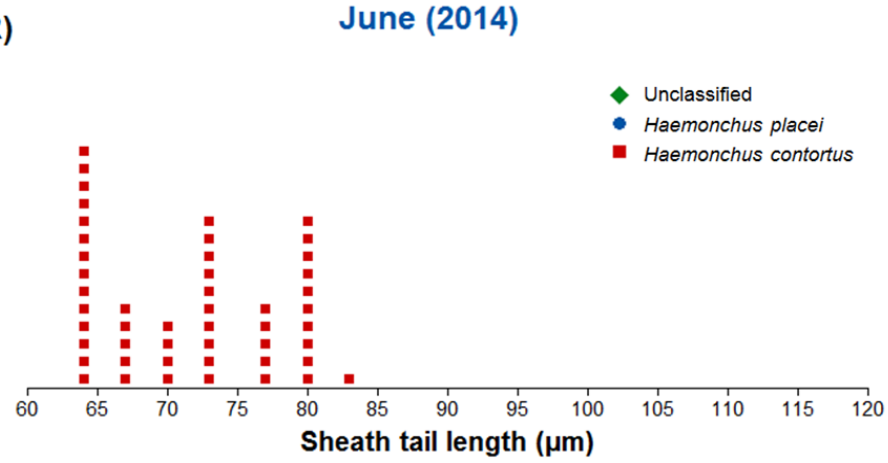

Each symbol represets up to 3 observations.

(Q)



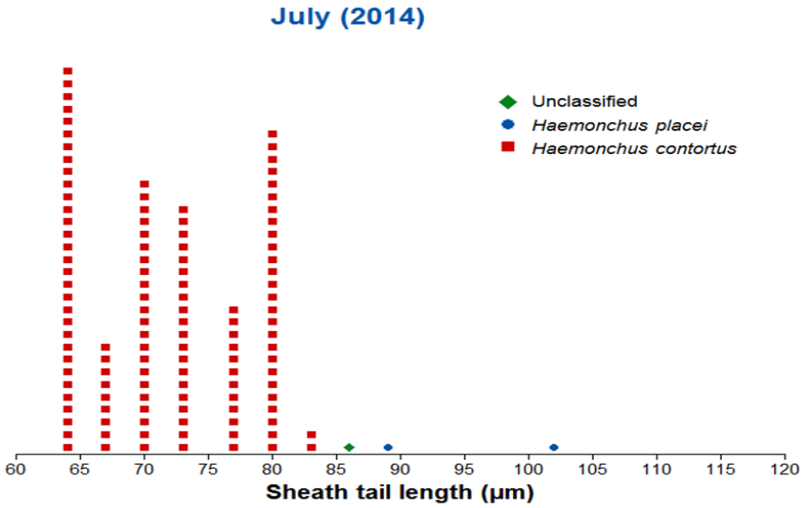

Each symbol represents up to 3 observations.

(R)



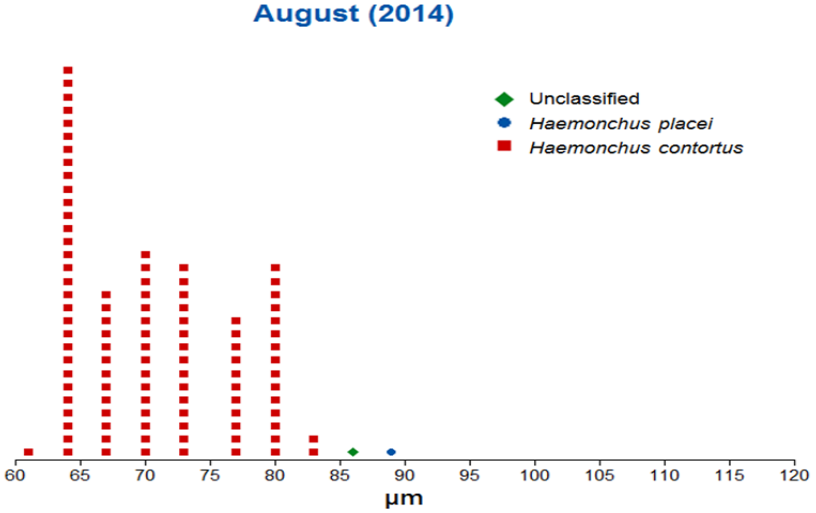

Each symbol represents up to 1 observation.

(S)



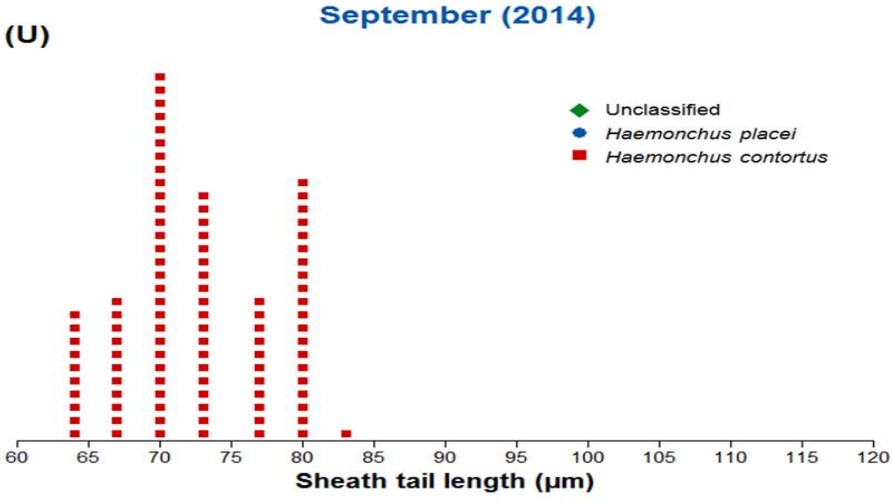

Each symbol represents up to 5 observations.

