UNIVERSIDADE ESTADUAL DE SANTA CRUZ PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA ANIMAL

KATHARINE COSTA DOS SANTOS

AVALIAÇÃO DA EFICÁCIA E PROTEÇÃO DOS PRINCÍPIOS ATIVOS AFOXOLANER/MILBEMICINA OXIMA E FLURALANER CONTRA *Tunga penetrans* EM CÃES NATURALMENTE INFESTADOS A CAMPO

ILHÉUS- BAHIA

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Tese apresentada à Universidade Estadual de Santa Cruz como exigência para a obtenção do título de Doutor em Ciência Animal.

Área de concentração: Clínica e Sanidade Animal

Orientadora: Profa. Dra. Renata Santiago Alberto Carlos

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"Sonhos determinam o que você quer. Ação determina o que você conquista."

Aldo Novak

RESUMO

AVALIAÇÃO DA EFICÁCIA E PROTEÇÃO DOS PRINCÍPIOS ATIVOS AFOXOLANER/MILBEMICINA OXIMA E FLURALANER CONTRA *Tunga penetrans* EM CÃES NATURALMENTE INFESTADOS A CAMPO

A tungíase é uma ectoparasitose tropical negligenciada, a qual acomete a pele dos hospedeiros através da penetração da pulga *Tunga penetrans.* Os cães em áreas endêmicas são considerados importantes reservatórios e disseminadores da doença, a qual apresenta alta morbidade à espécie. Devido a esses fatos estudos que colaboram para o controle e tratamento da tungíase em cães são necessários para a ampliação do conhecimento a cerca de fármacos no combate dessa zoonose. Objetivou-se com essa pesquisa, determinar a eficácia inseticida mensal de uma única administração oral de fluralaner na dose de 10–18 mg/kg (Defenza®) em cães naturalmente infestados com *T. penetrans* na Vila Juerana, Ilhéus, Bahia. Um total de 64 cães foram distribuídos em delineamento inteiramente casualizado entre um grupo tratado (GT) que recebeu uma dose única de Defenza® e um grupo controle negativo (GC). Cada grupo foi composto por 32 cães. As avaliações ocorreram nos dias 0, 7 ± 2, 14 ± 2, 21 ± 2, 28 ± 2, 35 ± 2 e 42 ± 2 pós-tratamento, nos quais os cães eram inspecionados (patas e pele). A eficácia primária do fármaco foi determinada a partir da porcentagem de cães tratados livres de pulgas (lesões de estágio II e III- Classificação de Fortaleza) após a administração da formulação em cada momento de avaliação. A eficácia secundária foi baseada no número de lesões ativas (estágios II e III) em cada grupo em cada momento de avaliação. A condição clínica dos animais foi definida com base no Pontuação de gravidade para tungíase canina (SCADT), que está relacionado ao número e à gravidade das lesões. A eficácia primária do produto foi maior que 95,0% dos dias 7 a 21 e atingiu 100,0% entre os dias 28 e 42, com uma associação significativa entre o tratamento e o declínio da infestação ($P < 0,025$) entre os dias 7 e 42. A eficácia secundária do medicamento foi maior que 99,9% dos dias 7 a 21, atingindo 100,0% entre os dias 28 e 42 ($P < 0,05$). Os cães tratados também pontuaram mais baixo no SCADT do que os animais de controle durante todo o período de avaliação clínica ($P < 0,05$). Uma única administração de Defenza® foi eficaz na eliminação de infestações de *Tunga penetrans*, bem como na prevenção do parasitismo por pelo

menos 42 dias após o tratamento. Objetivou-se com o segundo estudo, avaliar a eficácia 2.5 - 5.3 mg/kg de afloxalaner e 0.5–1.1 mg/kg milbemicina oxima (NexGard Spectra®), um ectoparasiticida mensal administrado por via oral para cães. Esse ensaio de campo cego e controlado negativamente na mesma comunidade do ensaio I. Sessenta e seis cães naturalmente infectados com *T. penetrans* foram alocados aleatoriamente em um grupo tratado (44 cães) e um grupo controle não tratado (22 cães). Em uma primeira fase, os cães do grupo tratado (GT) foram tratados nos dias 0, 30 e 60. A eficácia foi avaliada com base nas lesões cutâneas parasitárias macroscópicas (lesões de estágio II e III) nos dias 7, 14, 21, 30, 45, 60, 75 e 90. Em uma segunda fase, para avaliar reinfecções naturais, todos os cães foram tratados no dia 90 e avaliados a cada 2 semanas depois disso até que pelo menos 30% dos cães fossem infectados com pulgas de areia vivas. Na primeira fase, a eficácia (redução de pulgas da areia vivas) de 92,4% foi demonstrada no dia 7. Do dia 14 até o dia 90, a eficácia do NexGard Spectra® foi de 100%. Na segunda fase, todos os cães estavam livres de *T. penetrans* (II e III) de 15 a 45 dias após o tratamento do dia 90; 60 dias após o tratamento, 11% dos cães foram reinfectados e 75 dias após o tratamento, 40% dos cães foram reinfectados. O NexGard Spectra® também demonstrou ser altamente eficaz contra a tungíase canina. Além de um efeito benéfico na saúde e bem-estar do cão tratado, o uso deste produto pode ter um benefício à saúde em casos humanos ao controlar o principal reservatório de pulgas da areia. Essas opções de tratamento eficazes pode auxiliar nesse desafio de saúde pública e veterinária, que é a tungíase. A ampliação do conhecimento de opções de fármacos eficazes em cães colaboram no combate dessa zoonose.

Palavras-chave: bicho-de-pé; *Canis lupus familiaris*; fármaco; tratamento

ABSTRACT

EVALUATION OF THE EFFICACY AND PROTECTION OF THE ACTIVE INGREDIENTS AFOXOLANER/MILBEMICIN OXIME AND FLURALANER AGAINST *Tunga penetrans* IN NATURALLY INFESTED DOGS IN THE FIELD

Tungiasis is a neglected tropical ectoparasite that attacks the skin of hosts through penetration by the flea *Tunga penetrans.* Dogs in endemic areas are considered important reservoirs and disseminators of the disease, which presents high morbidity to the species. Due to these facts, studies that collaborate for the control and treatment of tungiasis in dogs are necessary to expand the knowledge about drugs to combat this zoonosis. The objective of this research was to determine the insecticidal efficacy of a single monthly oral administration of fluralaner at a dose of 10–18 mg/kg (Defenza®) in dogs naturally infested with *T. penetrans* in Vila Juerana, Ilhéus, Bahia. A total of 64 dogs were distributed in a randomized design between a treated group (TG) that received a single dose of Defenza® and a negative control group (CG). Each group consisted of 32 dogs. Evaluations occurred on days 0, 7 ± 2, 14 ± 2, 21 ± 2, 28 ± 2, 35 ± 2, and 42 ± 2 post-treatment, at which time the dogs were functional (paws and skin). Primary drug efficacy was determined from the percentage of treated dogs free of fleas (stage II and III lesions - Fortaleza Classification) after administration of the formulation at each evaluation time. Secondary efficacy was based on the number of active lesions (stages II and III) in each group at each evaluation time. The clinical condition of the animals was defined based on the Severity Score for Canine Tungiasis (SCADT), which is related to the number and severity of lesions. Primary efficacy of the product was greater than 95.0% from days 7 to 21 and reached 100.0% between days 28 and 42, with a significant association between treatment and infestation decline ($P < 0.025$) between days 7 and 42. Secondary efficacy of the drug was greater than 99.9% from days 7 to 21, reaching 100.0% between days 28 and 42 ($P < 0.05$). Treated dogs also scored lower on the SCADT than control animals throughout the clinical evaluation period ($P < 0.05$). A single administration of Defenza® was effective in eliminating Tunga penetrans infestations, as well as in preventing parasitism for at least 42 days after treatment. The aim of the second study was to evaluate the efficacy of 2.5–5.3 mg/kg afloxalaner and 0.5–1.1 mg/kg milbemycin oxime (NexGard Spectra®), a monthly orally administered ectoparasiticide for dogs. This was a blinded, controlled-level field

trial in the same community as trial I. Sixty-six dogs naturally infected with *T. penetrans* were randomly allocated to a treated group (44 dogs) and an untreated control group (22 dogs). In a first phase, dogs in the treated group (GT) were treated on days 0, 30 and 60. Efficacy was assessed based on macroscopic parasitic lesions (stage II and III lesions) on days 7, 14, 21, 30, 45, 60, 75 and 90. In a second phase, to assess natural reinfections, all dogs were treated on day 90 and assessed every 2 weeks thereafter until at least 30% of the dogs were infected with live sand fleas. In the first phase, efficacy (live flea reduction) of 92.4% was demonstrated on day 7. From day 14 to day 90, the efficacy of NexGard Spectra® was 100%. In the second phase, all dogs were free of *T. penetrans* (II and III) from 15 to 45 days after the day 90 treatment. At 60 days post-treatment, 11% of dogs were reinfected and at 75 days post-treatment, 40% of dogs were reinfected. NexGard Spectra® has also been shown to be highly effective against canine inflammatory bowel disease. In addition to the beneficial effects on the health and well-being of the treated dog, the use of this product may have a health benefit in humans by controlling the primary reservoir of sand fleas. These therapeutic treatment options may help address the public and veterinary health challenge of tungiasis. Expanding knowledge of effective drug options in dogs will help combat this zoonosis.

Keywords: jigger; Canis lupus familiaris; drug; treatment

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LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

UESC Universidade Estadual de Santa Cruz

mg Miligramas

kg quilos

SCADT Severity Score for Acute Dog Tungiasis

® Marca registrada

IC Intervalo de Confiança

OR Odds Ratio

SUMÁRIO

1. INTRODUÇÃO

A T*unga pentrans* é popularmente conhecida no Brasil como bicho de pé, uma espécie de pulga, que penetra a pele do hospedeiro humano ou animal em contato com o solo arenoso contaminado (HEUKELBACH et al., 2001; THIELECKE et al., 2013). O cão é apontado como principal hospedeiro reservatório, sendo um fator de risco para a tungíase (TAMENE., 2021), pricipalmente na América Latina (MUEHLEN et al., 2006). As infestações são autolimitadas, no entanto em áreas endêmicas a re-infestação ocorre constantemente e os indivíduos afetados apresentam grande número de pulgas em diferentes estágios de desenvolvimento (HEUKELBACH et al., 2004; ARIZA et al., 2007) .

A alta prevalência da doença está relacionada principalmente as populações mais pobres e marginalizadas em áreas tropicais e subtropicais (HEUKELBACH et al., 2001; HARVEY et al., 2017). Essas áreas endêmicas em sua maioria não possuem saneamento básico, pavimentação e infraestrutura, o que contribuem para manutenção do parasito e consequentemente da doença ((HEUKELBACH et al., 2001). A tungíase é considerada uma doença tropical negligenciada, sendo assim falta atenção por parte da mídia, das políticas públicas e da comunidade científica, apesar de impactarem milhões de pessoas e animais em todo o mundo (FELDMEIER et al., 2014; WHO, 2018).

A manutenção da sanidade dos cães em áreas endêmicas é fundamental para interromper o ciclo do parasita (MUTEBI et al., 2016), uma vez que o mesmo pode disseminar ovos por uma extensa área (DE JESUS et al., 2023). Após seu desenvolvimento ambiental, a pulga fêmea penetra a pele do hospedeiro e morre entre quatro seis semanas completando seu ciclo no hospedeiro (JOSEPH et al., 2006). Apesar de a tungíase se uma doença autolimitante na maioria das vezes ocorrem graves complicações, os cães geralmente apresentam-se altamente infestados resultando em diminuição da qualidade de vida, predispondo a complicações como infecções bacterianas secundárias, perda de membros e dígitos, necrose, gangrena e até a morte por sepse (HEUKELBACH, 2006; HARVEY., et al 2021).

Até o presente momento apenas um estudo comprovou a eficácia de um fármaco (Bravecto® - Fluralaner) disponível comercialmente para o tratamento da tungíase em cães (SANTOS., et al 2022). No estudo citado acima, a eficácia terapêutica e residual do fluralaner foi avaliada para tratar cães naturalmente infectados com *T. penetrans* em um desafio de teste de campo. A formulação, demonstrou eficácia mais de 90% dos cães no

grupo tratado, esses estavam livres de lesões de *T. penetrans* entre os dias 14 e 90, e o tratamento foi 100% eficaz nos dias 21, 28 e 60. No entanto, essa medicação é trimestral, o que eleva seu custo, não sendo na maioria das vezes uma opção viável para os tutores em áreas endêmicas (DOS SANTOS., et al 2022).

Esse estudo teve como objetivo avaliar a eficácia inseticida das formulações mensais de fluralaner (Defenza®) e afoxolaner + milbemicina oxima (NexGard Spectra®), em cães infestados com *T. penetrans* em condições de campo. Para que sejam opções de tratamento viáveis no tratamento e controle de *T. penetrans* em cães. Com uma maior disponibilidade em opções de tratamentos comerciais, aumentando facilitar a aquisição de tratamentos com as opções mensais para tratamento e controle da tungíase em cães. O controle do principal reservatório e indiretamente ambiental pode ser um importante passo para resolução desse problema de saúde única.

2. OBJETIVO GERAL

Avaliar a eficácia inseticida mensal das formulações orais fluralaner na dose de 10–18 mg/kg (Defenza®) e afoxolaner na dose 2,5 a 5,2mg/kg + 0,5 a 1,07 mg/kg de milbemicina oxima (NexGard Spectra ®) contra *Tunga penetrans* em cães naturalmente infestados.

3. OBJETIVO ESPECÍFICOS

I - Identificar, classificar (Classificação de Fortaleza) e contar lesões cutâneas associadas a *T. penetrans.*

II - Viabilizar novas formulações comerciais eficazes para tratamento da tungíase em cães.

III - Avaliar a condição clínica do cães tratados através da Pontuação de gravidade para tungíase canina (SCADT) em relacionar com o grupo não tratado.

IV - Estabelecer escore e descrever os sinais clínicos mais comuns relacionados a *T. penetrans* em cães.

V- Acompanhar a evolução das lesões cutâneas dos cães do grupo tratado e comparar com o grupo controle.

VI- Realizar um levantamento a cerca das opções tratamentos mais recentes para tungíase em animais domésticos.

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ARTIGO CIENTÍFICO

A revisão de literatura, metodologia e resultados obtidos serão apresentados em forma de artigo científico, o qual foram publicados nos periódicos Tropical Medicine and Infectious Disease e Parasites & Vectors. Desta forma, a formatação do manuscrito aqui apresentado seguirá as normas do periódico.

4. REVISÃO DE LITERATURA

Tropical Medicine and Infectious Diseas

MDPI

Review

Treatment of Animal Tungiasis: What's New?

Katharine Costa dos Santos [1], Paula Elisa Brandão Guedes [1], Jamille Bispo de Carvalho Teixeira [1], Tatiani Vitor Harvey [2] and Renata Santiago Alberto Carlos [1,*]

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Abstract: In tropical and subtropical countries, particularly in disadvantaged communities, tungiasis is a severe public health problem, which is often neglected by the authorities. The sand fleas *Tunga penetrans*, predominant in endemic areas, and *Tunga trimamillata*, whose cases in humans are less frequent, are the cause of this zoonosis. Domestic animals are potential reservoirs and disseminators of tungiasis, so controlling their infection would significantly advance the prevention of human cases. This literature review compiles the most recent studies and innovations in treating animal tungiasis. Studies of approaches to the treatment of animal tungiasis, as well as disease control and prevention, are described. Isoxazolines are highlighted as promising drugs to treat animal tungiasis, with high efficacy and pharmacological protection. The positive impacts of this discovery on public health are also discussed, since dogs are an essential risk factor for human tungiasis.

Keywords: animal tungiasis; control; drug; zoonosis

Introduction

Frequent infections caused by fleas belonging to the genus *Tunga* spp. directly affect the lives of communities in endemic areas in Latin America and sub-Saharan Africa [1,2]. Tungiasis mainly poses problems in favorable climatic conditions, such as in tropical regions, especially in dry and sandy soils in poor communities, such as on the outskirts of cities, in slums, fishing villages, and rural and indigenous communities [3]. Recently, tungiasis was included on the list of neglected tropical diseases by the World Health Organization (WHO) and the Pan American Health Organization, classified in the group of scabies and other parasitic skin diseases [4], given its importance in endemic areas.

Tunga penetrans is the zoonotic species

most often associated with tungiasis in humans and domestic and wild mammals [5,6]. Cases involving *Tunga trimamillata* are less frequent than those caused by *T. penetrans* [7]. Although, most of the time the host has a high parasite load, *T. penetrans* infections usually are self-limited [8]. Lesions caused by *T. penetrans* predispose to secondary bacterial infections [9,10], which may progress to deformity, loss of digits, self-mutilation, septicemia, and death [11,12].

Direct contact of the host with contaminated soil predisposes adult female fleas to penetrate the skin, usually in the region of the feet and hands in human cases, and the pads in affected animals. After penetration, female fleas undergo hypertrophy, forming neosomes, which mature and lay their eggs, remaining in situ until the parasite's death, which occurs four to six weeks after penetration [13,14]. The maintenance of *T. penetrans* in the environment occurs mainly through reservoirs, including dogs, cats, pigs, cattle, and rodents, which spread the parasite eggs in the soil [8–15]. This fact contributes to human cases of tungiasis, since most of these reservoirs are domestic animals that live directly with humans, thus being a risk factor for the disease [16].

Wild animals can also be seriously affected by tungiasis, as indicated in reports of infections in anteaters [17,18] (Figure 1), monkeys [19], and jaguars [20]. However, little is known about the importance and relationship of these wild species with human tungiasis, as well as the maintenance of the cycle of the flea *T. penetrans*, the epidemiological profile and the treatment of tungiasis in these wild species. This has given rise to the need for further studies of the disease in these species, because they can act as potential disseminators of these fleas, since most exotic species travel through large areas and are mainly prevalent in rural zones and indigenous communities that are potentially endemic areas for tungiasis [3,17–20].

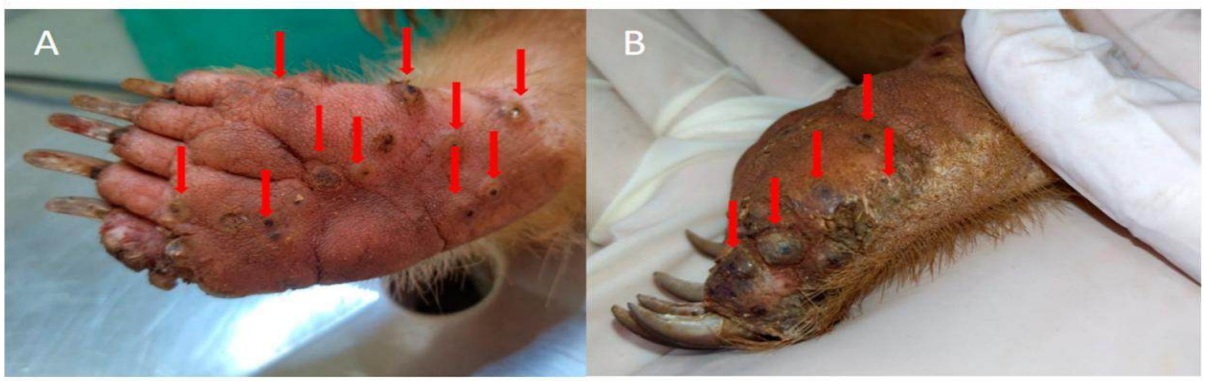

Figure 1. Anteater with *T. penetrans* in an endemic community for tungiasis in Brazil. (**A**,**B**) Paws of an anteater with numerous viable lesions caused by *T. penetrans.* Photos by Institute for Research and Conservation of Anteaters in Brazil.

Therefore, controlling *T. penetrans fleas* in animals can directly contribute to the prevention of human tungiasis [21], since animals favor the transmission and persistence of fleas in the environment [15,22]. In Brazil, dogs are considered one of the main carriers of fleas in the environment, while in African countries, pigs play this role [8,12]. As mentioned above, after the maturation of the eggs in the lesions present in the

parasitized hosts, the eggs are released into the environment, perpetuating the life cycle of fleas [15–21]. Due to the close relationship of environmental sharing between these animal species and humans, people are more exposed to tungiasis in places where infected hosts are present. These factors demonstrate the importance of treating infected animals, since reducing this parasitism will consequently reduce cases in humans, since there will be less or even no dispersal of fleas, which are the causative agents of tungiasis [2,8]. This would be a good strategy for public health authorities, through policies to control fleas in potential animal reservoirs. However, such policies cannot exclude other risk factors for tungiasis, such as poor housing conditions, substandard community hygiene, and traditional techniques for treating and controlling tungiasis [2,21,23]. Thus, control must be multifactorial and involve environmental and host control [15]. Manual flea extraction and treatment with topical antibiotic therapy to prevent complications is recommended for humans [3,9–24]. However, this method does not apply in most cases of animal tungiasis, since most animals are severely parasitized [21–25], as seen in Figure 2.

Concerning drug treatments for animals, which is discussed in this article, this occasionally has been used in animal tungiasis, such as the topical application of the organochlorine lindane (gamma-hexachlorocyclohexane) in pigs [26,27], the topical use of trichlorphone at 0.2% (Neguvon) in infested dogs [28], a 97% oily trichlorphone solution for dogs and cats, and collars impregnated with propoxur (carbamate), and flumethrin (pyrethroid) in dogs [22]. Other topical drugs have also been tested but showed toxic and carcinogenic potential [29,30]. There are also reports of using ivermectin to treat tungiasis in dogs [31,32]. However, controlled studies proving the effectiveness of drugs to treat and protect animals against *T. penetrans* are scarce [33]. Currently, a new perspective has emerged in treating tungiasis in dogs, the use of isoxazolines (e.g., fluralaner), which have demonstrated high efficacy [34,35].

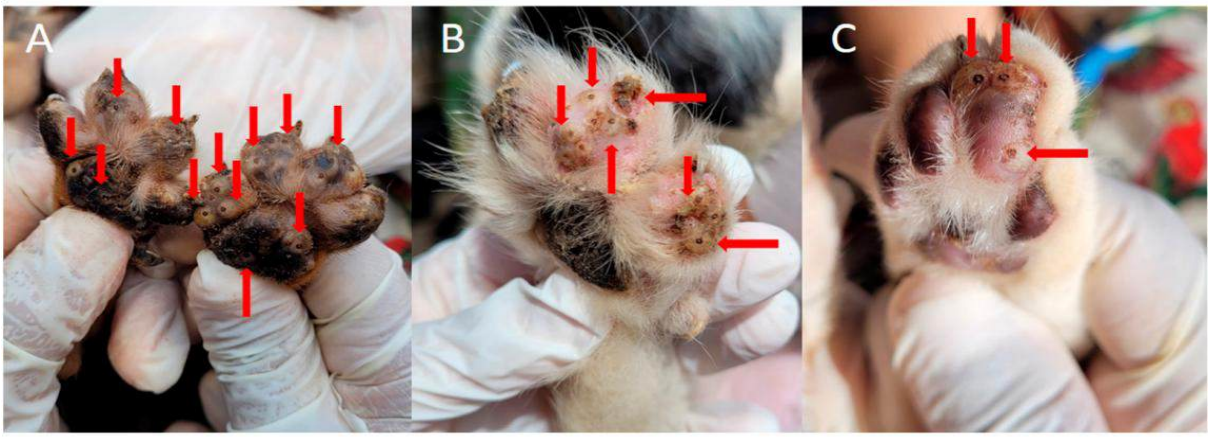

Figure 2. Animals infected with *T. penetrans* in an endemic community for tungiasis in Brazil. (**A**) Hind paws of a puppy with numerous viable lesions caused by *T. penetrans*. (**B**) Paw of a dog infected with several fleas, arrows demonstrating tungiasis lesions. (**C**) Cat paw with lesions compatible with viable tungiasis. Photos by Katharine Costa dos Santos.

This literature review provides a comprehensive critical assessment of the literary evidence regarding the treatment and prevention of animal tungiasis, as this disease is still a global public health challenge [6,36], presenting high morbidity and contributing to serious health problems in the affected individuals [16].

Challenges in the Treatment and Control of Animal Tungiasis

Tungiasis is a tropical parasitic disease that is generally neglected by public authorities, health professionals, and the pharmaceutical industry, even though it affects millions of individuals on different continents [6]. Public policies are scarce, and there needs to be more epidemiological, geographic, and clinical data on this zoonosis, which mainly affects socioeconomically excluded people [14,37]. Demographic and behavioral characteristics of the population directly influence tungiasis morbidity in endemic regions [2]. Therefore, the association of different prophylactic measures, contemplating the control of the main risk factors for tungiasis, is an assertive alternative to control this disease [21,38]. For effective tungiasis control, it is necessary to understand the dynamics of the

T. penetrans flea in the hosts and its development in the environment [12,13]. Flea penetration occurs on average within two days (stage I); after this period, abdominal hypertrophy begins (stage II), reaching maximum hypertrophy 2 to 3 days after complete penetration, with the formation of a white halo around the lesion 6 to 7 days after penetration (stage III). Subsequently, the lesion progresses to stage IV, which can remain for 3 to 4 weeks after penetration. At the end of the fourth week, the healing process begins (stage V), which can last until the end of the fifth or sixth week. During stages III and the beginning of stage IV, the parasite eggs are released and dispersed in the environment [14]. In the soil, the eggs hatch into larvae that feed on organic debris and develop, enabling reinfection to completing the cycle [13].

Sanitary and basic hygiene education is essential regarding tungiasis. For example, the habit of wearing shoes can increase the protection of the foot region against flea attacks in humans. However, in many endemic communities, this practice is uncommon [38,39], as seen in Figure 3. Moreover, the lack of paving, basic sanitation, selective garbage collection, and infrastructure in endemic areas contributes to the development of the parasite, since it adapts well to sandy soil and feeds on decomposed organic material [2,36]. Control measures must address all these issues, but the pharmacological control of fleas in reservoirs can promote a cascade effect, including control of the parasite in the environment and humans [15–24,40].

Figure 3. Relationship of environment, animals, and humans in a Brazilian community that is endemic for tungiasis. (**A**) Barefoot children playing on sandy soil next to a dog with tungiasis. (**B**) Barefoot child next to an infected dog. Photos by Katharine Costa dos Santos.

The climatic factor is also a significant challenge, since in some regions, the parasite's presence and reproduction occur in all seasons of the year [41], unlike others where peak infection mainly occurs in the dry period [42]. Thus, in regions with no seasonality for the disease, care needs to be constant, demanding higher investment and a better control strategy by public authorities and the affected population.

Another challenge in tungiasis control is that in endemic regions, it is common for most domestic animals to be semi-domiciled, with free access to the community, which enables infected animals to spread *T. penetrans eggs* throughout the environment [43]. Additionally, it is challenging to control parasites in animals in these areas due to the lack of financial resources by the population for the adequate treatment and control of fleas [3,25]. Because of this, people commonly treat animals with tungiasis using techniques that can sometimes harm their health, such as non-sterile removal [25,44]. As mentioned by Harvey et al. (2017), the instruments most used to remove fleas from dogs in an endemic area in Brazil included needles, pins, thorns, or pliers, used both for removing lesions in humans and animals [44]. This conduct predisposes to bacterial infections, increased inflammation, and in humans, potential transmission of viral pathogens such as HIV, hepatitis B, and hepatitis C [45,46]. In animals, complications such as concomitant infestation by other parasites (e.g., myiasis) can also occur in tungiasis lesions, acting as an aggravating factor [16], as observed in Figure 4. Thus, the challenges in controlling animal tungiasis also comprise the animal's well-being, which is sometimes seriously affected [11]. Disease control in animals considered reservoirs, such as dogs, can be a viable option to control tungiasis in endemic areas [44]. However, there are few clinical studies evaluating ectoparasiticides' effectiveness against *T. penetrans* to control and treat infections in domestic animals [35–39]. Thus, conducting new tests is vital to develop effective and economically viable options for society.

Figure 4. Complications of canine tungiasis. (**A**) Presence of myiasis in a lesion caused by *T. penetrans* in the paw of an infected dog. (**B**) Dog paw with tungiasis associated with secondary bacterial infection with necrotic tissue. (**C**) Dog paws with *T. penetrans* lesions showing suppuration and secondary infection. Photos by Katharine Costa dos Santos.

Tested Treatments for Animal and Human Tungiasis

The prominence of dogs and pigs in tungiasis has been evident since the first attempts at treating the disease. The first insecticides tested were based on organochlorines [26,27]. In 1967, lindane (gamma-hexachlorocyclohexane) was used topically on pigs infected with
T. penetrans [26]. In 1976, another report of the use of organochlorines in infected pigs was also described, with resolution of the cases [27]. These works reported the elimination of fleas with these drugs, but this class of pesticides is currently prohibited in many countries due to its toxicity and environmental contamination [26,27]. These studies did not present data to support the prevention of new infections, and the authors only reported an improvement in the tungiasis condition of the animal host.
In 1989, a case report also described the use of trichlorphone 0.2% (Neguvon) in cases of infected dogs, with reduction in fleas in the animals studied [28]. More recently, in 2008, another study tested a 97% solution of trichlorphone in oil and found it ineffective against *T. penetrans* in dogs and cats. In the same study, collars impregnated with propoxur (carbamate) and flumethrin (pyrethroid) were also tested and showed low efficacy against *T. penetrans* in infected dogs [22].
After this period, some studies of pharmacological treatments against tungiasis were reported in humans, such as oral ivermectin. In 2003, a human study evaluated the topical use of ivermectin (0.8%), metrifonate lotion (trichlorfon, 0.2%), thiabendazole lotion (5%), and thiabendazole ointment (5%). The authors observed that these active principles could significantly reduce the number of lesions. The groups were evaluated 3, 7, and 12 days after treatment, and on day 12, the authors reported that almost all fleas were dead. A decrease in viable lesions was observed during this period, with no significant difference between the treatment groups. However, the authors pointed out that further studies would be needed to optimize the doses and administration of these medications [47]. Another study, in 2004, demonstrated that oral ivermectin for humans did not show significant clinical efficacy against *T. penetrans* at the administered dose (300 µg/kg of body weight in a single dose, repeated after 24 h) [48].
In dogs, ivermectin for treatment of tungiasis was used for some time in isolated infections, as in the case report of a dog with tungiasis, in which ivermectin (Ivomec® 1% injection solution) was administered subcutaneously at a concentration of 0.3 mg/kg, causing total disappearance of the lesions about one month after treatment [31]. Ivermectin was also utilized in another case of infection in dogs in a rural area endemic to *T. penetrans*. In that study, the result after using the medication was not described [49].
Additionally, topical solutions tested in humans have proved to be effective in treating tungiasis, but their applicability and effectiveness have yet to be investigated in animals. They may also be economically viable options for the control of animal tungiasis [24,50–52], as demonstrated in a study of human tungiasis in 2009, with topical application of coconut oil (80%) associated with neem seed oil (20%) in the NC group and bathing the feet of patients with KMnO4 (single treatment; 15 min application on day 1). Both treatments led to the

elimination of fleas, although neem and coconut oil contributed to significant clinical improvement in acute pathology. On average, 67% of all live fleas in the NC group and 37% in the KMnO4 group showed abnormal development (early senility). Additionally, 64% of the patients treated with KMnO4 and 78% of those treated with NC showed a reduction in pain. Regarding itching, 67% of patients treated with NC and 60% with KMnO4 reported a decrease in itching within seven days. However, the neem/coconut oil mixture used in that trial was no longer effective in killing embedded *T. penetrans* after seven days, killing an average of 30–40% of fleas within six days [52].

Another example was a study conducted in Madagascar in 2013, which evaluated the effectiveness of applying an active plant-based repellent (Zanzarin®) against *T. penetrans* in infected humans. The formulation proved to be effective, and the intensity of the infestation decreased during the 10-week observation period, achieving an attack rate of zero (median) after using the repellent. Furthermore, the morbidity associated with tungiasis was reduced to an insignificant level [24]. However, the product is not commercially available in tungiasis-endemic countries.

In 2017, the topical administration of NYDA, an association of two dimethicones, was also evaluated against human tungiasis. The results revealed that seven days after treatment, 78% of those treated lost all signs of flea viability, and 90% of penetrated fleas showed abnormal development five days after treatment. In general, there were decreased signs of inflammation in the group treated with NYDA [51]. Mutebi et al. (2021) cited the treatment of a goat with paws infected by *T. penetrans* with the same active principle and obtained a positive result, as shown by the images presented in that study. Parasite death was described two days after using the formulation [11]. However, the authors stated that studies of the effectiveness of the formulation were needed before it could be recommended for animal use. Sometimes, effective drugs against human tungiasis are used to treat animal tungiasis due to the lack of commercial formulations tested for the treatment and control of this disease in domestic animals.

Advances in Treatments of Animal Tungiasis

Concerning the treatment of animal tungiasis, a study conducted in 2005 evaluating the combination of 10% imidacloprid and 50% permethrin (Advantix®) demonstrated effectiveness, according to the researchers, of this formulation against tungiasis lesions in dogs. In the field trial, 17 dogs infected with *T. penetrans fleas* were topically treated with the Advantix® formulation, while 17 remained untreated. Seven days after treatment, the authors observed a lower flea load in the treated dogs. An efficacy of 80% was achieved in the group treated on day 14 and 86% on day 21, but on day 28, there was already a decrease to 53%, while all dogs in the control group were parasitized. So, most dogs were free of tungiasis lesions in the treated group, while in the untreated group, the flea burden remained high [29].

Additionally, a case report described the treatment performed on a dog with lesions compatible with tungiasis identified on pads. The lesions were surgically removed, with the subsequent daily use of fipronil spray for seven days. There was complete recovery, but it was impossible to infer whether there was efficacy in the treatment with fipronil, since it was applied after the removal of the lesions [53].

In 2016, a topical aerosol containing chlorfenvinphos 4.8%, dichlorphos 0.75%, and gentian violet 0.145% (Supona® aerosol) showed some tungicidal efficacy by improving the morbidity associated with tungiasis in pigs. The study evaluated two groups, 29 in the treatment group and 26 in the control group. One week after treatment, 58.6% of treated pigs had no viable lesions compatible with tungiasis, while all control pigs had at least one viable lesion. The study demonstrated that topical treatment was influential in the treatment of porcine tungiasis [30]. However, the study did not evaluate the residual period of the drug to infer how long the combination of active principles would be effective against *T. penetrans*. Evaluations were only performed on days 0 (pre-treatment) and 7 (post-treatment).

In 2016, an aerosol product containing 4.8% chlorfenvinphos, 0.75% dichlorphos and 0.145% gentian violet (Supona® aerosol) was used to treat two severe cases of tungiasis in two infected goats in Uganda, Africa. In these cases, the kids presented viable lesions for tungiasis (stages II and III), and in the evaluation one week after the treatment, the animals no longer had viable lesions. They also had no clinical signs of bacterial infection and the hooves were in the process of re-epithelialization and moved normally. Despite reports of the

tungicidal effect of Supona® aerosol, this formulation was never validated through controlled studies for use in goats [54].

Regarding bovine tungiasis, a report of cattle infected by *T. penetrans* described the topical treatment of the hooves and teats with the direct application of a 4% trichlorfon solution in the footbath, where all the cattle recovered in approximately 20 days. However, the remaining fleas in the environment possibly contributed to re-infection [55].