(T)



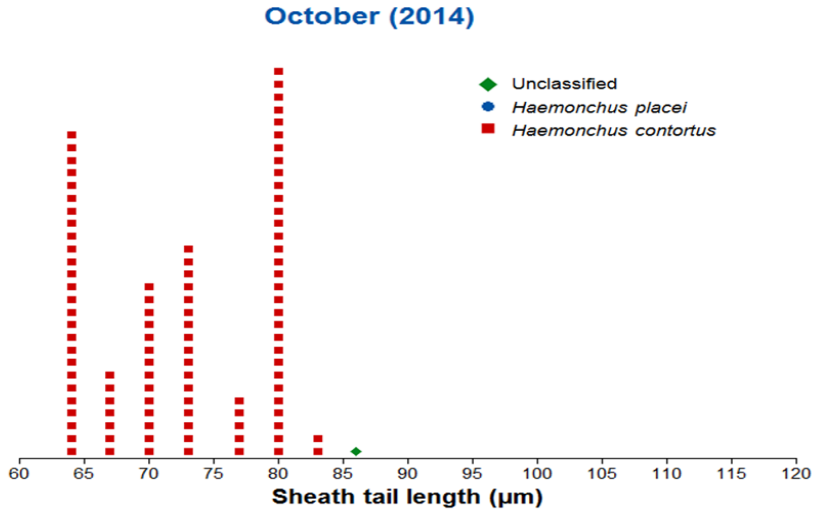

Each symbol represents up to 4 observations.

(U)



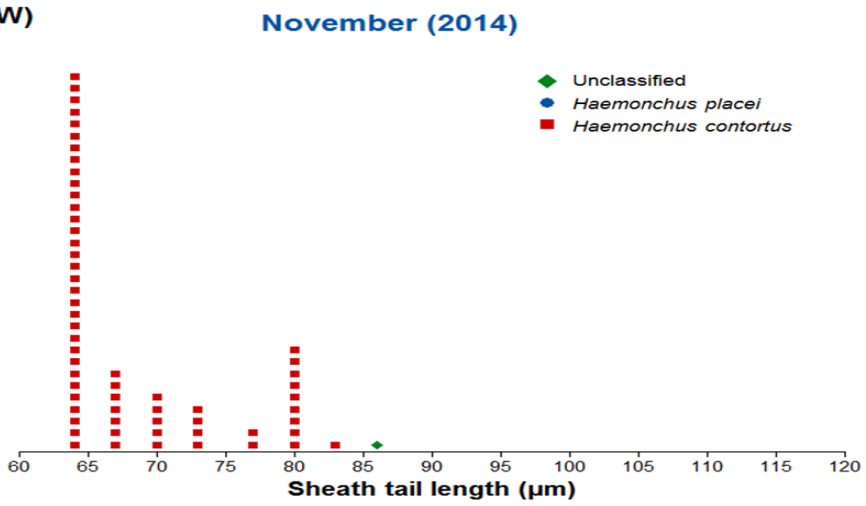

Each symbol represents up to 1 observation.

(V)



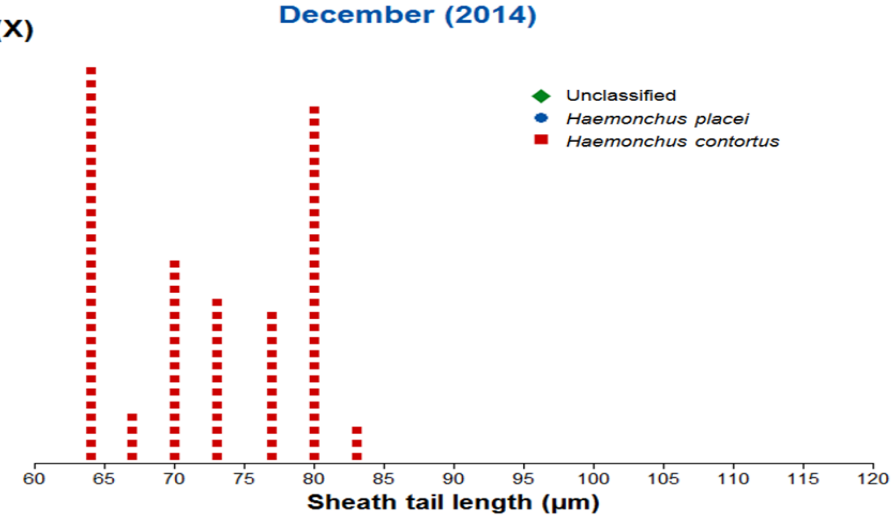

Each symbol represents up to 4 observations.

(W)




Each symbol represents up to 6 observations.

(X)




Each symbol represents up to 2 observations.