Tungiasis directly affects the productivity of livestock. Difficulty walking to feed; lesions in the mammary glands that make it impossible for the calves to feed, in turn causing malnutrition and inability to develop; infertility of males that have severe lesions in the testicles; and pain promote this drop in productivity [56,57].Currently, the options of ectoparasiticides for the treatment of livestock infested with

T. penetrans are limited. Hence, there is an urgent need to investigate the prophylactic effects of alternative formulations for the management of tungiasis in these animals, avoiding the decrease in their production and elimination of drug residues in their final products, with the overall goal of reducing the economic impacts of tungiasis in endemic regions severely affected by the disease.

Wild animals, as well as pets and livestock, can be directly affected by *T. penetrans* infections. The clinical signs are usually related to the severity of the infections and can interfere with the execution of common activities by these animals, such as difficulty in walking, making these animals more susceptible to their predators [17–20], or hampering their ability to hunt for food [19].

Although studies on tungiasis in wild animals are limited, some reports have described treatments in different wild species rescued or referred for medical care at specialized centers. In 2017, an anteater (*Myrmecophaga tridactyla*) was rescued and multiple lesions on the paws caused by *T. penetrans* fleas were observed. The animal was treated with ivermectin at 0.2 mg/kg, subcutaneously (Ivomec®), with a second dose applied 14 days after the surgical removal of the lesions [17]. In 2022, a monkey (*Alouatta guariba clamitans*) infected with *T. penetrans* was treated with topical application of 5.7 mg of nitenpyram (Capstar®) in a single dose, together with ivermectin at a dose of 0.2 mg/kg subcutaneously and 2.5 mL (SC) of anti-tetanus serum [20]. Both treatments caused improvement of lesions, but the authors did not report how many days it took after treatment for the animals to be free of viable lesions.

Although some previous works have reported effectiveness with the mentioned concentrations, the drugs cannot be considered to have clinical relevance as antiparasitics since, according to the guidelines for evaluating the effectiveness of antiparasitic substances for the treatment and prevention of ticks and fleas in dogs and cats, the European Medicines Agency (EMA) defines that the efficacy of the standard product must be at least 95% for adult fleas and at least 90% for the inhibition of the emergence of adult fleas. In addition, they must be nontoxic to the treated animal, and in the case of tungiasis, they must be resistant to water (e.g., rain) [58].

A Promising Discovery for the Treatment of Animal Tungiasis

A recent study has revealed the efficacy of an active ingredient belonging to the isoxazoline group, fluralaner, which showed excellent systemic and persistent insecticidal efficacy. This active ingredient blocks γ-aminobutyric acid (GABA) and glutamate-dependent chloride channels in neurons, interrupting the conduction of inhibitory stimuli and causing exacerbated excitation, paralysis, and death of the parasites [34,59]. Fluralaner is a systemic acaricide and insecticide with prolonged efficacy in animals. The drug has commercial oral formulation in the form of palatable tablets at a dosage equivalent to 25–56 mg/kg of bodyweight for dogs [35].

In the study cited above, fluralaner's therapeutic and residual efficacy was evaluated for treating dogs naturally infected with *T. penetrans* in a field trial challenge. Sixty-two dogs from an endemic community were randomized in a controlled double-blind study in which 31 dogs received oral fluralaner (*Bravecto*® chewable tablets) at doses of 25 to 56 mg of fluralaner/kg and 31 animals (control group) received no treatment. After the administration of the formulation, more than 90% of the dogs in the treated group were free of *T. penetrans lesions* between days 14 and 90, and the treatment was 100% effective on days 21, 28, and 60. The efficacy was about 97% on day 90, dropping on day 120 to 84%, reaching 6% on day 150 against *T. penetrans*. The flea counts on *fluralaner-treated* dogs were significantly lower ($p < 0.025$) than on the control dogs at intervals from day 7 to 120. The authors of this study concluded that a single oral dose of fluralaner was

effective for treatment, achieving long-term prevention (greater than 12 weeks) of tungiasis in dogs, without side effects on the animals [35].
In addition to evaluating the effectiveness of the Bravecto® in a chewable tablet formulation, the study clinically evaluated all dogs using a canine acute tungiasis severity score (SCADT), which related clinical signs common to tungiasis, such as pain, hyperemia, erythema, among others, as well as the number of viable lesions caused by *T. penetrans* according to the experimental group. It was observed that from day 7 to day 120, the mean SCADT scores were significantly reduced in treated dogs, with an average of 0.10 compared to 1.54 at day 120 for untreated dogs [35].

Conclusions

Since human and animal tungiasis are closely related, the effective treatment of domestic animals for *T. penetrans*, especially in endemic areas, can improve control of the disease in humans. Despite the existence of many studies on the treatment of animal tungiasis, most of these studies have shortcomings, such as the failure to apply randomized blinded designs to avoid biases, preventing readers from defining, for example, the percentage of effectiveness of the formulations tested.
Given the therapies tested so far to combat tungiasis in dogs, one of the main reservoirs and dispersers of the flea in the environment, the results of efficacy have not demonstrated effectiveness of the use of most of the substances tested so far. However, research carried out with fluralaner has shown it to be an effective alternative, although its high cost in some places often makes its use unfeasible for some people living in endemic areas. Thus, new studies with other isoxazolines and active ingredients focused on evaluating the effectiveness against animal tungiasis should be carried out, since empirical studies describing treatments do not contribute to controlling this zoonosis. In addition to this class, phenylpyrazoles can also be an option for treating *T. penetrans*, such as the commercial product fipronil, which has a topical pulicidal effect. However, its effectiveness against *T. penetrans* has yet to be *evaluated*.
Additionally, it is important to notice that fluralaner and other commercial medications have been shown to be effective for use in companion animals; however, there are currently no commercial products available that are effective for use in production for animals.

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6. CAPÍTULO I

6.1 ARTIGO CIENTIFÍCO II

RESEARCH **Open Access**

Efficacy of monthly treatment with oral fluralaner (Bravecto® 1-Month) against *Tunga penetrans* in dogs in Brazil: a randomized, double-blind, controlled field study

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Abstract

Background Tungiasis is a neglected tropical disease caused by the adult female sand flea (*Tunga penetrans*). Dogs are considered important reservoirs of *T. penetrans* in Brazil. The aim of this study was to determine the monthly insecticidal efficacy of a single oral administration of fluralaner at a dose of 10–18 mg/kg (Bravecto® 1-Month, also registered as Defenza in some countries; MSD Animal Health) in dogs naturally infested with *T. penetrans*.

Methods This clinical trial was conducted in a rural community located in Ilhéus, Bahia, Brazil. A total of 64 dogs were selected and distributed in a completely randomized design between a treated group (TG) that received one® single dose of Bravecto® 1-Month (Defenza®) and a negative control group (CG) that received no treatment. Each group was composed of 32 dogs. The evaluations took place on days 0, 7 ± 2, 14 ± 2, 21 ± 2, 28 ± 2, 35 ± 2, and 42 ± 2 post treatment, in which the dogs were inspected to evaluate the infestation stage and classify lesions associated with tungiasis. The primary efficacy was determined from the percentage of treated dogs free of fleas (stage II and III lesions) after administration of the formulation at each evaluation time. Secondary efficacy was based on the number of active lesions (stages II and III) in each group at each evaluation time. The clinical condition of the animals was defined based on the Severity Score for Acute Dog Tungiasis (SCADT), which is related to the number and severity of lesions.

Results The primary efficacy of the product was greater than 95.0% from days 7 to 21 and reached 100.0% between days 28 and 42, with a significant association between treatment and infestation decline ($P < 0.025$) between days 7 and 42. Secondary drug efficacy was greater than 99.9% from days 7 to 21, reaching 100.0% between days 28 and 42 ($P < 0.05$). The treated dogs also scored lower on the SCADT than the control animals did during the entire clinical evaluation period ($P < 0.05$).

Conclusions A single administration of Bravecto® 1-Month (Defenza®) was effective in eliminating *Tunga penetrans* infestations, as well as in preventing parasitism for at least 42 days after treatment.

Keywords Canine tungiasis, Ectoparasitosis, Treatment, Rural communities, Zoonosis

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Background

Tungiasis is a neglected tropical disease (NTD) [1, 2], caused by the adult female sand flea (*Tunga penetrans*) [3]. Dogs can also be infested by *T. penetrans* [4, 5]. Direct contact with contaminated soil predisposes the host to penetration, which usually occurs in the paw regions. After penetration, fleas hypertrophize their neosomes, mature, and eliminate their eggs, remaining in situ until their death, which occurs 4–6 weeks after penetration [4–7]. In most cases, the hosts have a high parasitic load [8], predisposing them to complications such as secondary bacterial infections [9, 10] that progress to deformity and/or loss of digit(s), self-mutilation, septicemia, and death in dogs [11].

In Brazil, dogs are the most affected domestic reservoir and are considered the main disseminators of these ectoparasites [8–14]. Moreover, close contact with this species is considered one of the main risk factors for *T. penetrans* infestation in humans [15]. The high prevalence of tungiasis in humans and animals is associated with low socioeconomic indicators in endemic communities [4, 7], especially in fishing villages [8, 10], indigenous communities [5], slums [9], and rural areas [8].

The participation of dogs in the dynamics of the disease is evident in endemic locations, as these animals have a wide circulation area and cover large perimeters, spreading flea eggs in the environment and facilitating the establishment of the parasite's biological cycle [15, 16]. Environmental contamination caused by infested animals can be prevented by controlling the fleas in the host. This would cause a decrease in reservoirs and a subsequent reduction in prevalence rates in humans and animals [17, 18].

In both humans and animals, the distal regions of the limbs are most affected by infestations because of frequent contact with contaminated soil [14]. However, ectopic lesions on the lips, muzzles, elbows, mammary glands, and genitals are not rare [11]. Infested dogs commonly harbor tens or hundreds of sand fleas, which favor the formation of lesion clusters and aggravate the disease [15, 17–19].

Currently, only a few treatments are available for canine tungiasis. The combination of 10.0% imidacloprid and 50.0% permethrin (Advantix®) has approximately
80.0% efficacy in treating and preventing *T. penetrans* reinfestations for 14 days; however, the efficacy decreases significantly to approximately 30.0% in the following week [20]. In addition, subcutaneous treatment with ivermectin (Ivomec 1.0%) healed the lesions in infested dogs 1 month after administration [21]. However, none of these treatments are acceptable for pesticide use according to the European Medicines Agency, which defines the minimum efficacy of a standard product as 95.0% for adult fleas and at least 90.0% for emerging adult fleas [22].

Recently, treatments with a single dose of 25–56 mg/kg fluralaner (Bravecto®), a molecule from the isoxazoline group, showed high and persistent systemic insecticidal efficacy against *T. penetrans* in dogs from day 7 to 120 post treatment. The number of live fleas in treated dogs was reduced by > 90.0% on day 7, > 95.0% between days 14 and 90, and 100.0% from day 21 to 60 [23].

However, considering the cost and feasibility of administering fluralaner with quarterly effectiveness, administration of the same molecule at a shortened duration would enable its use in cases of low environmental pressure or low resources, as is the case in deprived communities. Moreover, the treatment may serve as a tool

for public managers of future programs to control this disease and a feasible option for use in dogs that travel to endemic areas for a short period of time, ensuring the protection of these animals [23].

BravectoThis study presents results of a clinical study on the ® 1-Month (also registered as Defenza® in some countries; MSD Animal Health) formulation to control and prevent canine tungiasis. The study sought to determine the efficacy of the formulation against *T. penetrans* in naturally infested dogs, treated once orally, as well as the impact of the treatment on the clinical conditions of the treated animals. Additionally, acute clinical signs of tungiasis during the study period are listed.

Methods

Study area and population

The study was carried out in the semi-rural community ′ ′ Vila Juerana, Ilhéus, Bahia, Brazil (14° 47 00" S, 39° 03 00" W). The village is located at a mangrove and beach region that is considered as a tourist area of the Costa do Cacau region, with an annual temperature variation of 22–25 °C, regular rainfall distributed throughout the year, and a humid tropical climate [24]. Most of the village community have precarious sanitary and social conditions. The streets are unpaved and consist of sandy and clay soil, and some buildings are unfinished without concrete floors [8, 25]. Moreover, the study area had previously shown a high year-round prevalence of tungiasis in dogs [8, 16, 23–26].

Prior to the start of the study, dog owners were informed about the research objectives and methodology and provided written consent for the inclusion of their dogs.

As inclusion criteria, dogs were ≥ 8 weeks in age, had body weights ≥ 2.5 kg, had adequate temperament to allow clinical and parasitological evaluations, and presented at least one live flea at stage II or III lesion in the Fortaleza classification [3]. The selected dogs underwent clinical evaluation, and only animals with no clinical signs other than tungiasis were enrolled in the study, regardless of sex or breed. Dogs treated with fluralaner less than 90 days before day 0 or with other ectoparasiticides with short acting activity within 14 days before day 0 (isoxazolines, amitraz, fipronil, macrocyclic lactones, or pyrethroids), as well as pregnant or lactating female dogs, were not allowed to participate in the trial. Throughout the study period, the dogs remained under the care of their owners and maintained their usual routines.

Study design

This field study was randomized, negatively controlled, and double-masked. On day 0, 64 dogs were randomly distributed (using a prior computer-generated list) into one of two experimental groups, each composed of 32 dogs. The dogs were implanted with numerically coded microchips for individual identification. The clinical history of each dog was recorded, and each dog underwent a complete physical examination, including ectoparasite assessment.

Study activities were distributed between the two veterinary teams. One team assigned the animals to groups, administered the treatments, and did not participate in the clinical evaluations. The other team performed the clinical evaluations and inspections of the dogs and was blinded to the treatment assignments. Dogs in the treatment group (TG) received a single oral fluralaner dose (Bravecto® 1-Month/Defenza®) at the approved dose rate of 10–18 mg fluralaner/kg body weight, according to label instructions, on day 0. The dogs in the control group (CG) remained untreated.

All animals were treated on the same day, received wet commercial dog food, and the owners were not informed of the group to which their dogs were assigned. During the study, if necessary, dogs were treated for any observed secondary complications of tungiasis as the treatment presented no insecticide activity or any other routine health needs. The administration of any other parasiticidal drug or product with insecticidal activity was prohibited. In the end of the study, all 64 dogs were treated with a dose of ® 25–56 mg/kg of fluralaner (Bravecto).

Clinical evaluation, skin inspection, and lesion documentation

Dogs were evaluated weekly with seven scheduled examinations for each dog including days 0 (enrolment and treatment), 7 ± 2, 14 ± 2, 21 ± 2, 28 ± 2, 35 ± 2, and 43 ± 2.

At each visit, the dogs underwent a general physical examination and detailed skin inspection. The entire body of the animal was examined, with special attention paid to the paws, limbs, tail, mammary glands, abdomen, testicles, and nose. Before the examination, the dogs' paws were cleaned using water and a brush to improve the detection of all lesion stages. The identified lesions were counted and staged according to the Fortaleza classification [3].

Severity Score for Acute Dog Tungiasis (SCADT) were also assigned to each dog to record the severity of tungiasis lesions throughout the study. Each clinical sign was scored for each affected area and the results were added to obtain the SCADT (Table 1). The maximum possible score for this classification was 27. If the SCADT score exceeded 22, the dog would be treated by surgical removal of sand fleas and excluded from the study for ethical reasons; however, this procedure was not required during the trial. All lesions were classified, quantified, photographed, and documented in the parasitological skin examination records.

Table 1 Severity score for acute clinical signs of designated topographic tungiasis

Clinical signs	Number of locations affected	Score
Hyperemia and/or edema[a]	1–5	1
	6–10	2
	11–16	3
Pain on digital pressure	1–5	1
	6–10	2
	11–16	3
Suppuration and/or formation of abscesses[a]	1–5	1
	6–10	2
	11–16	3
Clustering of lesions [b]	1–5	1
	6–10	2
	11–16	3
Fissure(s) [a]	1–5	1
	6–10	2
	11–16	3
Skin ulceration	1–5	1
	6–10	2
	11–16	3
Mutilation of lesions regardless of the sites involved [c]		2
Altered gait/lameness		3
Ectopy of lesions		0.5 [d]

[a] Regardless of the number of foci and the size of the area involved [b] Three or more lesions close together (1–2 mm apart) [c] Mutilation of lesions indicates severe itching [d] For each ectopic body part involved, up to a maximum of eight ectopic sites; maximum four points

Therefore, the maximum individual score (SCADT) for a dog was 27 (23 + 4)

Statistical analysis

Statistical analysis was performed to assess the treatment efficacy using R version 3.6.1 software, with dogs as the experimental unit. The primary efficacy assessment of the product was based on the percentage of dogs free of live fleas (stages II and III) in the treatment group. The 95.0% confidence limits for the percentage of dogs free of live *T. penetrans* were calculated as Wilson scoring intervals. At each time point of post treatment evaluation, Fisher's exact test (unilateral, using Casagrande, Pike, and Smith [27] continuity correction) was used with a significance level of $\alpha = 0.025$ to compare the percentage of dogs free of parasites between the TG and the CG. The resulting *P* values, odds ratios (OR), and 95.0% confidence limits for the OR were obtained from the PROC FREQ using the Taylor series approach.

Secondary efficacy was calculated according to the number of live fleas (stages II and III) in each group on each evaluation day by calculating geometric and arithmetic means using the following formula:

$$\text{Secondary efficacy}(\%) = 100\times \frac{\text{Dc at day X} - \text{Dt at day X}}{\text{Dc at day X}}$$

where *Dc* is the mean number of live fleas (geometric and arithmetic) in the control group (total lesions/number of animals), *Dt* is the mean number of live fleas (geometric and arithmetic) in the treated group (total lesions/number of animals), and *X* is the experimental day.

Notably, the geometric mean is used in cases of counting zero fleas on a dog, according to the following equation:

$$\underset{Geometricmean}{[X_g]} = \left(\prod_{i=1}^{n}(X+1)\right)^{\frac{1}{n}} - 1$$

where *Xg* is the geometric mean and *n* is the number of individuals in the group.