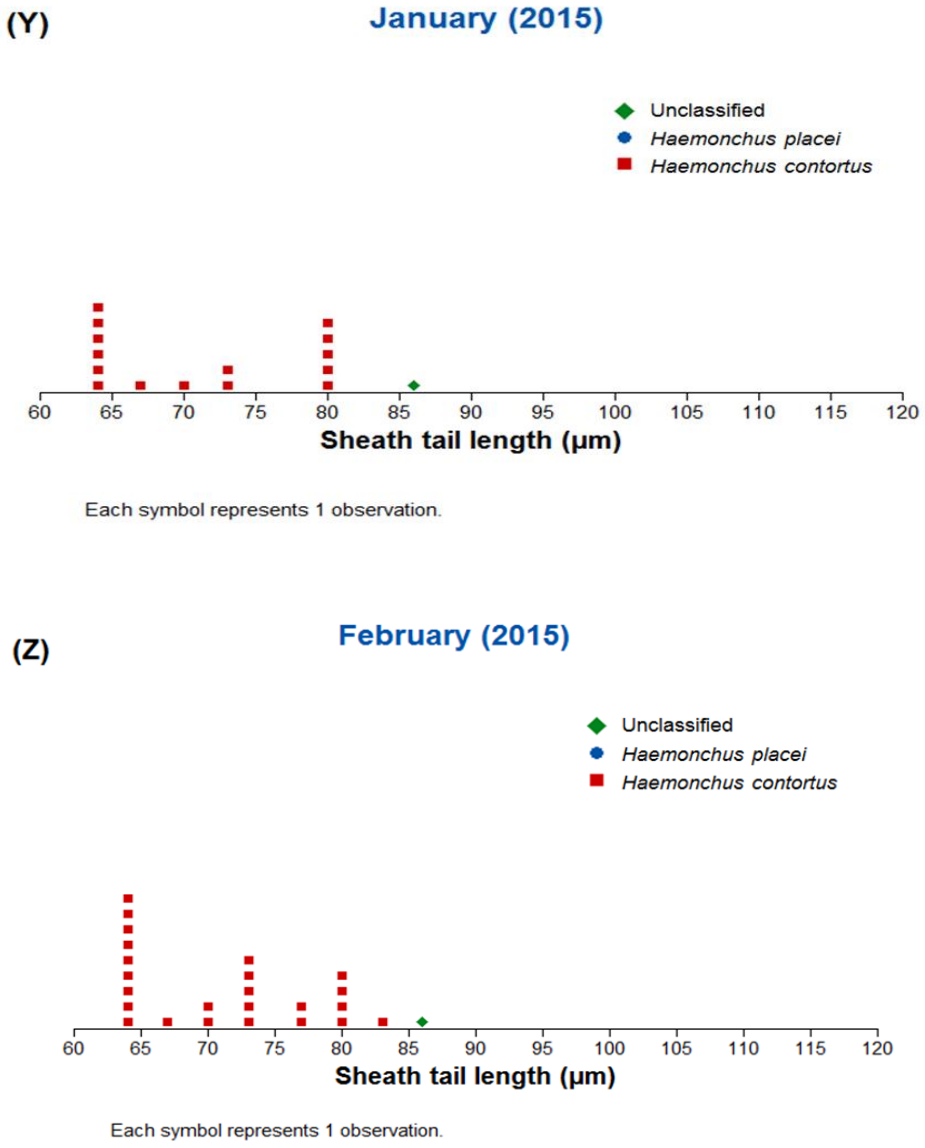



Figure 3. Dot plot of sheath tail length (µm) of *Haemonchus contortus* and *Haemonchus placei* third stage larvae (L3) produced monthly (February 2014 to February 2015). The Sheath tail length comprises the distance between the tip of the larval tail and the end of the sheath tail of the L3. Initially, a group of animals received a single artificial infection with 4,000 L3 of *H. placei* (day 0,) figures N to Z or 4,000 L3 of *H. contortus* (day 11), figures A to M. These animals shared the same pasture until the day 392 after *H. placei* infection. Infective larvae with measures between 60µm to 85µm were identified as *H. contortus*, between 85.1µm to 88.9µm as not confirmed and between 89µm to 120µm as *H. placei*.

Table 1. Total worm burden of the lambs artificially infected with 4,000 L3 of *Haemonchus placei* (SpHpl1 laboratory isolate, n=16) on January 14 (2014) or with *Haemonchus contortus* (SpHco2 laboratory isolate, n=16) on January 25, 2014. Sheep shared the same pasture during 357 days starting on February 18, 2014, being exposed to re-infection by both *Haemonchus* species.

| Euthanasia day | Initial infection | Adults | | Juveniles | Total worm burden |
|---|---|---|---|---|---|
| | | Males | Females | | |
| Feb 24, 2014 | *H. contortus* | 956 | 1237 | 10 | 2203 |
| | *H. contortus* | 1262 | 1466 | 0 | 2728 |
| | *H. placei* | 1251 | 1319 | 30 | 2600 |
| | *H. placei* | 2173 | 1656 | 40 | 3869 |
| May 22, 2014 | *H. contortus* | 1 | 0 | 10 | 11 |
| | *H. contortus* | 1200 | 2329 | 2238 | 5767 |
| | *H. contortus* | 1 | 0 | 0 | 1 |
| | *H. placei* | 0 | 0 | 0 | 0 |
| | *H. placei* | 765 | 1390 | 348 | 2503 |
| | *H. placei* | 11 | 18 | 4 | 33 |
| Aug 23, 2014 | *H. contortus* | 274 | 300 | 171 | 745 |
| | *H. contortus* | 40 | 38 | 61 | 139 |
| | *H. contortus* | 541 | 962 | 54 | 1557 |
| | *H. placei* | 0 | 2 | 0 | 2 |
| | *H. placei* | 510 | 854 | 30 | 1394 |
| | *H. placei* | 196 | 241 | 73 | 510 |
| Nov 19, 2014 | *H. contortus* | 4 | 13 | 4 | 21 |
| | *H. contortus* | 42 | 57 | 16 | 115 |
| | *H. contortus* | 231 | 461 | 2 | 694 |
| | *H. placei* | 1272 | 1376 | 44 | 2692 |
| | *H. placei* | 4 | 30 | 3 | 37 |
| | *H. placei* | 0 | 0 | 7 | 7 |
| Feb 11, 2015 | *H. contortus* | 0 | 0 | 4 | 4 |
| | *H. contortus* | 189 | 224 | 120 | 533 |
| | *H. contortus* | 11 | 178 | 6 | 195 |
| | *H. contortus* | 8766 | 9949 | 40 | 18755 |
| | *H. contortus* | 0 | 0 | 1 | 1 |
| | *H. placei* | 255 | 1141 | 214 | 1610 |
| | *H. placei* | 15 | 44 | 1 | 60 |
| | *H. placei* | 190 | 482 | 1 | 673 |
| | *H. placei* | 18 | 93 | 96 | 207 |
| | *H. placei* | 2 | 34 | 17 | 53 |

Table 2. Total worm burden of the tracer animals introduced together with the flock where they remained for 14 days. After this period, the tracer animals were kept indoors during 20 days and at the end of this period they were euthanized for the recovery and quantification of the parasites.

| Tracer animal number | Starting grazing | Day of euthanasia | Adults | | Juveniles | Total worm burden |
|---|---|---|---|---|---|---|
| | | | Males | Females | | |
| 9 | May 8, 2014 | Jun 12, 2014 | 223 | 345 | 51 | 619 |
| 22 | May 8, 2014 | Jun 12, 2014 | 342 | 336 | 28 | 706 |
| 51 | Aug 8, 2014* | Sep 11, 2014 | 73 | 80 | 11 | 164 |
| 62 | Nov 4, 2014 | Dec 10, 2014 | 413 | 534 | 0 | 947 |
| 42 | Nov 4, 2014 | Dec 10, 2014 | 715 | 1057 | 0 | 1772 |
| 25 | Jan 27, 2015 | March 2, 2015 | 848 | 1026 | 166 | 2040 |
| 45 | Jan 27, 2015 | March 2, 2015 | 449 | 580 | 0 | 1029 |

*On August 08, 2014 only one lamb was euthanized. The other tracer lamb was found dead due to undetermined cause.