To be considered effective, a flea product must reduce parasites by more than 90.0% [28]; therefore, this percentage was used in this study to assess formulation effectiveness.

Arithmetic mean and mean SCADT scores in the two groups were compared at each post-treatment evaluation timepoint using Shapiro–Wilk test to identify the normality of data. If the data distribution was nonnormal, the Mann–Whitney *U* test was performed with the level of significance set to $\alpha = 0.05$ (two-sided).

Results

The study dogs, aged between 3 months and 11 years, were all mixed breed, intact, and weighed between 2.5 and 33.3 kg. On day 0, before treatment, *T. penetrans* mean live flea counts were 10.3 ± 9.8 in treated dogs and 11.5 ± 13.9 in untreated dogs, whereas the flea count per dog was 1–94 fleas on treated dogs and 1–108 on control dogs. The study groups were comparable on day zero in terms of age, weight, sex, and SCADT distribution. The data from the animals in each group are presented in Table 2.

hree dogs died during the study, but none of the deaths were related to the administered treatment. One dog from the control group died due to the ingestion of a toxic substance applied at home by the owner, and the other two animals (one from the control group and one from the treated group) were hit by cars. However, data collected until the time of their deaths were considered in the analyses. No adverse clinical effects such as systemic allergic responses, vomiting, or diarrhea were reported.

Table 2 Age, weight, sex, breed, flea count, and SCADT on day 0.

	Treated group	Group control
Number of dogs (*n*)	32	32
Age (years) (mean ± SD)	3.4 ± 2.4	3.9 ± 3.1
Age (median)	3	3
Age range	3 months to 10 years	3 months to 11 years

Body weight (kg) (mean ± SD)	9 ± 4.7	9.4 ± 6.8
Body weight (median)	8.1	7.7
Body weight range (kg)	3–19.7	2.5–33.3
Intact females	14 (43.2%)	15 (46.8%)
Intact males	18 (56.2%)	17 (53.1%)
Mixed breed	32 (100.0%)	32 (100.0%)
Flea count (mean ± SD)	10.3 ± 9.8	11.5 ± 13.9
Flea count (median)	7.5	6
Flea count range	1–94	1–108
SCADT (mean ± SD)	3.0 ± 2.6	2.3 ± 2.2
SCADT (median)	2.5	2.0
SCADT range	0–14	0–9

SD standard deviation

Table 3 Number and percentage of flea-free dogs by study day and study group

Day	Treated group		Group control		*P* value [b](one-sided)	OR
	Free from fleas (*n*)	Percentage (%)	Free from fleas (*n*)	Percentage (%)		OR [c] [95% CI[a]]
7	31 (32)	96.9 (84.2–99.4)	1 (31)	3.2 (5.5–15.7)	< 0.0001	930.0 [55.1; 15,554.1]
14	31 (32)	96.9 (84.2–99.4)	7 (31)	22.6 (11.4–39.8)	< 0.0001	106.3 [12.2; 923.5]
21	31 (32)	96.9 (84.2–99.4)	7 (30)	20.0 (11.7–40.9)	< 0.0001	101.7 [11.1; 886.4]
28	31 (31)	100.0 (88.9–100.0)	11 (30)	36.7 (21.8–54.4)	< 0.0001	50.5 [6.4; 448.4]
35	31 (31)	100.0 (88.9–100.0)	10 (30)	33.3 (19.2–51.2)	< 0.0001	52.0 [7.4; 552.3]
42	31 (31)	100.0 (88.9–100.0)	8 (30)	26.7 (14.1–44.5)	< 0.0001	85.2 [9.9; 731.5]

[a] *CI* confidence interval [b] Fisher's exact test, $\alpha = 0.025$ [c] *OR* Odds ratio, *CI* Confidence Interval

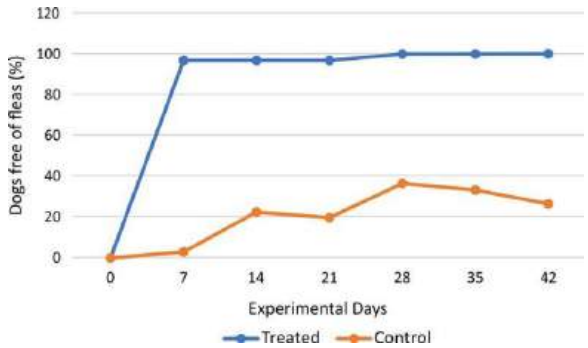

Fig. 1 Percentage of flea-free dogs on each assessment day by study group

Primary efficacy

Data related to primary efficacy (calculated based on the number of animals without fleas) are shown in Table 3 and illustrated in Fig. 1. From days 7–21, the primary efficacy remained above 95.0%, reaching 100.0% (31/31) between days 28 and 42. In the CG, the proportion of parasite-free animals did not exceed 40.0% throughout the study period. Statistical comparison of parasite-free individuals between the TG and CG demonstrated that treatment resulted in significant differences between the groups on each experimental day throughout the study from day 0 to 42 ($P < 0.0001$, statistical values of each evaluation day are in Table 3). Therefore, the treatment was effective in preventing *T. penetrans* infestation in dogs.

Secondary efficacy

The secondary efficacy (based on the number of live fleas) of fluralaner on day 7 was 99.7%, remaining above 99.0% from days 7 to 21, and reaching 100.0% from days 28 to 42. The arithmetic and geometric means were similar on all experimental days in the treated group. The arithmetic mean of live fleas was significantly low in the treated dogs ($P < 0.0001$, statistical values of each evaluation day are in Table 4) from days 7 to 42. The arithmetic means of live fleas in the CG varied between 3.7 and 7.1 and in the TG varied between 0.0 and 0.4, during all the experimental days of the study.

The geometric means of live flea counts for each study group at all evaluation times are shown in Fig. 2.

Table 4 *Tunga penetrans* counts (percentage of efficacy and *P*-values) in treated and untreated dogs on all treatment days

Day	Geometric mean live flea counts			Arithmetic mean live flea counts			*P* value	Statistical value (*W*) W
			Efficacy %			Efficacy %		
	TG	CG		TG	CG			TG
0	6.84	7.07	–	10.34	11.56	–	0.839	472.5
7	0.01	4.08	99.7	0.01	6.17	99.8	< 0.0001	975
14	0.02	4.00	99.5	0.03	5.00	99.7	< 0.0001	874.5
21	0.03	4.86	99.4	0.04	7.10	99.4	< 0.0001	856
28	0.00	6.87	100.0	0.00	5.00	100.0	< 0.0001	759.5
35	0.00	5.40	100.0	0.00	4.50	100.0	< 0.0001	775
42	0.00	3.52	100.0	0.00	3.70	100.0	< 0.0001	821.5

Wilcoxon test (Mann–Whitney *U* test), $\alpha = 0.05$

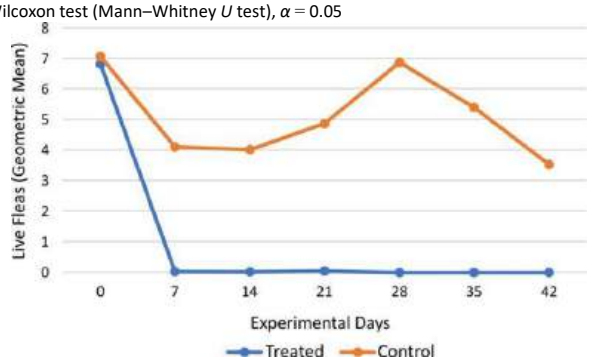

Fig. 2 Geometric means of live flea counts by study group over time

The presence of lesions before and after treatment was monitored using the photographic records of the enrolled dogs. Examples of these three animals are shown in Figs. 3, 4 and 5. **Total SCADT**

The means of the SCADT by study day and study group as well as the amplitude ranges are shown in Table 5. The mean SCADT score differed significantly between the groups from days 7 to 42 ($P < 0$ 0.05, statistical values of each evaluation day are in Table 5). On days 35 and 42, the mean severity scores in the treatment group were zero.

Discussion

The Bravecto® 1-Month (Defenza®) formulation, with monthly indication, was chosen for the study due to its potent systemic active compound from the isoxazoline family with acaricidal and insecticidal action. The formulation is effective against infestations caused by several species of ectoparasites [29–31], including *T. penetrans*® fleas [23], and has a lower cost than that of B ravecto chewable tablet, which is effective for 3 months.

Fluralaner is a potent inhibitor of the GABA-controlled chloride channel with specific action on arthropods, consequently causing the death of the ectoparasite [32]. The systemic action of fluralaner is an important pharmacological characteristic because its effect is not affected by rain or bathing, which represents an advantage when compared to topical products with the same pharmacological action [33, 34]. This particularity is relevant for dogs raised in semi-domesticated areas, as they are exposed to rainwater and swim in rivers and seas, as was the case for the animals in this study. Fluralaner at a dose of 10–18 mg/kg (BravectoWe observed that a single oral administration of fluralaner ®) effectively protected dogs against ® 1-Month/*T. penetrans* infestation for at least 6 weeks under field conditions in an endemic region with high infestation rates (i.e., high environmental exposure). In the first 3 weeks after treatment, the primary efficacy was greater than 95.0%, reaching 100.0% protection in the 4th week and remaining for the following 2 weeks. Notably, this is one of the first monthly formulations tested with proven efficacy against *T. penetrans* in dogs. Considering that the monthly formulation of fluralaner presented similar results to those achieved by the quarterly formulation for the treatment of canine tungiasis [23], we inferred that even at a low dosage, the effectiveness of the active principle in the monthly formulation would remain high.

Fig. 3 Evolution of the lesions through the study for dog 36 of the TG. **a**, **b** On day 0, the dog had multiple lesions caused by *T. penetrans* in stages II and III located on the pads (arrows). **c** On day 7, after treatment, the footpads no longer present any vital lesions; it is possible to observe lesions in stages IV and V in the anterior and posterior paws (arrows). **d** On day 21, the pads still remain without vital lesions. **e**, **f** On the 35th and 42th day of evaluation, respectively, the pads were free of *T. penetrans* lesions and completely re-epithelialized. Source: Personal archive

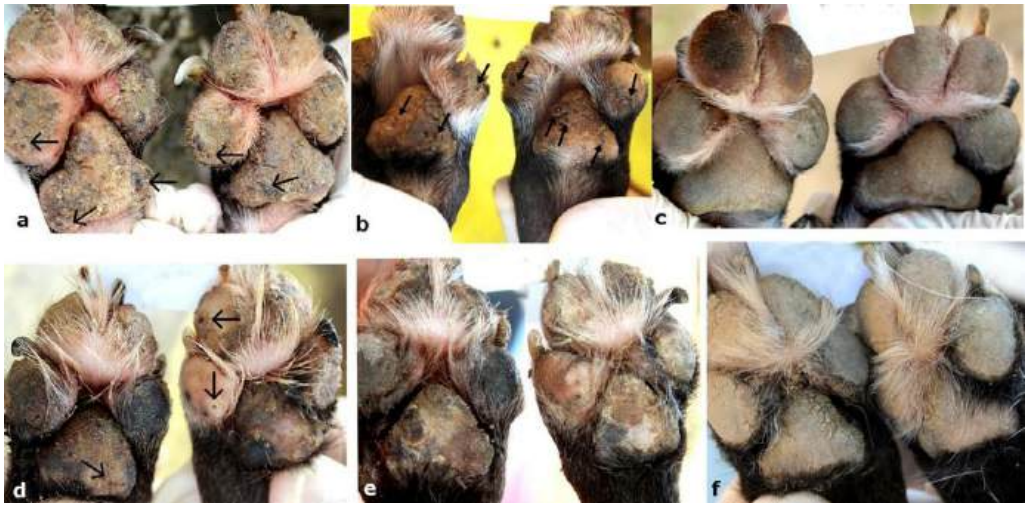

Fig. 4 Evolution of vital lesions in two treated dogs. **a** Animal 12 of the TG on day 0, with multiple lesions in stages II and III in the forepaws (arrows). **b** On day 7 of the evaluation, dog 12 after treatment, the footpads no longer present any vital lesions, it is possible to observe lesions in stages IV and V on the paws (arrows). **c** The same dog 12 on day 42 post-treatment with completely healthy front paws and no lesions caused by *T. penetrans*. **d** Animal 24 of the TG on day 0 with several vital lesions on the hind paws (arrows); **e** Animal 24 on day 7 of the study showing hind paws with desquamated epithelium, in the phase of re-epithelialization. **f** The same dog on day 42 of evaluation without the presence of lesions or scars from lesions caused by *T. penetrans* on the hind paws. Source: Personal archive

Fig. 5 Evolution of vital lesions in two untreated dogs (arrows). **a–c** Animal 58 from the CG in evaluation days 0, 7, and 42, respectively, with stages II and III on forepaws. **d–f** Animal 46 of the CG, on evaluation days 0, 7, and 42 with several vital lesions (II and III) on the front paws. Source: Personal archive.

Additionally, secondary efficacy ranged from 99.0% to 100.0%, thereby revealing the treatment's impact on the *T. penetrans* reproductive cycle, which was previously demonstrated in a similar article [23]. The reduction in the number of flea-releasing eggs in the environment may favor the reduction of infestation in animals that share the same environment as the treated dogs. This environmental effect is a possible cause for the reduction in the number of tungiasis-positive dogs in the untreated group [35].

Table 5 : SCADT by study day and study group

Day	Median		Median		*P* value (two-sided)	Statistical value (*W*)
	Treated		Control			
	Mean ± SD		Mean ± SD			
0	2.90 ± 2.50	2.5	2.30 ± 2.20	2	0.15	634 7
	0.84 ± 1.03	0	2.47 ± 2.15	2	0.00	259 14
	1.59 ± 1.76	1.50	2.60 ± 1.94	2	0.04	335 21
	0.62 ± 1.49	0	2.54 ± 2.31	2.5	0.00	226
28	0.14 ± 0.56	0	1.50 ± 1.80	1	0.00	234.5
35	0.00 ± 0.00	0	1.28 ± 1.40	1	0.00	108
42	0.00 ± 0.00	0	1.00 ± 1.36	0	0.00	246.5

Wilcoxon test (Mann–Whitney *U* test), $\alpha = 0.05$

Furthermore, the low SCADT averages observed in the treated animals demonstrated that they showed few acute local clinical signs such as pain, edema, hyperemia, and ulceration, compared with untreated dogs, as observed in similar studies [23, 35]. In view of this, the present results allow us to infer that the treatment provides an improved quality of life and consequently improves the well-being of animals by eliminating sand fleas. Furthermore, no dog treated with B ravecto® 1-Month (Defenza®) manifested any adverse reactions after administration, demonstrating that the formulation is safe for dogs [23, 36–38].

The formulation is effective in combating *T. penetrans* for at least 42 days; hence, we also suggest the formulation's use as a disease control tool for owners who wish to medicate their dogs for a short period of time.

This will aid in treating or preventing infestations, especially in the case of tourists traveling to tungiasis-endemic areas, such as the area in this study. We emphasize that the control of tungiasis is a public health issue, especially in endemic areas of Brazil, where dogs are identified as one of the (main risk factors for tungiasis.DefenzaWe suggest the potential use of Bravecto®) in public health programs aimed at control® 1-Month ling tungiasis, as well as in endemic areas by dog owners. This is owing to the formulation's commercial availability and low cost compared with that of other proven effective options.

ConclusionsThe Bravecto® 1-Month (Defenza®) formulation, orally administered in a single dose of 10–18 mg/kg, was effective against natural *T. penetrans* flea infestations in dogs 7–42 days after administration. The dogs treated in this study showed an improvement in SCADT associated with tungiasis.

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Author contributions

Project administration was performed by FAB, SNDR, FBB and RSAC. Investigation was carried out by KCS, PEBG, TVH, JBCT, JTSO, APS, AVJ, TVB, RSAC, and GRA. Writing—original draft preparation was performed by KCS Writing— review and editing was carried out by SNDR, FBB, RSAC, PEBG, GRA and FAB Supervision was performed by RSAB, GRA, and FAB. All authors read and approved the final manuscript. **Funding**

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Availability of data and materials

The authors confirm that the data supporting the findings of this study are available within the article.

Declarations

Ethics approval and consent to participate

This study was approved by the Animal Use Ethics Committee (CEUA) of State University of Santa Cruz (UESC), Ilhéus, Bahia, Brazil (020/2019). This study was conducted considering the principles of "Good Clinical Practice" VICH GL9 (GCP) adopted by the Committee for Medicinal Products for Veterinary Use
(CVMP), European Medicines Agency (EMA) in June 2000 (CVMP/VICH/595/98Final). Informed consent was obtained from the owners of all animals included in the study.

Consent for publication Not applicable.

Competing interests

The following authors have a conflict of interest: Sofia Nadir Sanches Ramos and Francisco Bonomi Barufi are employees at MSD Animal Health. The other authors declare no conflicts of interest.

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Publisher's Note

7. CAPÍTULO II

7.1 ARTIGO CIENTIFÍCO III

RESEARCH **Open Access**

Efficacy of an oral formulation of afoxolaner and milbemycin oxime against *Tunga penetrans* in naturally infected dogs

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Abstract

Background The sand flea *Tunga penetrans* is one of the agents of tungiasis, an important parasitic skin disease affecting humans and several mammalian species. Tungiasis is mainly observed in disadvantaged rural and peripheral urban communities in Latin America and sub-Saharan Africa. The dog is a major reservoir of *Tunga* fleas. Hematophagous adult female *Tunga*® spp. embed and grow in their host's epidermis and cause cutaneous inflammatory disorders. NexGard® Spectra is an orally administered endectocide for dogs, a co-formulation of the isoxazoline afoxolaner and the macrocyclic lactone milbemycin oxime. The objective of this study was to assess the efficacy of this product against canine tungiasis.