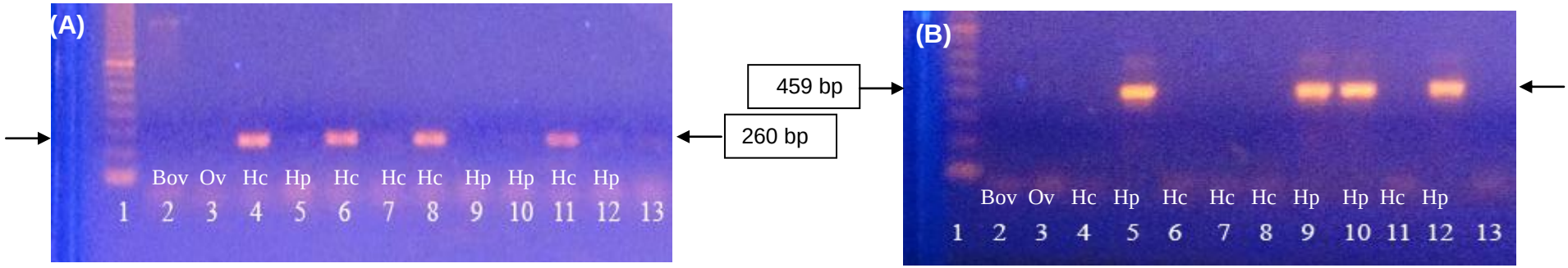



Figure 4. PCR reactions with species-specific primers HcBotuF1/R2 for *Haemonchus contortus* (A), and HpBotuF/R for *Haemonchus placei* (B) and DNA samples. Line 1 show 100 bp molecular markers (GE Healthcare); 2 and 3-bovine and ovine DNA, respectively (hosts); 4 – *H. contortus* (control); 5 - *H. placei* (control*);* 6, 8 and 11 - *H. contortus*, 9, 10, 12 - *H. placei*, 7 – not amplified and 13 - reagents without DNA.

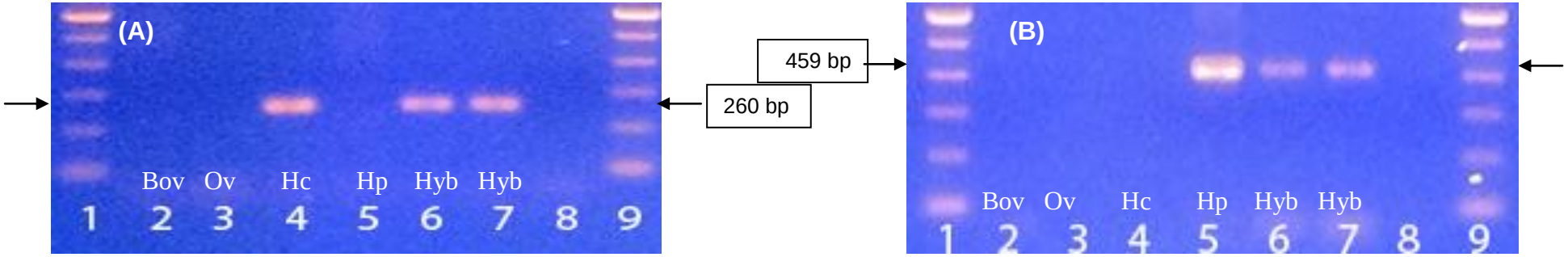



Figure 5. PCR reactions with the species-specific primers HcBotuF1/R2 for *Haemonchus contortus* (A), and HpBotuF/R for *Haemonchus placei* (B) and DNA samples. Lines 1 and 9 show 100 bp molecular markers (GE Healthcare); 2 and 3-bovine and ovine DNA, respectively (hosts); 4 – *H. contortus* (control); 5 - *H. placei* (control*);* 6 – hybrid from tracer lamb n. 22 (grazing period May 2014); 7 – hybrid from experimental animal n. 25 (euthanized on August 2014) and 8 - reagents without DNA.

Table 3. Morphological and molecular evaluation of *Haemonchus* specimens from the experimental animals. Females and juveniles were added when it was not possible to complete 10 adult males per animal. These parasites were recovered from lambs artificially infected with 4,000 L3 of *Haemonchus placei* (SpHpl1 laboratory isolate, n=16) on January 14, 2014 or with *Haemonchus contortus* (SpHco2 laboratory isolate, n=16) on January 25, 2014. Lambs shared the same pasture during 357 days starting on February 18, 2014, being exposed to re-infection by both *Haemonchus* species.

| Day of euthanasia | Initial infection | Morphology | | | Molecular analysis (PCR) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | n* | *H. contortus* | *H. placei* | n | *H. contortus* | *H. placei* | Hybrid | No amplification** |
| February 24, 2014 | *H. contortus* | 20 | 20 | 0 | 20 | 20 | 0 | 0 | 0 |
| February 24, 2014 | *H. placei* | 20 | 0 | 20 | 20 | 0 | 20 | 0 | 0 |
| May 22, 2014 | *H. contortus* | 12 | 12 | 0 | 21 | 20 | 0 | 0 | 1 |
| May 22, 2014 | *H. placei* | 20 | 19 | 1 | 20 | 19 | 1 | 0 | 0 |
| August 23, 2014 | *H. contortus* | 30 | 30 | 0 | 30 | 29 | 0 | 1 | 0 |
| August 23, 2014 | *H. placei* | 20 | 20 | 0 | 22 | 22 | 0 | 0 | 0 |
| November 19, 2014 | *H. contortus* | 24 | 24 | 0 | 30 | 30 | 0 | 0 | 0 |
| November 19, 2014 | *H. placei* | 14 | 14 | 0 | 27 | 26 | 0 | 0 | 1 |
| February 11, 2015 | *H. contortus* | 30 | 30 | 0 | 35 | 34 | 0 | 0 | 1 |
| February 11, 2015 | *H. placei* | 42 | 42 | 0 | 50 | 50 | 0 | 0 | 0 |

*n= number of worms with morphology per animal.

**Samples that not yield amplification with both primers.

Table 4. Morphological and molecular evaluation of worms from tracer animals. These animals were introduced together with the flock where they remained for 14 days. After this period, the tracer animals were kept indoors during 20 days and at the end of this period the animals were euthanized for the recovery and quantification of the parasites.

| | | Morphology | | | Molecular evaluation (PCR) | | | |
|---|---|---|---|---|---|---|---|---|
| Grazing period | Tracer animal number | n** | *H. contortus* | *H. placei* | n | *H. contortus* | *H. placei* | Hybrid |
| May 08 to 22, 2014 | 9 | 10 | 5 | 5 | 20 | 15 | 5 | 0 |
| May 08 to 22, 2014 | 22 | 7 | 3 | 4 | 20 | 5 | 14 | 1 |
| Aug 08 to 22, 2014* | 51 | 20 | 14 | 6 | 40 | 40 | 0 | 0 |
| Nov 04 a 20, 2014 | 42 | 10 | 10 | 0 | 20 | 20 | 0 | 0 |
| Nov 04 a 20, 2014 | 62 | 10 | 10 | 0 | 20 | 20 | 0 | 0 |
| Jan 27 to Feb 10, 2015 | 25 | 10 | 8 | 2 | 20 | 20 | 0 | 0 |
| Jan 27 to Feb 10, 2015 | 45 | 10 | 7 | 3 | 20 | 20 | 0 | 0 |

*During the month of August just one animal grazing.

**n=number of worms with morphology per animal.

Table 5. Agreement between PCR and discriminate function analysis for the diagnosis of *Haemonchus contortus* (SpHco2 laboratory isolate) and *Haemonchus placei* (SpHpl1 laboratory isolate) adult male obtained from experimental and tracer animals.

| | | PCR | | |
|---|---|---|---|---|
| | | *Haemonchus contortus* | *Haemonchus placei* | total |
| Discriminate function* | *Haemonchus contortus* | 267 | 0 | 267 |
| | *Haemonchus placei* | 15 | 26 | 41 |
| | Total | 282 | 26 | 308 |

Kappa coefficient: 0.7503 (95% lower confidence limit = 0.6310 and 95% upper confidence limit = 0.8697)

*Discriminate function calculated based on descriptions by Achi et al. (2003).

# Capítulo 4

*Manuscrito redigido de acordo com as normas do periódico Veterinary Parasitology.

**Molecular evaluation of levamisole resistance in *Haemonchus* isolates and its F1 (*Haemonchus contortus* X *Haemonchus placei*)**

**Abstract**

This study aimed to characterize levamisole resistance in laboratory isolates and the resistance profile in a first filial generation (F1) in sheep. The SpHco2 laboratory isolate (*Haemonchus contortus*) with multidrug resistance was used to infect a donor sheep, while the SpHpl1 laboratory isolate (*Haemonchus placei*) with susceptibility to levamisole was used to infect a donor calf in order to produce infective larvae (L3). Infective larvae from SpHpl1 isolate were used to infect the experimental sheep of the parental group (n=6) on day 0 and 11 days later the same animals received the SpHco2 isolate. Fecal cultures were performed individually and the F1 of L3 were used to infect the animals from groups F1-42 DPI (n=6) and F1-84 DPI (n=6). All animals were euthanized and the parasites recovered for molecular evaluation. Levamisole resistance status was evaluated based on an insertion/deletion (indel) of 63 bp which is associated with levamisole resistance. Parasites from sheep donor (n=20), infected with SpHco2 isolate, were 85% homozygous resistant and 15% were heterozygous. The *H. contortus* specimens were also mostly homozygous resistant (97.6% of 126 samples). Five of the eight hybrids recovered presented the 256 bp amplification band and three specimens did not show any bands. An interesting observation was that none of the 129 *H. placei* DNA samples extract from specimens from donor calf and from all experimental groups of sheep presented amplification bands. In summary, the primer pair evaluated in this study no amplification in PCR products with *H. placei* DNA samples. In addition, *H. contortus* SpHco2 laboratory isolate showed amplification bands that corroborated with a high level of levamisole resistance observed in a previous controlled euthanized trial in sheep.

Keywords: anthelmintic resistance, laboratory isolate, levamisole, PCR.

## 1. Introduction

*Haemonchus contortus* population shows enormous genetic diversity, allowing anthelmintic resistance alleles to be rapidly selected (reviewed by Prichard, 2001). In contrast, Brasil et al. (2012) observed a lower genetic diversity among *Haemonchus placei* compared with *Haemonchus contortus* populations in ruminants from Brazil.

The classical methods as fecal egg count reduction test (FECRT), egg hatch test (EHT), and larval development test (LDT) are widely used to evaluate the anthelmintic resistance (Echevarria et al., 1996; Coles et al., 2006; Soutello et al., 2007; Neves et al., 2014; Chagas et al., 2016). However, molecular analysis contributes to find mutations present even at low frequencies in the population and allow the comprehension of the genes and the physiological expression of anthelmintic sensitivity (Martin et al., 2012; Chagas et al., 2016).

Primers have been developed to evaluate the insertion/deletion (indel) of 63 bp in the acr-8 gene, this deletion promotes the formation of the truncated ACR-8b protein, and this protein is correlated with the loss of levamisole susceptibility (Barrerè et al., 2014; Kotze and Prichard, 2016). *Haemonchus contortus* loci differences between susceptible and resistant populations that are genetically independent might simply be a reflection of genetic isolation of the populations (reviewed by Prichard, 2001). The anthelmintic resistance is frequently polygenic and can be associated with mechanisms of drug transport and these mechanisms are only partially understood for many drug classes (reviewed by Roeber et al., 2013). Thus, this study aimed to evaluate the levamisole resistance in *H. contortus* and *H. placei* isolates, F1 produced by mixed infections and in their hybrids by PCR technique.