Methods A blinded, negative-controlled field trial was conducted in a Brazilian community known to be highly endemic for tungiasis. Sixty-six dogs naturally infected with live *T. penetrans* were randomly allocated to a treated group (44 dogs) and an untreated control group (22 dogs). In a first phase, dogs from the treated group were treated on days 0, 30, and 60. Efficacy was evaluated on the basis of the macroscopic parasitic skin lesions (Fortaleza classification) on days 7, 14, 21, 30, 45, 60, 75, and 90. In a second phase, to evaluate natural reinfections, all dogs were treated on day 90 and evaluated every 2 weeks thereafter until at least 30% of dogs were infected with live sand fleas. **Results** During the first phase, efficacy (reduction in live sand fleas) of 92.4% was demonstrated on day 7. From day 14® until day 90, the efficacy of NexGard® Spectra was 100%. In the second phase, all dogs were free of live *T. penetrans* from 15 until 45 days after the day 90 treatment; 60 days post-treatment, 11% of dogs were reinfected, and 75 days post-treatment, 40% of dogs were reinfected.

Conclusions NexGard® Spectra was demonstrated to be highly effective against canine tungiasis. In addition to an obvious beneficial effect on the health and welfare of the treated dog, the use of this product may have a onehealth benefit on human cases by controlling the main reservoir of sand fleas.

Keywords Afoxolaner, Dog, Efficacy, Field, *Tunga penetrans*

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Background

Sand fleas of the genus *Tunga* (Siphonaptera, Tungidae, Tunginae), also called chigoe fleas and jigger fleas, are among the agents of tungiasis, a zoonotic parasitic skin disease [1]. Tungiasis is an important and severe public health concern in tropical and subtropical regions such as Latin America, the Caribbean, and sub-Saharan Africa [2]. Tungiasis is included in the list of neglected tropical diseases by the World Health Organization, in the category of scabies and parasitic skin diseases [3]. High human prevalence can occur in disadvantaged rural and peripheral urban communities [4–8].

The 1-mm-long adult *Tunga* spp. inhabit the floor and infest a mammalian host mainly by direct skin contact. They usually affect feet in humans, pads in animals, or other skin areas with frequent floor contact. After minutes to hours of host contact, the hematophagous sand flea takes a blood meal, and within a few hours, the female penetrates the epidermis, remains embedded, and matures into a globular pea-size neosome growing by abdominal hypertrophy. The neosomy period lasts approximately 5–6 weeks before parasite death, and causes a highly morbid and intense inflammatory reaction, increasing in intensity at the late stage [9–13]. When the epidermis penetration phase is complete, during stage III of the Fortaleza classification, (Table 1), hundreds of eggs are released into the environment by each female. Similar to other Siphonaptera, the eggs hatch into larvae that feed on organic debris and molt into pupae that contribute to efficient and lasting environmental contamination in sand, soil, dust, dirt, and cracks in floors [5].

Fourteen *Tunga* species are known, including the zoonotic species *Tunga penetrans*, the most prevalent and widely described sand flea species in Brazil [14]. *Tunga penetrans* frequently infests humans and domestic, semi-domestic, and wildlife mammals such as dogs, cats, pigs, cattle, goats, and rats [14–18]. Endemicity in humans is exacerbated in poor communities where the standards of medical care and hygiene are low, and where there is close proximity to domestic, semi-domestic, or stray reservoir animals. [1, 2, 19]. Dogs in Brazil and pigs in Africa are considered the main reservoirs [19, 20]. A prevalence of infection as high as 86% has been described in Brazilian dogs in highly endemic areas [21, 22]. The incidence of human tungiasis can reach 50% in highly endemic Brazilian areas [23]. Besides environmental infection by the off-host developing stage, inter-host transmission of adult *Tunga* is hypothesized to occur in crowded areas [5]. Some cases have been detected in travelers returning from endemic areas [23–26] but are probably underdiagnosed, as they generally do not require specialist treatment [23].

Table 1 Description of the Fortaleza classification

Stage	Characteristics
I	Penetration phase (30 min to a few hours). Reddish spot of approximately 1 mm
II	Hypertrophy beginning (1–2 days post-penetration). Dark spot of approximately 1–2 mm usually in the middle of a hyperemic area
III	Hypertrophy maximum (2 days–3 weeks post-penetration). Round glassy yellow spot, often raised, with a central dark spot about 4–13 mm in diameter
IV	Dead parasite (3–5 weeks post-penetration). Brown to black raised circular patches surrounded by necrotic tissue
V	Residual scar (6 weeks to several months post-penetration). A shallow circular crater in the skin with necrotic edges

The control strategies for tungiasis are challenging and require an integrated approach [11, 17, 27]. They include environmental sanitation and control of immature off-host stages, human treatment, and animal reservoir management including treatment and animal– human proximity control [28]. The epidemiology is also influenced by the climate, as infection peaks are observed in dry seasons [27]. In areas with consistent climatic conditions, control is further complicated as contamination is constant throughout the year.

Afoxolaner is a systemic insecticide and acaricide compound belonging to the isoxazoline group, acting on gamma-aminobutyric acid (GABA)-gated chloride ion channels, and resulting in electrophysiological disruption of the central nervous system and death of the arthropod [formulations for dogs (NexGard®29, 30 Plus, Boehringer Ingelheim Animal]. Afoxolaner is available in three oral ®, NexGard Spectra®, and NexGard Health) and is indicated for the treatment and control of flea (*Ctenocephalides felis* and *C. canis*), tick, and mite infestations [31–33]. Afoxolaner was also demonstrated efficacious against the screwworm *Cochliomyia hominivorax* in dogs in a field trial in Brazil [34]. After oral administration, alone or in combination with

milbemycin oxime (MO), afoxolaner is rapidly absorbed, with plasma peak levels (T_{max}) observed between 2 and 6 h, and is slowly eliminated, with an elimination half-life of 15 ± 8 days, resulting in rapid onset of efficacy, and sustained efficacy lasting 1 month [35, 36]. Afoxolaner is highly bound to plasma proteins, therefore acting through a systemic pathway on hematophagous arthropods.

The efficacy of fluralaner, another isoxazoline compound, has already been demonstrated against *T. penetrans* in dogs [37].

This manuscript describes a field study designed to assess the afoxolaner insecticidal activity against *T. penetrans* in naturally infected dogs in an endemic region of Brazil.

Methods

This study was a blinded, negative-controlled, and randomized field trial conducted in one site in Brazil from November 2021 to May 2022. It was conducted in accordance with the principles of good clinical practice (VICH GL 9).

The study site was located in a resource-poor rural community (district of Aritaguá, Ilhéus, Bahia) known to have a high tungiasis prevalence [21, 22]. The local climate is steady throughout the year, with average annual temperatures ranging from 22 to 25 °C, and with a regular and consistent rainfall regime. The village was populated with 368 residents and approximately 100 dogs. Some residences were unfinished and did not have concrete floors, and streets were unpaved and consisted of sand and earth. Dogs were semi-domestic and roamed freely, but were habituated to return to and dwell in the same house or house yard.

The investigated veterinary product (IVP) was NexGard Spectra®, a palatable tablet formulation for oral administration to dogs, containing afoxolaner and MO. It is recognized that MO, the nematicide compound of the product, has a negligible effect on arthropods when given orally once a month [38, 39], and thus this compound does not bring any additional ectoparasiticide efficacy to that of afoxolaner. Depending on body weight, the administered doses range from 2.5 to 5.3 mg/kg afoxolaner and 0.5–1.1 mg/kg MO. NexGard Spectra® is indicated for the treatment of *Ctenocephalides felis* and *C. canis* fleas, ticks, mites, gastrointestinal nematodes, lungworms, and eyeworms, and for the prevention of heartworm disease [39]. During the study, all treatments were administered according to label instructions.

Table 2 Description of severity score for acute dog tungiasis (SCADT)

Clinical signs	Number of affected locations	Score
Hyperemia and edema	1–5	1
	6–10	2
	11–16	3
Pain at the site when pressed	1–5	1
	6–10	2
	11–16	3
Suppuration and formation of abscesses[a]	1–5	1
	6–10	2
	11–16	3
Cluster of lesions[b]	1–5	1
	6–10	2
	11–16	3
Fissure(s)[a]	1–5	1

	6–10	2
	11–16	3
Skin ulcer[a]	1–5	1
	6–10	2
	11–16	3
Mutilation of lesions regardless of the sites involved[c]		2
Altered gait/lameness		3
Ectopy of lesions		0.5[d]

The maximum individual score (SCADT) for a dog is 27 (23 + 4) [a] Regardless of the number of foci and the size of the area involved [b] Three or more lesions close together (1–2 mm apart) [c] Mutilation of lesions indicating severe itching [d] For each ectopic body part involved, up to a maximum of eight ectopic sites; maximum 4 points

The canine tungiasis was primarily evaluated using the Fortaleza classification [12] (Table 1) and secondarilyusing the severity score for acute dog tungiasis (SCADT) classification (Table 2).

At each visit, a detailed skin inspection was performed, including paws, limbs, tail, mammary glands, abdomen, testes, and nose, to search for tungiasis lesions. Before examination, the dogs' paws were cleaned using a brush and water to improve lesion detection and scoring. Identified lesions were staged according to the Fortaleza classification. Each clinical sign was scored for each affected area, and the results added to obtain the SCADT. If an animal exceeded a SCADT score of 22, the dog was treated by surgical removal of *Tunga* spp. neosomes and removed from the study for ethical reasons, as it was not needed in this trial.

To be eligible for inclusion, dogs had to be affected with at least three tungiasis lesions of stage II or III of the Fortaleza classification [12], corresponding to the live embedded stages of the fleas.

The dogs had to comply with the NexGard Spectra® label (e.g., weighing at least 2 kg, aged at least 8 weeks), and had to be healthy and have a suitable temperament. Ten neosomes were collected from 10 different dogs for morphological speciation and underwent identification in the Veterinary Parasitology Laboratory of Santa Cruz State University, Brazil. All neosomes were identified as being from *T. penetrans*.

Throughout the study, usual husbandry conditions were maintained, and commercial canine feed was provided by study personnel. At the beginning of the study, dogs from the untreated control group were administered a dewormer containing febantel, pyrantel pamoate, and praziquantel (Therax Plus, UCB Pharma) by unblinded personnel. All study activities were performed at the respective dogs' house yard and with documented owner consent. Personnel responsible for the parasitic and clinical evaluations were blinded to study group.

Dogs were assigned to the treated group or the untreated control group on a 2:1 ratio, per order of inclusion and on the basis of a random allocation list. Each individual dog was an experimental unit, and dogs from the same house yard were assigned independently to the treated or the untreated control group.

Forty-four dogs were assigned to the IVP-treated group, and 41 completed the study. Twenty-two dogs were assigned to the untreated control group, and 21 completed the study. The four dogs that did not complete the study were removed for reasons unrelated to the study design or the IVP; all interim data obtained from these dogs were nevertheless included in the analyses. All dogs were mongrels and were identified with a subcutaneous microchip. The main characteristics of the dogs and baseline measurements are described in Table 3.

The study was conducted in two phases. Phase I was designed to evaluate the efficacy of three monthly treatments with the IVP against tungiasis. Phase II was designed to evaluate natural reinfections after a single treatment in this specific environment.

In phase I, dogs from the treated group were administered IVP orally on days 0, 30, and 60. The control group remained untreated. Dogs were evaluated for tungiasis weekly after the first treatment and then every 2 weeks after the second and third treatments, i.e., on days (±2) 7, 14, 21, 30, 44, 60, 74, 90. In phase II, on day 90, all dogs (including the previously treated group and the control group) received an IVP treatment and were

evaluated every 2 weeks until reappearance of tungiasis lesions in at least 30% of dogs. Dogs were thus evaluated on days (±2) 105, 120, 135, 150, and 165, when the 30% reinfection threshold had been exceeded.

Data analysis

Primary variable The *T. penetrans* lesion counts of stages II and III of the Fortaleza classification were the primary variable, as they corresponded to the live embedded stages.

Efficacy was calculated on each evaluation time point using two methods:

- Group comparison, where the groups were compared at each post-treatment evaluation time point, using the formula:

$$\text{Efficacy}(\%) = (M^{*}\ \text{control} - - M^{*}\ \text{treated}) / (M^{*}\ \text{control}) \times 100$$

*M = GM (geometric mean) of combined stage II and III lesions (Fortaleza classification). For information, efficacy based on AM (arithmetic means) was also calculated.

The data obtained were analyzed per the Shapiro– Wilk method to determine whether the distribution was parametric or non-parametric. Considering that the distribution was non-parametric, the Wilcoxon test was used.

- Comparison of the percentage of dogs infected with parasites in each group, at each post-treatment evaluation time point, using the formula:

Table 3 Distribution of age, weight, sex, flea count, and total severity score (SCADT) on day 0

	Treated group	Control group
Number of dogs [*n*]	44	22
Age [years]: mean (median) [range]	3.8 (3) [0.25–15]	3.4 (2) [0.7–9]
Body weight [kg]: mean (median) [range]	9.5 (8) [2.6–28.2]	10.9 (10) [3.7–22.0]
Sex	14 females, 30 males	12 females, 10 males
Flea count mean of lesions on stages II and III (median)	18.8 (8.5)	19.4 (10.5)
Flea count lesions on stages II and III range	3–68	3–63
SCADT mean (median)	2.1 (2)	2.6 (2.75)
SCADT interval	0–8.5	0–4.5

All males and females were intact (none were spayed or neutered)

There was no statistical difference between the SCADT of the two groups on day 0 ($P = 0.264$)

$$\text{Efficacy}(\%) = [(Nc - - Nt)/(Nc] \times 100$$

Nc = % of animals with active lesions in the control group, Nt = % of animals with active lesion in the treated group.

The 95% confidence limits for the percentage of dogs free of live *T. penetrans* were calculated as Wilson scoring intervals.

Total severity scores (SCADT) were the secondary variable and were calculated by study day and study group.

The evaluation of the SCADT for canine tungiasis was based on the mean and median of the clinical signs on each assessment day. Mean SCADT scores in the two groups were compared at each post-treatment evaluation time point using the Wilcoxon test (exact), with the level of significance set to $\alpha = 0.05$ (two-sided).

Results

Phase I: evaluation of the efficacy of three monthly treatments with NexGard Spectra®

Primary variable

The efficacy results based on Fortaleza classification II or III lesions in the treated group compared to the untreated control group are detailed in Table 4.

On day 0 before treatment, the average baseline sum of both levels of lesion was 18.8 in the treated group (n = 44) and 19.4 in the untreated control group (n = 22). On day 7 the efficacy (percent reduction) observed in the treated group in comparison to the untreated control group was 92.4% by geometric mean (94.5% by arithmetic mean). From day 14 until day 90, the efficacy of the IVP was maintained at 100%.

The percentage of flea-free dogs in the treated group and the untreated control group are shown in Table 5.

As per inclusion requirements, on day 0, 100% of dogs from each group were infected by live embedded *T. penetrans* (i.e., were affected with Fortaleza level II and/ or III lesions). On day 7, 25/44 dogs (57%) in the treated group and 2/22 (9%) dogs in the untreated control group were free of live *T. penetrans*. From day 14 to day 90, all dogs in the treated group were free of live *T. penetrans*. The infection levels improved somewhat in the untreated control dogs, especially during the third month (on days 60, 75, and 90), when 52–62% of dogs were diagnosed free of live *T. penetrans,* nevertheless remaining significantly lower than the treated group over the 3 months ($P < 0.0001$ at all time points).

Total severity scores (SCADT)

The SCADT by study day and study group and the corresponding intervals are shown in Table 6. Mean total SCADT differed significantly between groups on days 7, 14, 21, 30, 60, and 90, whilst on days 44 and 74 there was no significant difference between groups.

Table 4 Phase I, efficacy results based on means of Fortaleza classification II or III lesions in the IVP-treated group and the untreated control group

Day	GM[a]			AM[b]			*P*-value[c]
	IVP	Control	% Eff[d]	IVP	Control	% Eff[d]	
0	11.84	13.66	–	18.84	19.41	–	0.483
7	0.59	7.77	92.43	0.955	17.41	94.6	< 0.0001
14	0	8.58	100	0	20.05	100	< 0.0001
21	0	8.38	100	0	20.86	100	< 0.0001
30	0	5.65	100	0	13.59	100	< 0.0001
44	0	2.10	100	0	4.86	100	< 0.0001
60	0	0.93	100	0	4.86	100	< 0.0001
74	0	1.14	100	0	4.19	100	< 0.0001
90	0	1.81	100	0	9.95	100	< 0.0001

[a] Geometric mean of combined stage II and III lesions (Fortaleza classification) [b] Arithmetic mean of combined stage II and III lesions (Fortaleza classification) [c] Wilcoxon test (Mann–Whitney) [d] Efficacy (%) = (GM/AM control − GM/AM treated)/(GM/AM control) × 100

Table 5 Phase I, efficacy results based on the percentage of *Tunga*-free dogs in the IVP-treated group and the untreated control group

Day	IVP group	Control group	*P*-value[c]	% Eff[d]

	n_a	%$_b$	n_a	%$_b$		
0	0 in 44	0.0	0 in 22	0.0	NA	NA
7	25 in 44	56.8	2 in 22	9.0	< 0.0001	52.5 (31.6–67.0)[b]
14	44 in 44	100	2 in 22	9.0	< 0.0001	100
21	43 in 43	100	3 in 22	13.6	< 0.0001	100
30	43 in 43	100	4 in 22	18.1	< 0.0001	100
44	43 in 43	100	10 in 21	47.6	< 0.0001	100
60	42 in 42	100	12 in 21	57.1	< 0.0001	100
74	41 in 41	100	13 in 21	61.9	< 0.0001	100
90	41 in 41	100	11 in 21	52.4	< 0.0001	100

[a] *n* = number of animals free of *Tunga* lesions in the group [b] % = percentage of animals free of *Tunga* lesions in the group [c] *t*-test

[d] Efficacy (%) = (Nc − Nt)/(Nc)] × 100, where Nc = % of animals with active lesions in the control group, Nt = % of animals with active lesion in the treated group

Table 6 Phase I, SCADT severity scores per group and time point

Day	Treated group			Control group			*P*-value
	Mean ± SD			Mean ± SD			
Median			Interval		Median		Interval
0	2.1 ± 2.12	2	0–8.5	2.6 ± 2.20	2.75	0–4.5	0.264
7	0.61 ± 1.28	0	0–6	1.00 ± 1.31	0	0–4	0.121
14	0.47 ± 1.13	0	0–5	2.38 ± 2.96	1	0–11	< .0001
21	0.37 ± 0.81	0	0–3	1.77 ± 2.59	1	0–9	0.003
30	0.09 ± 0.42	0	0–2	0.95 ± 1.30	0	0–4	< .0001
44	0.16 ± 0.30	0	0–3	0.19 ± 0.80	0	0–4	0.785
60	0.09 ± 0.43	0	0–2	0.71 ± 1.60	0	0–7	0.024
74	0.21 ± 0.88	0	0–5	0.52 ± 1.03	0	0–3	0.078
90	0.00 ± 0.00	0	0–0	1.22 ± 2.40	0	0–8	< .0001

Table 7 Phase II, percentage of *Tunga*-free dogs after a single administration of NexGard® Spectra on day 90

Day	Previously IVP-treated group[a]		Previously untreated control group[a]		All dogs	
	n_b	%$_c$	n_b	%$_c$	n_b	%$_c$
90	41 in 41	100	11 in 21	52.4	52 in 62	84
105	41 in 41	100	21 of 21	100	62 in 62	100
120	41 in 41	100	21 of 21	100	62 in 62	100
135	41 in 41	100	21 of 21	100	62 in 62	100
150	36 in 41	87.8	19 of 21	90.5	55 in 62	89
165	22 in 41	53.65	15 of 21	76.2	37 in 62	60

[a] Previous group denomination, not adequate for phase II, as all dogs received an IVP treatment on day 90 [b] *n* = number of animals free of *Tunga* lesions in the group

[c] % = percentage of animals free of *Tunga* lesions in the group

Phase II: evaluation of natural reinfections after a single treatment every 2 weeks for the reappearance of active tungiasis lesions (i.e., level II or III Fortaleza classification).
On day 90, all dogs were treated and then monitored

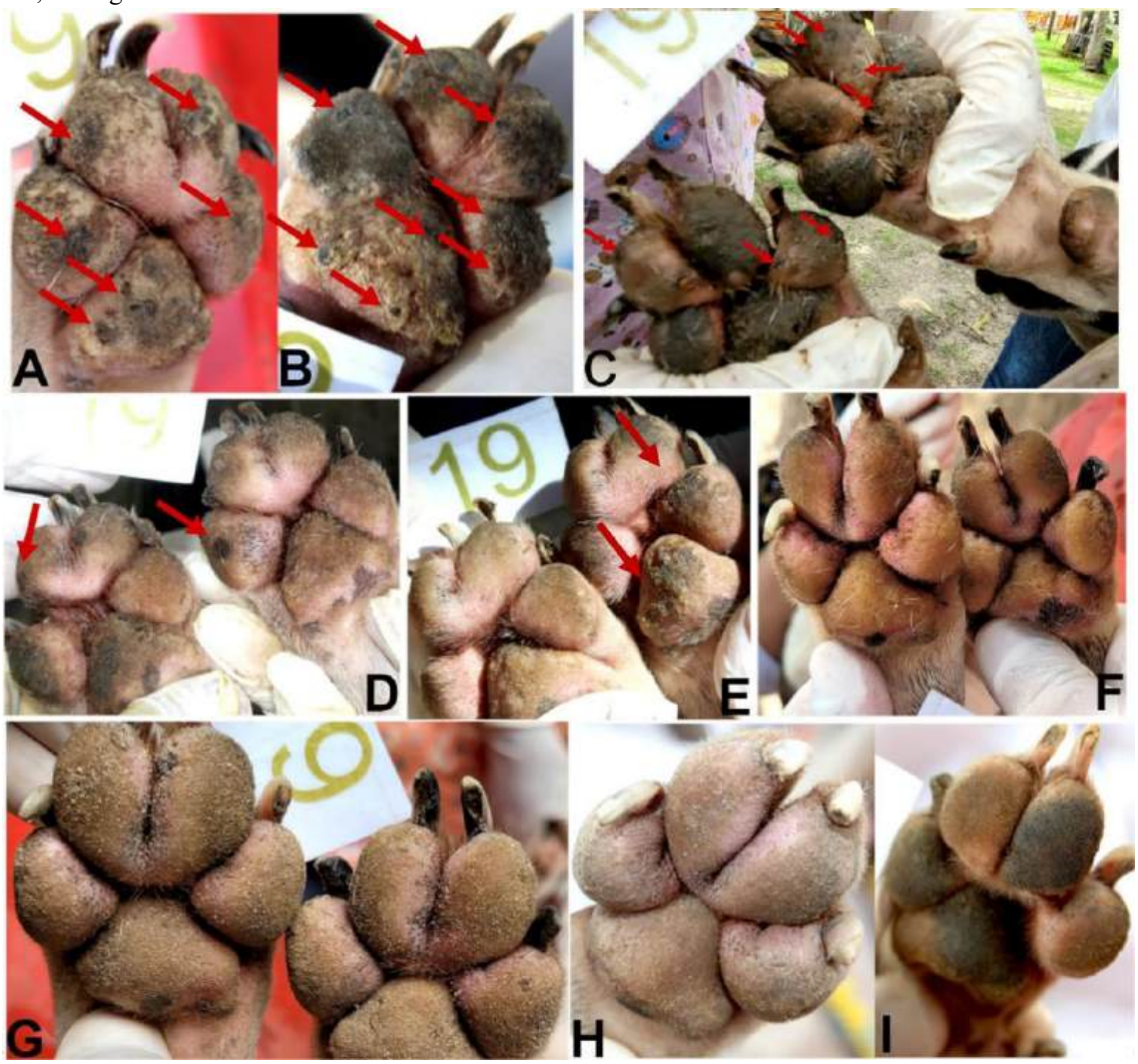

Fig. 1 Evolution of *T. penetrans* lesions for dog 19 of the treated group (*source*: personal collection). **A**, **B** Day 0, the dog had multiple stage II and III lesions located on the front feet pads; **C** day 7, the pads had no longer stages II and III lesions, stage IV lesions were visible (arrows); **D** day 14, **E** day 21, stage IV lesions were visible (arrows); **F** day 30; **G** day 60, the pads had no longer any *T. penetrans* lesions; **H**, **I** day 90, the pads were free of *T.penetrans* lesions and complety re-epithelialized.

The percentages of dogs that remained free of active tungiasis lesions are shown in Table 7.

The 10 dogs that previously belonged to the untreated control group and were affected with active tungiasis lesions before the day 90 treatment confirmed the results of phase I, as they were cleared from active lesions on day 105, 15 days after their IVP treatment. All 62 dogs remained free of new infection with *T. penetrans* until 45 days after treatment (day 135). Eleven percent of dogs were reinfected 60 days after treatment (day 150) and 40% were reinfected 75 days after treatment (day 165) when the study was closed.

In both phases, no adverse reaction related to treatment was observed in any dog.

Discussion

The results of this field trial demonstrated a high level of efficacy of the IVP for the treatment and control of tungiasis in dogs, in a highly endemic area. Phase I demonstrated that 100% efficacy was achieved within 2 weeks after the first treatment and was maintained at 100% with monthly treatments. Phase II demonstrated that

a single treatment provided sustained efficacy of 100% for at least 45 days, but that a regular treatment regimen was necessary, as environmental reinfection occurred afterwards. This study also demonstrated that the treatment significantly improved the dermal skin lesions associated with *T. penetrans* infections.

During phase I, even though significantly lower than in the IVP group, the number of *Tunga*-free dogs increased somewhat in the untreated control dogs, in particular 6 weeks (from day 44) after the first afoxolaner administration to the IVP-treated group (Table 4). The three monthly treatments with afoxolaner in an important proportion of the local dog population may have impacted the parasite turnover of the environmental contamination, thus reducing the infection rate in the untreated dogs. Figures 1 and 2 illustrate the evolution of *T. penetrans* lesions in a treated and an untreated control dog living in the same house yard. Figures 3 and 4 illustrate the evolution of *T. penetrans* lesions in a treated dog and an untreated control dog living alone in different house yards, respectively.

The most common treatment of tungiasis in humans is the mechanical extraction of the parasitic lesion followed by local/systemic symptomatic treatment of the inflammatory and secondary infectious consequences. Nevertheless, a sustained and efficient reduction in the local *Tunga* spp. prevalence in impoverished endemic areas requires drastic environmental control measures, which may be inefficient, difficult, and unaffordable in such areas [2, 11]. Besides the environmental hygienic measures and human–animal proximity controls, the direct treatment of *T. penetrans* in dogs, a major reservoir of *Tunga* spp., may contribute to the control of the disease in humans and provide an efficient one-health strategy to public health authorities [17, 28].

In the present study, afoxolaner provided a convenient and efficacious solution for the control of ® is a highly palatable [*T. penetrans*40] in dogs. NexGard S pectra chewable tablet and therefore easily accepted by dogs, which simplifies oral administration, including to

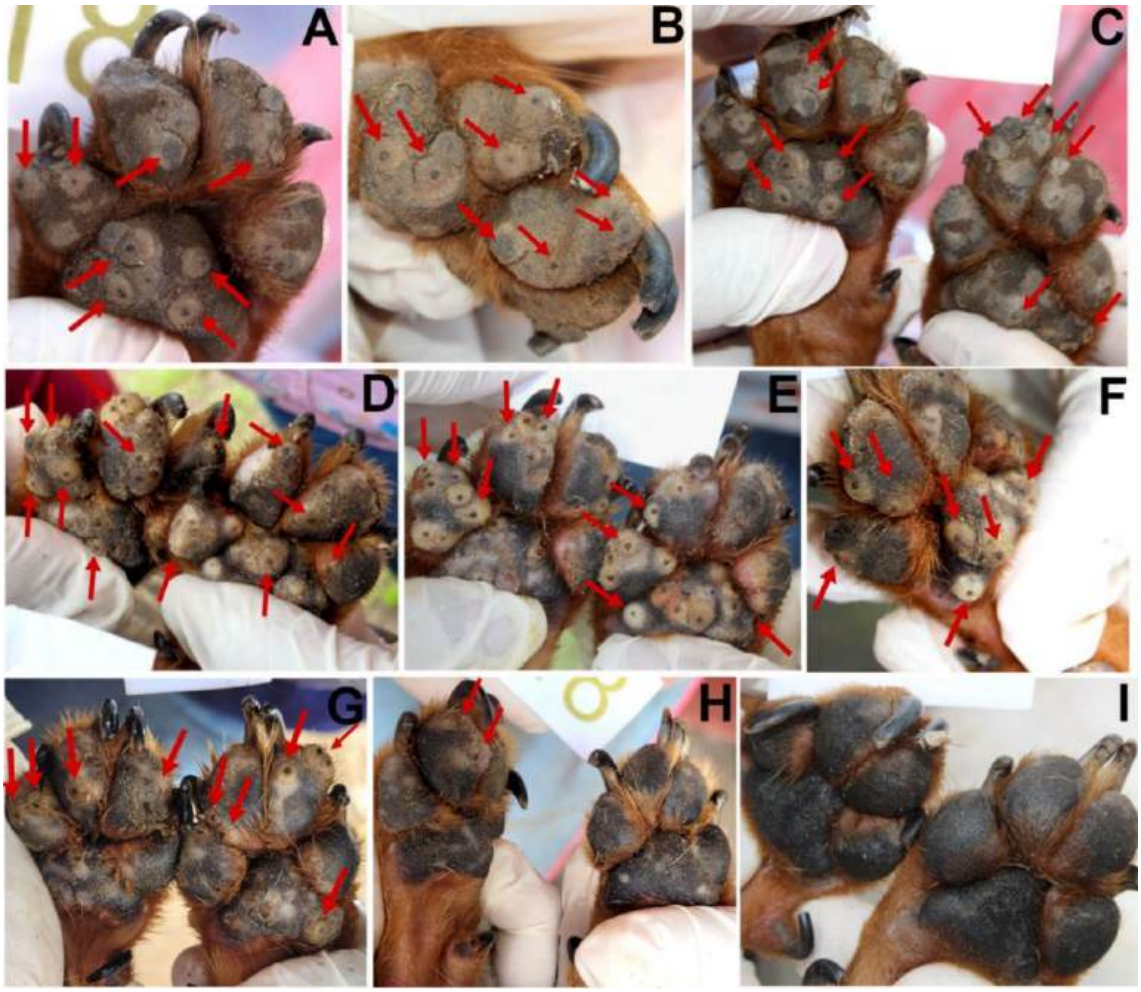

Fig. 2 Evolution of *T. penetrans* lesions for dog 18 of the untreated control group (co-living with dog 19, Fig. 3) (*source*: personal collection). **A**–**C** Day 0; **D** day 7; **E** day 14, **F** day 21; **G** day 30; **H** day 60, the dog had multiple stage II and III lesions located on the front feet pads (arrows); **I** day 90, the pads were free of stage II and III lesions.

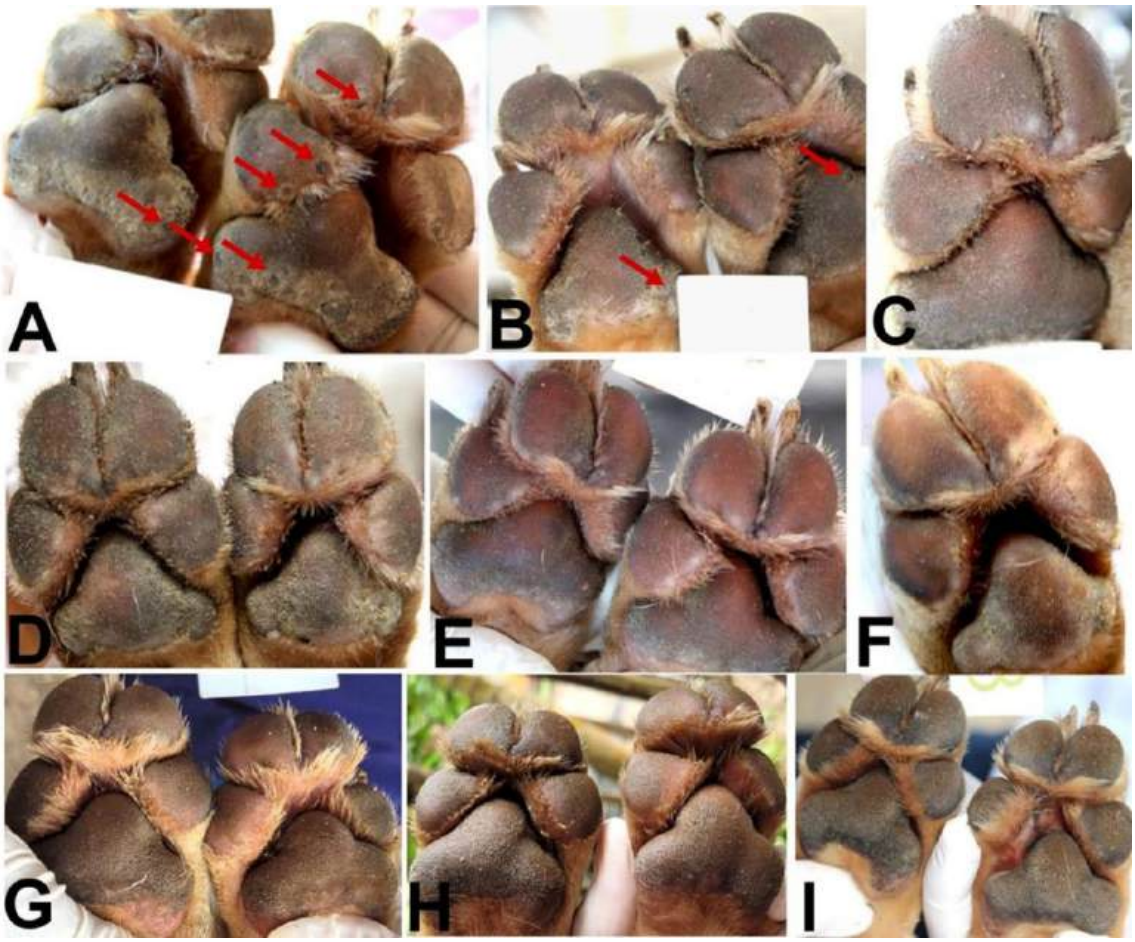

Fig. 3 Evolution of *T. penetrans* lesions for dog 23 of the treated group (*source*: personal collection). **A** Day 0, the dog had multiple stage II and III lesions located on the front feet pads; **B**, **C** day 7, stage II and III lesions were no longer visible, stage V lesions were visible (arrows in **B**); **D** day 14 and **E**, **F** day 21, the pads remained free of stages II and III lesions; **G** day 30, **H** day 60, and **I** day 90, the pads were free of *T. penetrans* lesions and were completely re-epithelialized.

individuals with a lower level of domestication that typically populate *T. penetrans* endemic areas. Besides palatability, which facilitates a high level of compliance, the monthly regimen of this product allows the treatment duration to be adapted in areas with seasonal peaks of infection.NexGard Spectra® combines afoxolaner and MO. The effect of MO on arthropods is believed to be negligible [39]; however, no specific data were obtained on its effect on embedded *T. penetrans.* It is possible that MO also contributed to the efficacy observed in this study, but only for a short duration after each IVP oral administration, because of its short half-life, i.e., 1.6 days ± 0.4 days for the A3 form and 3.3 days ± 1.4 days the A4 form [36, 38], and therefore in a negligible way relative to afoxolaner. Nevertheless, the decision was made to avoid the use of MO in the control group in this study, to avoid any confusion about the observations made on the untreated control animals. The dewormer used in the control group, a combination of pyrantel pamoate, fenbendazole, and praziquantel, did not have any active ingredient with a potential effect on *T. penetrans*.