## 2. Material and Methods

This trial was approved and conducted in accordance with the experimental protocol approved by the local Ethical Committee (protocol number 449-CEEA, Institute of

Biosciences, UNESP, Botucatu – Sao Paulo).

Details about SpHpl1 (*H. placei*) and SpHco2 (*H. contortus*) laboratory isolates, donor animal management and infections are described by Santos et al. (unpublished). Briefly, donor sheep was infected exclusively with 25,000 L3 of SpHco2 laboratory isolate (*H. contortus*) with multidrug resistance, confirmed in a previous study by Almeida et al. (2010).

A donor calf received 25,000 L3 of SpHpl1 laboratory isolate (*H. placei*). In a previous study, levamisole presented 100% efficacy in sheep infected with the SpHpl1 isolate based on fecal egg count reduction test (Santos et al., 2014). The SpHco2 isolate (*H. contortus*) after the treatment with levamisole showed no significant reduction in worm burden (Almeida et al., 2010).

Fecal cultures from the feces of donor animals (calf and sheep) were performed separately for the L3 production and the species identity were confirmed by morphological and molecular evaluations (Santos et al., unpublished data).

2.1. Experimental design

Details about the experimental design have been explained by Santos et al. (unpublished data). A total of 18 Suffolk male lambs were maintained in pens during all trial. The parental group (n=6) received artificial infection with 2,000 L3 of SpHpl1 on day zero and eleven days later the same animals received 2,000 L3 of SpHco2 (Figure 1).

Feces were recovered from parental group between 35 and 50 days post-infection (DPI) with SpHco2. Individual fecal cultures were performed individually for the production of L3 first filial generation (F1), these L3 were assigned for infection of animals from F1 group (n=12). Each animal from the parental group was used to infect two animals of the F1 group, this group was subdivided in F1-42 DPI (n=6) and F1-84 DPI (n=6). Animals from parental, F1-42 DPI and F1-84 DPI groups were euthanized at 50 DPI, 42 DPI or 84 DPI

(Table 1).

For the recovery of adult parasites, the animals were fasted for 12 hours before being euthanized. The abomasums were opened and its content was placed in a graduated beaker. The volume was brought up to 1 L with saline solution. The content was homogenized and divided into two containers of 500 mL each, which were immediately frozen for the preservation of parasites.

2.2. Molecular evaluation of levamisole resistance

Twenty adult male parasites were evaluated from donor sheep and donor cattle, infected exclusively with SpHco2 laboratory isolate or SpHpl1 laboratory isolate, respectively. From the experimental animals a total of 279 adult males were evaluated: 60 from the parental group, 120 from the F1-42 DPI group and 99 from the F1-84 DPI group. To identify the *H. contortus* and *H. placei*, as well as the hybrids, these parasites were previously evaluated and the results are described by Santos et al. (unpublished data), using species-specific primer-pairs described by Amarante et al. (2017).

Genomic DNA was obtained from cattle and sheep blood samples (hosts) and from specimens of *H. contortus* and *H. placei* previously identified based on morphological analysis and used as positive and negative PCR controls. All DNA samples were extracted using a QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

The DNA samples were subjected to PCR analysis with the primer pairs ForInsert/RevInsert described by Barrère et al. (2014). The presence of this indel (63 bp) in the exon 3b sequence of the acr-8 gene is associated with levamisole susceptibility in *H. contortus*, while its absence indicates levamisole resistance. Susceptible homozygous parasites present a PCR product of 319 bp amplification band (presence of the 63 bp indel);

resistant homozygous show a 256 bp band (deletion of the 63 bp indel) and heterozygous nematodes present both 256 and 319 bp bands (insertion and deletion of the 63 bp indel).

PCR products were electrophoresed on 2% agarose gel in 1% TAE buffer containing ethidium bromide and photographed under UV light using a Sony Cyber-shot DSC-HX1 camera (Sony Electronics, San Diego, California, USA). All PCR reactions were performed at least in duplicate. Results of molecular analyses are presented descriptively.

## 3. Results

*Donor animals*

*Haemonchus contortus* specimens (n=20) from donor sheep were evaluated for levamisole resistance (Fig. 1) and 85% (n=17) were identified as homozygous resistant (256 bp) while only 15% (n=3) were identified as heterozygous (256 e 319 bp). Although DNA samples from all 20 *H. placei* specimens had been analyzed, the ForInsert/RevInsert primer pair did not amplify any bands (Fig. 1).

*Experimental animals*

Considering the parental, F1-42 DPI and F1-84 DPI groups, none of the 129 *H. placei* DNA samples evaluated showed amplification, 126 of the 142 *H. contortus* samples displayed amplification, 97.6% were homozygous resistant (256 bp band) and 2.4% were heterozygous (256 and 319 bp bands) (Table 2). Eight hybrids were identified and five showed the 256 bp amplification band which is associated with levamisole resistance (Table 1).

## 4. Discussion

A total of 129 DNA samples from *H. placei* specimens were evaluated in this study and none showed amplification band with the ForInsert/RevInsert primer pair (Barrère et al.,

2014). Therefore, this primer pair could be useful for the distinction between populations of *H. placei* and *H. contortus* in cases of mixed infection, associated with the species-specific primer pairs described by Amarante et al. (2017).

This non-amplification in *H. placei* specimens can also be related to the non-annealing of the primers pairs due to differences in nucleotide sequence between *H. placei* and *H. contortus* and, for this reason, a new design of primers specific to *H. placei* is indicated.

Further studies are necessary to clarify this issue because it is still unclear how resistance alleles arise, are selected and spread in parasite populations and also whether the mechanisms of resistance differ between different parasite species or different isolates of the same species (Gilleard, 2006). In São Paulo state, where the *H. placei*, used in the present study, was isolated from cattle, *H. placei* species usually shows resistance to ivermectin, but susceptibility to levamisole and albendazole (Soutello et al., 2007; Neves et al., 2014). In contrast, in the same state, multidrug resistance (including levamisole) is widespread in *H. contortus* from sheep (Almeida et al., 2010; Veríssimo et al., 2012).