Even though dogs are the main reservoir species of *T. penetrans*, the environment may be loaded with infective stages for a significant period, and other animal species may also play a reservoir role. Therefore, it would be highly valuable to further assess the correlation between human cases and the control of the parasite in dogs in endemic communities.

use of NexGard SpectraIf confirmed, the obvious one-health benefit of the ® in dogs in relation to tungiasis may be further sustained by its nematicidal spectrum that includes several other zoonotic agents for which dogs are a reservoir [41]. For example, *Ancylostoma* spp. and *Toxocara* spp. are, through their larva migrans effect, another significant public health concern in many regions of the world including areas of tungiasis [® 42–45], and NexGard Spectra has registered efficacy against them [38].

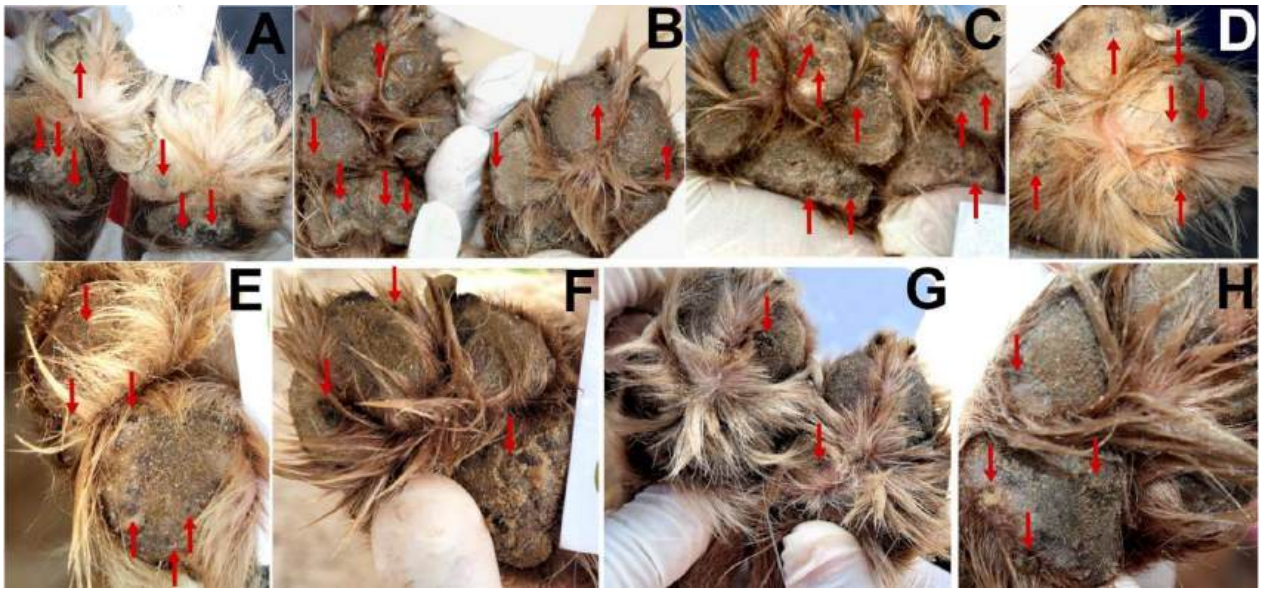

Fig. 4 Evolution of *T. penetrans* lesions for dog 36 of the untreated control group (not co-living with a treated dog) (*source*: personal collection). **A** Day 0, **B** day 7, the dog had multiple stage II and III lesions located on the front feet pads; **C** day 14, in addition to the stage II and III lesions, stage IV lesions can be observed (arrows); **D**, **E** day 21, **F** day 30, **G** day 60, and **H** day 90, the pads remained infected with *T. penetrans* lesions

Conclusions

This study demonstrated that monthly oral administration of afoxolaner was highly effective for the treatment and control of tungiasis in dogs, the main reservoir of *T. penetrans* in many endemic areas in the world. Apart from an obvious beneficial effect on the health and welfare of the treated dog, the use of this product may also have a one-health benefit.

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Author contributions
Project administration: FB, RSAC, ET, AAC, KDB. Investigation: KCS, PEBG, TVH, JBCT, RCV, AWML, ACRL, TVB, GRA, APS, RSAC. Writing—original draft preparation: ET. Writing—review and editing: FB, RSAC. Supervision: RSAC, KDB, AAC. All authors read and approved the final manuscript.

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Availability of data and materials All available data are within the manuscript.

Declarations

Ethics approval and consent to participate
All animal procedures in these studies were reviewed and approved by the Ethics Committee for Animal Experimentation (CEUA) of the State University of Santa Cruz (UESC), Ilhéus, Bahia, Brazil, under protocol number 017/2021, and were in compliance with all applicable sections of the Final Rules of the Animal Welfare Act regulations (9 CFR), the current AVMA Guidelines, and ETS 123 (European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes). Informed consent was obtained from owners of all animals included in the study.

Consent for publication Not applicable.

Competing interests
As they are employees of Boehringer Ingelheim Animal Health, the following authors have a conflict of interest: Frederic Beugnet, Eric Tielemans, Andre Antonio Cutolo, and Karin Denise Botteon. The other authors declare no conflict of interest.

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Publisher's Note

8 CONCLUSÃO

Conclui-se que a administração mensal do Denfenza® (fluralaner 10–18 mg/kg) foi eficaz na proteção e tratamento em cães infestados por *T. penetrans* por até 42 dias como observado no estudo. Bem como, a formulação Nexgard Spectra® (2.5-5.3 mg/kg de afloxalaner e 0.5–1.1 mg/kg milbemicina oxima) foi eficaz mensalmente por ao longo do período experimental, com administração mensal do fármaco. A condição clínica dos cães tratados foi melhor do que o grupo controle nos estudos. Em ambos os estudos as duas formulações testadas não aprestaram efeitos adversos.

9 CONSIDERAÇÕES FINAIS

A partir dos resultados obtidos, é possível o desenvolvimento de políticas públicas para o controle da tungíase com base no tratamento em cães, principal hospedeiro resevatório da *T. penetrans.* Uma opção de controle indireto da doença em humanos e outras espécies animal em regiões endêmicas.

As medicações eficazes testadas nesses estudos proporcionam qualidade de vida para os cães que sofrem com as infestações por *T. penetrans.* Previnem complicações em decorrência da tungíase, sendo possível comprovar através do Pontuação de gravidade para tungíase canina (SCADT), que apresentou sempre menor nos grupos tratados em ambos os estudos.

A eficácia primária de foi de 95% na primeira semana após tratamento com Defenza®, estudo com o Nexgard Spectra® na primeira semana apresentou eficácia primária de 92,4%. Na primeira semana de avaliação o Defenza® apresentou maior eficácia que o Nexgard Spectra®. No entanto, nos D30 ambos os fármacos apresentaram 100% de eficácia e está foi diminuindo ao longo do tempo como observado nos estudos.

Através dos presentes estudos publicados a comprovação da eficácia dos fármacos são novas opções de tratamento para tungíase animal comercialmente mais viáveis, por conta do menor custo. A possibilidade da apresentação mensal é oportuno para turistas em áreas endêmicas que podem querer previnir a tungíase em seus cães.

ANEXO A - Normas da Revista Tropical Medicine and Infectious Disease - Artigo I

Manuscript Preparation

General Considerations

- **Research manuscripts** should comprise:
    - **Front matter**: Title, Author list, Affiliations, Abstract, Keywords.
    - **Research manuscript sections**: Introduction, Materials and Methods, Results, Discussion, Conclusions (optional).
    - **Back matter**: Supplementary Materials, Acknowledgments, Author Contributions, Conflicts of Interest, **References**.
- **Review manuscripts** should comprise the **front matter**, literature review sections and the **back matter**. The template file can also be used to prepare the front and back matter of your review manuscript. It is not necessary to follow the remaining structure. Structured reviews and meta-analyses should use the same structure as research articles and ensure they conform to the **PRISMA** guidelines.
- **Case reports** should include a succinct introduction about the general medical condition or relevant symptoms that will be discussed in the case report; the case presentation including all of the relevant de-identified demographic and descriptive information about the patient(s), and a description of the symptoms, diagnosis, treatment, and outcome; a discussion providing context and any necessary explanation of specific treatment decisions; a conclusion briefly outlining the take-home message and the lessons learned.
- **Graphical Abstract:**

  A graphical abstract (GA) is an image that appears alongside the text abstract in the Table of Contents. In addition to summarizing the content, it should represent the topic of the article in an attention-grabbing way. Moreover, it should not be exactly the same as the Figure in the paper or just a simple superposition of several subfigures. Note that the GA must be original and unpublished artwork. Any postage stamps, currency from any country, or trademarked items should not be included in it.

  The GA should be a high-quality illustration or diagram in any of the following formats: PNG, JPEG, TIFF, or SVG. Written text in a GA should be clear and easy to read, using one of the following fonts: Times, Arial, Courier, Helvetica, Ubuntu or Calibri.

  The minimum required size for the GA is 560 × 1100 pixels (height × width). The size should be of high quality in order to reproduce well.
- **Acronyms/Abbreviations/Initialisms** should be defined the first time they appear in each of three sections: the abstract; the main text; the first figure or table. When defined for the first time, the acronym/abbreviation/initialism should be added in parentheses after the written-out form.
- **SI Units** (International System of Units) should be used. Imperial, US customary and other units should be converted to SI units whenever possible.
- **Accession numbers** of RNA, DNA and protein sequences used in the manuscript should be provided in the Materials and Methods section. Also see the section on **Deposition of Sequences and Expression Data**.

- **Equations:** If you are using Word, please use either the Microsoft Equation Editor or the MathType add-on. Equations should be editable by the editorial office and not appear in a picture format.
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Front Matter

These sections should appear in all manuscript types

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- **Keywords:** Three to ten pertinent keywords need to be added after the abstract. We recommend that the keywords are specific to the article, yet reasonably common within the subject discipline.

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- All Figures, Schemes and Tables should be inserted into the main text close to their first citation and must be numbered following their number of appearance (Figure 1, Scheme I, Figure 2, Scheme II, Table 1, *etc.*).
- All Figures, Schemes and Tables should have a short explanatory title and caption.
- All table columns should have an explanatory heading. To facilitate the copy-editing of larger tables, smaller fonts may be used, but no less than 8 pt. in size. Authors should use the Table option of Microsoft Word to create tables.

- Authors are encouraged to prepare figures and schemes in color (RGB at 8-bit per channel). There is no additional cost for publishing full color graphics.

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Original Images for Blots and Gels Requirements

For the main text, please ensure that:

- All experimental samples and controls used for one comparative analysis are run on the same blot/gel.
- Image processing methods, such as adjusting the brightness or contrast, do not alter or distort the information in the figure and are applied to every pixel. High-contrast blots/gels are discouraged.
- Cropped blots/gels present in the main text retain all important information and bands.
- You have checked figures for duplications and ensured the figure legends are clear and accurate. Please include all relevant information in the figure legends and clearly indicate any re-arrangement of lanes.

In order to ensure the integrity and scientific validity of blots (including, but not limited to, Western blots) and the reporting of gel data, original, uncropped and unadjusted images should be uploaded as Supporting Information files at the time of initial submission.

A single PDF file or a zip folder including all the original images reported in the main figure and supplemental figures should be prepared. Authors should annotate each original image, corresponding to the figure in the main article or supplementary materials, and label each lane or loading order. All experimental samples and controls used for one comparative analysis should be run on the same blot/gel image. For quantitative analyses, please provide the blots/gels for each independent biological replicate used in the analysis.

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Supplementary Materials, Data Deposit and Software Source Code

MDPI Research Data Policies

MDPI is committed to supporting open scientific exchange and enabling our authors to achieve best practices in sharing and archiving research data. We encourage all authors of articles published in MDPI journals to share their research data. Individual journal guidelines can be found at the journal 'Instructions for Authors' page. Data sharing policies concern the minimal dataset that supports the central findings of a published study. Generated data should be publicly available and cited in accordance with journal guidelines.

MDPI data policies are informed by **TOP Guidelines** and **FAIR Principles**.

Where ethical, legal or privacy issues are present, data should not be shared. The authors should make any limitations clear in the Data Availability Statement upon

submission. Authors should ensure that data shared are in accordance with consent provided by participants on the use of confidential data.

Data Availability Statements provide details regarding where data supporting reported results can be found, including links to publicly archived datasets analyzed or generated during the study.

Below are suggested Data Availability Statements:

- Data available in a publicly accessible repository
  The data presented in this study are openly available in [repository name e.g., FigShare] at [**doi**], reference number [reference number].
- Data available in a publicly accessible repository that does not issue DOIs
  Publicly available datasets were analyzed in this study. This data can be found here: [link/accession number]
- Data available on request due to restrictions eg privacy or ethical
  The data presented in this study are available on request from the corresponding author. The data are not publicly available due to [insert reason here]
- 3rd Party Data
  Restrictions apply to the availability of these data. Data was obtained from [third party] and are available [from the authors/at URL] with the permission of [third party].
- Data sharing not applicable
  No new data were created or analyzed in this study. Data sharing is not applicable to this article.
- Data is contained within the article or supplementary material
  The data presented in this study are available in [insert article or supplementary material here]

Data citation:

- [dataset] Authors. Year. Dataset title; Data repository or archive; Version (if any); Persistent identifier (e.g., DOI).

Computer Code and Software

For work where novel computer code was developed, authors should release the code either by depositing in a recognized, public repository such as **GitHub** or uploading as supplementary information to the publication. The name, version, corporation and location information for all software used should be clearly indicated. Please include all the parameters used to run software/programs analyses.

Supplementary Material

Additional data and files can be uploaded as "Supplementary Files" during the manuscript submission process. The supplementary files will also be available to the referees as part of the peer-review process. Any file format is acceptable; however, we recommend that common, non-proprietary formats are used where possible. For more information on supplementary materials, please refer to **https://www.mdpi.com/authors/layout#_bookmark83**.

References in Supplementary Files

Citations and References in Supplementary files are permitted provided that they also appear in the reference list of the main text.

Unpublished Data

Restrictions on data availability should be noted during submission and in the manuscript. "Data not shown" should be avoided: authors are encouraged to publish all observations related to the submitted manuscript as Supplementary Material. "Unpublished data" intended for publication in a manuscript that is either planned, "in preparation" or "submitted" but not yet accepted, should be cited in the text and a reference should be added in the References section. "Personal Communication" should also be cited in the text and reference added in the References section. (see also the MDPI reference list and citations style guide).

Remote Hosting and Large Data Sets

Data may be deposited with specialized service providers or institutional/subject repositories, preferably those that use the DataCite mechanism. Large data sets and files greater than 60 MB must be deposited in this way. For a list of other repositories specialized in scientific and experimental data, please consult **databib.org** or **re3data.org**. The data repository name, link to the data set (URL) and accession number, doi or handle number of the data set must be provided in the paper. The journal **Data** also accepts submissions of data set papers.

Deposition of Sequences and Expression Data

New sequence information must be deposited to the appropriate database prior to submission of the manuscript. Accession numbers provided by the database should be included in the submitted manuscript. Manuscripts will not be published until the accession number is provided.

- *New nucleic acid sequences* must be deposited into an acceptable repository such as **GenBank**, **EMBL**, or **DDBJ**. Sequences should be submitted to only one database.
- *New high throughput sequencing (HTS) datasets* (RNA-seq, ChIP-Seq, degradome analysis, …) must be deposited either in the **GEO database** or in the NCBI's **Sequence Read Archive (SRA)**.
- *New microarray data* must be deposited either in the **GEO** or the **ArrayExpress** databases.The "Minimal Information About a Microarray Experiment" (MIAME) guidelines published by the Microarray Gene Expression Data Society must be followed.
- *New protein sequences* obtained by protein sequencing must be submitted to UniProt (submission tool **SPIN**). Annotated protein structure and its reference sequence must be submitted to **RCSB of Protein Data Bank**.

All sequence names and the accession numbers provided by the databases must be provided in the Materials and Methods section of the article.

Deposition of Proteomics Data

Methods used to generate the proteomics data should be described in detail and we encourage authors to adhere to the "**Minimum Information About a Proteomics Experiment**". All generated mass spectrometry raw data must be deposited in the appropriate public database such as **ProteomeXchange**, **PRIDE** or **jPOST**. At the time of submission, please include all relevant information in the materials and methods section, such as repository where the data was submitted and link, data set identifier, username and password needed to access the data.

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Research and Publication Ethics

Research Ethics

Research Involving Human Subjects

When reporting on research that involves human subjects, human material, human tissues, or human data, authors must declare that the investigations were carried out following the rules of the Declaration of Helsinki of 1975 (**https://www.wma.net/what-we-do/medical-ethics/declaration-of-helsinki/**), revised in 2013. According to point 23 of this declaration, an approval from the local institutional review board (IRB) or other appropriate ethics committee must be obtained before undertaking the research to confirm the study meets national and international guidelines. As a minimum, a statement including the project identification code, date of approval, and name of the ethics committee or institutional review board must be stated in Section 'Institutional Review Board Statement' of the article.

Example of an ethical statement: "All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of XXX (Project identification code)."

For non-interventional studies (e.g. surveys, questionnaires, social media research), all participants must be fully informed if the anonymity is assured, why the research is being conducted, how their data will be used and if there are any risks associated. As with all research involving humans, ethical approval from an appropriate ethics committee must be obtained prior to conducting the study. If ethical approval is not required, authors must either provide an exemption from the ethics committee or are encouraged to cite the local or national legislation that indicates ethics approval is not required for this type of study. Where a study has been granted exemption, the name of the ethics committee which provided this should be stated in Section 'Institutional Review Board Statement' with a full explanation regarding why ethical approval was not required.