The *H. contortus* laboratory isolate (SpHco2) assessed in this trial was previously evaluated by Almeida et al. (2010) in controlled euthanized trial of sheep and showed a high degree of resistance to levamisole. Thus, our results also confirmed the levamisole resistance status in SpHco2 isolate, the present molecular results that show predominance of homozygous resistant bands (256 bp) are in agreement with the findings of Barrère et al. (2014), who reported the 319 bp band (presence of the indel) was more frequently observed in susceptible isolates, while the 256 bp band, are presented in resistant isolates.

Different results were reported by Chagas et al. (2016) in a study using the same levamisole resistance primer pair (described by Barrère et al., 2014). They observed in susceptible McMaster isolate the presence of the 256 bp at a higher frequency (marker of resistance). The discrepancy between our results and those obtained by Chagas et al. (2016)

could be due a different mechanism from the one involving Hco-acr-8b expression (Barrère et al., 2014). According to Kotze et al. (2014), resistance to cholinergic anthelmintics in parasitic nematodes is polygenic rather than a simple single-gene mechanism, and involves changes in expression of nAChR subunits, truncated receptor subunits, and mutations in receptor subunits, as well as a possible contribution from P-glycoproteins (P-gps). For this reason, it is difficult to develop effective molecular-based diagnostic tools for the detection of resistance to levamisole.

Eight hybrids from F1 groups previously described by Santos et al. (unpublished) were also analyzed and five specimens (62.5%), showed the levamisole resistant band and three specimens (37.5%) did not amplify. Probably, if a new primer capable to detect the levamisole gene resistance in *H. placei* will be developed, will be possible to characterize the resistance profile in these hybrids. Chaudhry et al. (2015) observed one hybrid (product of the crossing between *H. contortus* and *H. placei*) containing a *H. contortus* allele (Hc10) with the F200Y isotype-1 β-tubulin benzimidazole resistance mutation, suggesting there is a potential for interspecies introgression of drug resistance loci. It would be interesting to evaluate in future studies if the *H. placei* susceptible background could be retained through backcrossing into a *H. contortus* population, reversing its resistant status.

*Haemonchus contortus* laboratory isolate evaluated in this trial showed a high frequency of levamisole resistance gene confirming findings of a previous controlled euthanized trial. The *H. placei* DNA samples did not yield any amplification band with the same primer pair, while the hybrids displayed results similar to those of the parental *H. contortus*.

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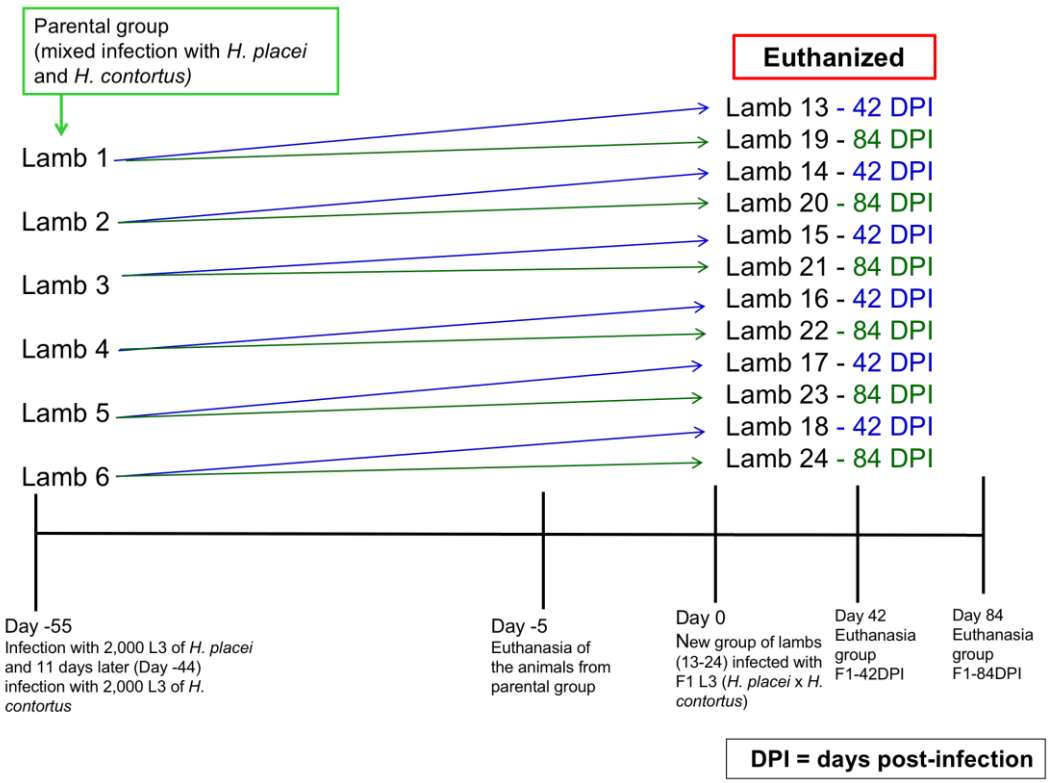


Figure 1. Sheep from the parental group (1 to 6) were artificially infected with 2,000 infective larvae (L3) of *Haemonchus placei* (day 0) and the same animals were infected with 2,000 *Haemonchus contortus* L3 11 days later. First filial generation (F1) of L3 produced from parental animals (from 35 DPI until 50 DPI) were used to infect lambs from F1 group (n=12, lambs 13 to 24). Each animal from F1 group received 4,000 L3 (*H. contortus* + *H. placei* from parental animals), each animal from the parental group was L3 donor to two animals from F1 group. These lambs were subdivided into two groups and euthanized at 42 DPI (F1-42 DPI, n=6) or 84 DPI (F1-84 DPI, n=6). (Santos et al. unpublished data, adapted scheme). Animals from 7 to 12 remained uninfected during the trial (control group).

Table 1. PCR reactions to evaluate levamisole resistance in SpHco2 laboratory isolate (*Haemonchus contortus*) and SpHpl1 laboratory isolate (*Haemonchus placei*) with the primer pair described by Barrère et al. (2014). The parental group received 2,000 infective larvae (L3) of *Haemonchus placei* (day 0) and 11 days later were infected with 2,000 L3 of *Haemonchus contortus*. First filial generation (F1) of L3 produced by these animals were used to infect the animals from group F1-42 DPI and F1-84 DPI (4,000 L3 per animal). Parental, F1-42 DPI and F1-84 DPI groups were euthanized 50, 42 and 84 days post-infection, respectively.

| Group | n | *Haemonchus contortus* | | | *Haemonchus placei* | | | Hybrids | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Homozygous Resistant | Heterozygous | No amplified | Homozygous resistant | Heterozygous | No amplified | Homozygous resistant | Heterozygous | No amplified |
| Parental | 60 | 20 | 0 | 11 | 0 | 0 | 29 | 0 | 0 | 0 |
| F1-42 DPI | 120 | 53 | 2 | 5 | 0 | 0 | 55 | 3 | 0 | 2 |
| F1-84 DPI | 99 | 50 | 1 | 0 | 0 | 0 | 45 | 2 | 0 | 1 |
| Total | 279 | 123 | 3 | 16 | 0 | 0 | 129 | 5 | 0 | 3 |

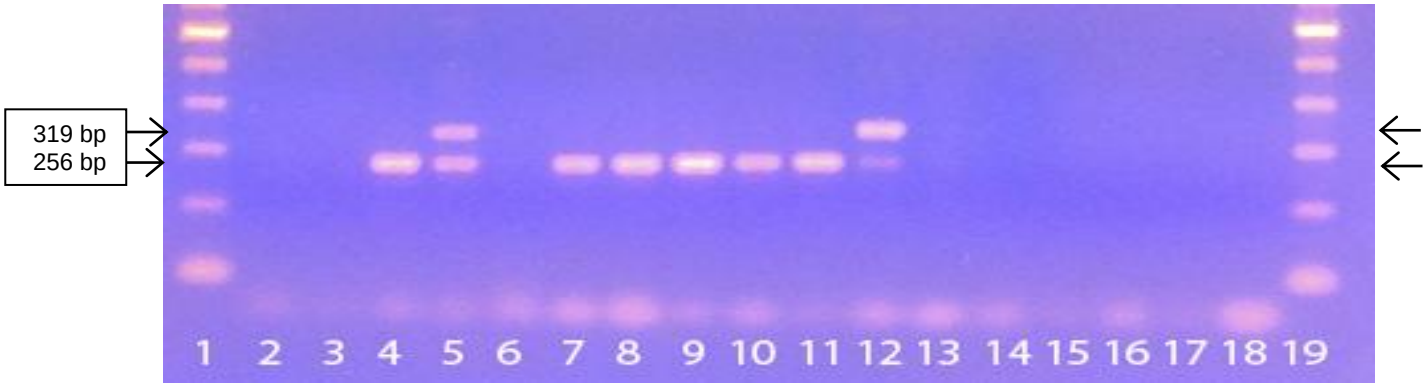


Figure 1. PCR reactions with the primer pair ForInsert/RevInsert described by Barrère et al. (2014) to detect levamisole resistance. PCR products with this primer show amplification bands with 319bp (susceptible), 256bp (resistant) and 256bp and 319bp (heterozygous). Line 1 and 19 show 100bp molecular markers (GE Healthcare); 2 and 3-bovine and ovine DNA, respectively (hosts); 4 - *Haemonchus contortus* homozygous levamisole resistant (control); 5 - *H. contortus* heterozygous levamisole (control); 6 - *Haemonchus placei* (control); 7 to 11 - *H. contortus* homozygous resistant; 12 - *H. contortus* heterozygous; 13 to 17 - *H. placei* and 18-reagents without DNA.

**Considerações finais**

As técnicas moleculares são mais específicas para a diferenciação dessas espécies e podem ser utilizadas para a identificação de híbridos interespecíficos, bem como na avaliação de fluxo gênico.

A biologia molecular pode ser empregada como ferramenta para a avaliação dos genes candidatos, que estão relacionados à resistência anti-helmíntica, e esses estudos vêm sendo amplamente difundidos nos últimos dez anos.

Os presentes estudos contribuíram para a compreensão do comportamento de infecções mistas em ovinos. No primeiro capítulo foi observado que os marcadores moleculares avaliados podem ser aplicados para a diferenciação das espécies *H. contortus* e *H. placei*. Nos capítulos 2 e 3 observamos a ocorrência do estabelecimento de infecções mistas (*H. contortus* + *H. placei*) em ovinos com a produção de híbridos em condições de infecção controlada, em confinamento, ou em condições de reinfecções, em pastagem. No capítulo 2 também foi observado que quando a competição interespecífica é possível houve a eliminação de *H. placei* (parasita específico de bovinos) em menos de 12 meses, em condições de pastejo somente com ovinos.

Ao avaliarmos o gene de resistência ao levamisol, o isolado utilizado no estudo SpHco2 (*H. contortus*) apresentou elevado grau de resistência à ação do levamisol, contudo, o primer avaliado não apresentou banda de amplificação ao ser utilizado com o isolado SpHpl1 (*H. placei*). Contudo, mais pesquisas são necessárias para avaliar o fluxo dos genes que conferem resistência ao levamisole sua expressão e introgressão.

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# *Certificado*

Certificamos que o Protocolo nº **449-CEUA**, sobre "Interação entre as infecções por Haemonchus contortus e Haemonchus placei em ovinos", sob a responsabilidade de **Alessandro Francisco Talamini do Amarante**, está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA) e foi aprovado "*Ad referendum*" da ***COMISSÃO DE ÉTICA NO USO DE ANIMAIS*** (CEUA), nesta data.

Botucatu, 10 de abril de 2013.

**Prof. Dr. Wellerson Rodrigo Scarano**
Presidente da CEUA

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