A written informed consent for publication must be obtained from participating patients. Data relating to individual participants must be described in detail, but private information identifying participants need not be included unless the identifiable materials are of relevance to the research (for example, photographs of participants' faces that show a particular symptom). Patients' initials or other personal identifiers must not appear in any images. For manuscripts that include any case details, personal information, and/or images of patients, authors must obtain signed

informed consent for publication from patients (or their relatives/guardians) before submitting to an MDPI journal. Patient details must be anonymized as far as possible, e.g., do not mention specific age, ethnicity, or occupation where they are not relevant to the conclusions. A **template permission form** is available to download. A blank version of the form used to obtain permission (without the patient names or signature) must be uploaded with your submission. Editors reserve the right to reject any submission that does not meet these requirements.

You may refer to our sample form and provide an appropriate form after consulting with your affiliated institution. For the purposes of publishing in MDPI journals, a consent, permission, or release form should include unlimited permission for publication in all formats (including print, electronic, and online), in sublicensed and reprinted versions (including translations and derived works), and in other works and products under open access license. To respect patients' and any other individual's privacy, please do not send signed forms. The journal reserves the right to ask authors to provide signed forms if necessary.

If the study reports research involving vulnerable groups, an additional check may be performed. The submitted manuscript will be scrutinized by the editorial office and upon request, documentary evidence (blank consent forms and any related discussion documents from the ethics board) must be supplied. Additionally, when studies describe groups by race, ethnicity, gender, disability, disease, etc., explanation regarding why such categorization was needed must be clearly stated in the article.

Ethical Guidelines for the Use of Animals in Research

The editors will require that the benefits potentially derived from any research causing harm to animals are significant in relation to any cost endured by animals, and that procedures followed are unlikely to cause offense to the majority of readers. Authors should particularly ensure that their research complies with the commonly-accepted '3Rs [1]':

- Replacement of animals by alternatives wherever possible,
- Reduction in number of animals used, and
- Refinement of experimental conditions and procedures to minimize the harm to animals.

Authors must include details on housing, husbandry and pain management in their manuscript.

For further guidance authors should refer to the Code of Practice for the Housing and Care of Animals Used in Scientific Procedures [2], American Association for Laboratory Animal Science [3] or European Animal Research Association [4].

If national legislation requires it, studies involving vertebrates or higher invertebrates must only be carried out after obtaining approval from the appropriate ethics committee. As a minimum, the project identification code, date of approval and name of the ethics committee or institutional review board should be stated in Section 'Institutional Review Board Statement'. Research procedures must be carried out in accordance with national and institutional regulations. Statements on animal welfare should confirm that the study complied with all relevant legislation. Clinical studies involving animals and interventions outside of routine care require ethics committee

oversight as per the American Veterinary Medical Association. If the study involved client-owned animals, informed client consent must be obtained and certified in the manuscript report of the research. Owners must be fully informed if there are any risks associated with the procedures and that the research will be published. If available, a high standard of veterinary care must be provided. Authors are responsible for correctness of the statements provided in the manuscript.

If ethical approval is not required by national laws, authors must provide an exemption from the ethics committee, if one is available. Where a study has been granted exemption, the name of the ethics committee that provided this should be stated in Section 'Institutional Review Board Statement' with a full explanation on why the ethical approval was not required.

If no animal ethics committee is available to review applications, authors should be aware that the ethics of their research will be evaluated by reviewers and editors. Authors should provide a statement justifying the work from an ethical perspective, using the same utilitarian framework that is used by ethics committees. Authors may be asked to provide this even if they have received ethical approval.

MDPI endorses the ARRIVE guidelines (**arriveguidelines.org/**) for reporting experiments using live animals. Authors and reviewers must use the ARRIVE guidelines as a checklist, which can be found at **https://arriveguidelines.org/sites/arrive/files/documents/ARRIVE%20Compliance%20Questionnaire.pdf**. Editors reserve the right to ask for the checklist and to reject submissions that do not adhere to these guidelines, to reject submissions based on ethical or animal welfare concerns or if the procedure described does not appear to be justified by the value of the work presented.

1. NSW Department of Primary Industries and Animal Research Review Panel. Three Rs. Available online: **https://www.animalethics.org.au/three-rs**
2. Home Office. Animals (Scientific Procedures) Act 1986. Code of Practice for the Housing and Care of Animals Bred, Supplied or Used for Scientific Purposes. Available online: **https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/388535/CoPanimalsWeb.pdf**
3. American Association for Laboratory Animal Science. The Scientific Basis for Regulation of Animal Care and Use. Available online: **https://www.aalas.org/about-aalas/position-papers/scientific-basis-for-regulation-of-animal-care-and-use**
4. European Animal Research Association. EU regulations on animal research. Available online: **https://www.eara.eu/animal-research-law**

Research Involving Cell Lines

Methods sections for submissions reporting on research with cell lines should state the origin of any cell lines. For established cell lines the provenance should be stated and references must also be given to either a published paper or to a commercial source. If previously unpublished *de novo* cell lines were used, including those gifted from another laboratory, details of institutional review board or ethics committee approval must be given, and confirmation of written informed consent must be provided if the line is of human origin.

An example of Ethical Statements:

The HCT116 cell line was obtained from XXXX. The $MLH1^{+}$ cell line was provided by XXXXX, Ltd. The DLD-1 cell line was obtained from Dr. XXXX. The DR-GFP and SA-GFP reporter plasmids were obtained from Dr. XXX and the Rad51K133A expression vector was obtained from Dr. XXXX.

Research Involving Plants

Experimental research on plants (either cultivated or wild) including collection of plant material, must comply with institutional, national, or international guidelines. We recommend that authors comply with the **Convention on Biological Diversity** and the **Convention on the Trade in Endangered Species of Wild Fauna and Flora**.

For each submitted manuscript supporting genetic information and origin must be provided. For research manuscripts involving rare and non-model plants (other than, e.g., *Arabidopsis thaliana, Nicotiana benthamiana, Oryza sativa*, or many other typical model plants), voucher specimens must be deposited in an accessible herbarium or museum. Vouchers may be requested for review by future investigators to verify the identity of the material used in the study (especially if taxonomic rearrangements occur in the future). They should include details of the populations sampled on the site of collection (GPS coordinates), date of collection, and document the part(s) used in the study where appropriate. For rare, threatened or endangered species this can be waived but it is necessary for the author to describe this in the cover letter.

Editors reserve the rights to reject any submission that does not meet these requirements.

An example of Ethical Statements:

Torenia fournieri plants were used in this study. White-flowered Crown White (CrW) and violet-flowered Crown Violet (CrV) cultivars selected from 'Crown Mix' (XXX Company, City, Country) were kindly provided by Dr. XXX (XXX Institute, City, Country).

Arabidopis mutant lines (SALKxxxx, SAILxxxx,…) were kindly provided by Dr. XXX, institute, city, country).

Clinical Trials Registration

Registration

MDPI follows the International Committee of Medical Journal Editors (ICMJE) **guidelines** which require and recommend registration of clinical trials in a public trials registry at or before the time of first patient enrollment as a condition of consideration for publication.

Purely observational studies do not require registration. A clinical trial not only refers to studies that take place in a hospital or involve pharmaceuticals, but also refer to all studies which involve participant randomization and group classification in the context of the intervention under assessment.

Authors are strongly encouraged to pre-register clinical trials with an international clinical trials register and cite a reference to the registration in the Methods section. Suitable databases include **clinicaltrials.gov**, **the EU Clinical Trials Register** and those listed by the World Health Organisation **International Clinical Trials Registry Platform**.

Approval to conduct a study from an independent local, regional, or national review body is not equivalent to prospective clinical trial registration. MDPI reserves the right to decline any paper without trial registration for further peer-review. However, if the study protocol has been published before the enrolment, the registration can be waived with correct citation of the published protocol.

CONSORT Statement

MDPI requires a completed CONSORT 2010 **checklist** and **flow diagram** as a condition of submission when reporting the results of a randomized trial. Templates for these can be found here or on the CONSORT website (**http://www.consort-statement.org**) which also describes several CONSORT checklist extensions for different designs and types of data beyond two group parallel trials. At minimum, your article should report the content addressed by each item of the checklist.

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Sex and Gender in Research

We encourage our authors to follow the **'Sex and Gender Equity in Research – SAGER – guidelines'** and to include sex and gender considerations where relevant. Authors should use the terms sex (biological attribute) and gender (shaped by social and cultural circumstances) carefully in order to avoid confusing both terms. Article titles and/or abstracts should indicate clearly what sex(es) the study applies to. Authors should also describe in the background, whether sex and/or gender differences may be expected; report how sex and/or gender were accounted for in the design of the study; provide disaggregated data by sex and/or gender, where appropriate; and discuss respective results. If a sex and/or gender analysis was not conducted, the rationale should be given in the Discussion. We suggest that our authors consult the full **guidelines** before submission.

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Borders and Territories

Potential disputes over borders and territories may have particular relevance for authors in describing their research or in an author or editor correspondence address, and should be respected. Content decisions are an editorial matter and where there is a potential or perceived dispute or complaint, the editorial team will attempt to find a resolution that satisfies parties involved.

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Publication Ethics Statement

TropicalMed is a member of the Committee on Publication Ethics (**COPE**). We fully adhere to its **Code of Conduct** and to its **Best Practice Guidelines**.

The editors of this journal enforce a rigorous peer-review process together with strict ethical policies and standards to ensure to add high quality scientific works to the field of scholarly publication. Unfortunately, cases of plagiarism, data falsification, image manipulation, inappropriate authorship credit, and the like, do arise. The editors of *TropicalMed* take such publishing ethics issues very seriously and are trained to proceed in such cases with a zero tolerance policy.

Authors wishing to publish their papers in *TropicalMed* must abide to the following:

- Any facts that might be perceived as a possible conflict of interest of the author(s) must be disclosed in the paper prior to submission.
- Authors should accurately present their research findings and include an objective discussion of the significance of their findings.
- Data and methods used in the research need to be presented in sufficient detail in the paper, so that other researchers can replicate the work.
- Raw data should preferably be publicly deposited by the authors before submission of their manuscript. Authors need to at least have the raw data readily available for presentation to the referees and the editors of the journal, if requested. Authors need to ensure appropriate measures are taken so that raw data is retained in full for a reasonable time after publication.
- Simultaneous submission of manuscripts to more than one journal is not tolerated.
- The journal accepts exact translations of previously published work. All submissions of translations must conform with our **policies on translations**.
- If errors and inaccuracies are found by the authors after publication of their paper, they need to be promptly communicated to the editors of this journal so that appropriate actions can be taken. Please refer to our **policy regarding Updating Published Papers**.
- Your manuscript should not contain any information that has already been published. If you include already published figures or images, please obtain the necessary permission from the copyright holder to publish under the CC-BY license. For further information, see the **Rights and Permissions** page.
- Plagiarism, data fabrication and image manipulation are not tolerated.
    - **Plagiarism is not acceptable** in *TropicalMed* submissions.

      Plagiarism includes copying text, ideas, images, or data from another source, even from your own publications, without giving any credit to the original source.

      Reuse of text that is copied from another source must be between quotes and the original source must be cited. If a study's design or the manuscript's structure or language has been inspired by previous works, these works must be explicitly cited.

      All MDPI submissions are checked for plagiarism using the industry standard software iThenticate. If plagiarism is detected during the peer review process, the manuscript may be rejected. If plagiarism is

detected after publication, an investigation will take place and action taken in accordance with our policies.

- **Image files must not be manipulated or adjusted in any way** that could lead to misinterpretation of the information provided by the original image.

  Irregular manipulation includes: 1) introduction, enhancement, moving, or removing features from the original image; 2) grouping of images that should obviously be presented separately (e.g., from different parts of the same gel, or from different gels); or 3) modifying the contrast, brightness or color balance to obscure, eliminate or enhance some information.

  If irregular image manipulation is identified and confirmed during the peer review process, we may reject the manuscript. If irregular image manipulation is identified and confirmed after publication, we may correct or retract the paper.

Our in-house editors will investigate any allegations of publication misconduct and may contact the authors' institutions or funders if necessary. If evidence of misconduct is found, appropriate action will be taken to correct or retract the publication. Authors are expected to comply with the best ethical publication practices when publishing with MDPI.

Citation Policy

Authors should ensure that where material is taken from other sources (including their own published writing) the source is clearly cited and that where appropriate permission is obtained.

Authors should not engage in excessive self-citation of their own work.

Authors should not copy references from other publications if they have not read the cited work.

Authors should not preferentially cite their own or their friends', peers', or institution's publications.

Authors should not cite advertisements or advertorial material.

In accordance with COPE guidelines, we expect that "original wording taken directly from publications by other researchers should appear in quotation marks with the appropriate citations." This condition also applies to an author's own work. COPE have produced a discussion document on **citation manipulation** with recommendations for best practice.

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Reviewer Suggestions

During the submission process, please suggest three potential reviewers with the appropriate expertise to review the manuscript. The editors will not necessarily approach these referees. Please provide detailed contact information (address,

homepage, phone, e-mail address). The proposed referees should neither be current collaborators of the co-authors nor have published with any of the co-authors of the manuscript within the last three years. Proposed reviewers should be from different institutions to the authors. You may identify appropriate Editorial Board members of the journal as potential reviewers. You may suggest reviewers from among the authors that you frequently cite in your paper.

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English Corrections

MDPI provides minor English editing by native English speakers for all accepted papers, included in the APC. The APC does not cover extensive English editing. Submitted papers should be written in good English and require no more than minor English editing before publication. Your paper could be returned to you at the English editing stage of the publication process if extensive editing is required, which could delay the publication of your work. You may choose to use a paid language-editing service, such as MDPI's **Author Services**, before submitting your paper for publication. If you use an alternative service that provides a confirmation certificate, please send a copy to the Editorial Office. Authors from economically developing countries or nations should consider registration with **AuthorAid**, a global research community that provides networking, mentoring, resources and training for researchers.

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Preprints and Conference Papers

TropicalMed accepts submissions that have previously been made available as preprints provided that they have not undergone peer review. A preprint is a draft version of a paper made available online before submission to a journal.

MDPI operates ***Preprints***, a preprint server to which submitted papers can be uploaded directly after completing journal submission. Note that *Preprints* operates independently of the journal and posting a preprint does not affect the peer review process. Check the *Preprints* **instructions for authors** for further information.

Expanded and high-quality conference papers can be considered as articles if they fulfill the following requirements: (1) the paper should be expanded to the size of a research article; (2) the conference paper should be cited and noted on the first page of the paper; (3) if the authors do not hold the copyright of the published conference paper, authors should seek the appropriate permission from the copyright holder; (4) authors are asked to disclose that it is conference paper in their cover letter and include a statement on what has been changed compared to the original conference paper. *TropicalMed* does not publish pilot studies or studies with inadequate statistical power.

Unpublished conference papers that do not meet the above conditions are recommended to be submitted to the **Proceedings Series journals**.

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Authorship

MDPI follows the International Committee of Medical Journal Editors (ICMJE) guidelines which state that, in order to qualify for authorship of a manuscript, the following criteria should be observed:

- Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
- Drafting the work or reviewing it critically for important intellectual content; AND
- Final approval of the version to be published; AND
- Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Those who contributed to the work but do not qualify for authorship should be listed in the acknowledgments. More detailed guidance on authorship is given by the International Council of Medical Journal Editors (ICMJE).

Any change to the author list should be approved by all authors including any who have been removed from the list. The corresponding author should act as a point of contact between the editor and the other authors and should keep co-authors informed and involve them in major decisions about the publication. We reserve the right to request confirmation that all authors meet the authorship conditions.

For more details about authorship please check MDPI ethics website.

Reviewers Recommendation

Authors can recommend potential reviewers. Journal editors will check to make sure there are no conflicts of interest before contacting those reviewers, and will not consider those with competing interests. Reviewers are asked to declare any conflicts of interest. Authors can also enter the names of potential peer reviewers they wish to exclude from consideration in the peer review of their manuscript, during the initial submission progress. The editorial team will respect these requests so long as this does not interfere with the objective and thorough assessment of the submission.

Editorial Independence

Lack of Interference with Editorial Decisions

Editorial independence is of utmost importance and MDPI does not interfere with editorial decisions. All articles published by MDPI are peer reviewed and assessed by our independent editorial boards, and MDPI staff are not involved in decisions to accept manuscripts. When making an editorial decision, we expect the academic editor to make their decision based only upon:

- The suitability of selected reviewers;
- Adequacy of reviewer comments and author response;
- Overall scientific quality of the paper.

In all of our journals, in every aspect of operation, MDPI policies are informed by the mission to make science and research findings open and accessible as widely and rapidly as possible.

Editors and Editorial Staff as Authors

Editorial staff or editors shall not be involved in processing their own academic work. Submissions authored by editorial staff/editors will be assigned to at least two independent outside reviewers. Decisions will be made by other Editorial Board Members who do not have a conflict of interest with the author. Journal staff are not involved in the processing of their own work submitted to any MDPI journals.

Conflicts of Interest

According to The International Committee of Medical Journal Editors, "Authors should avoid entering into agreements with study sponsors, both for-profit and non-profit, that interfere with authors' access to all of the study's data or that interfere with their ability to analyze and interpret the data and to prepare and publish manuscripts independently when and where they choose."

All authors must disclose all relationships or interests that could inappropriately influence or bias their work. Examples of potential conflicts of interest include but are not limited to financial interests (such as membership, employment, consultancies, stocks/shares ownership, honoraria, grants or other funding, paid expert testimonies and patent-licensing arrangements) and non-financial interests (such as personal or professional relationships, affiliations, personal beliefs).

Authors can disclose potential conflicts of interest via the online submission system during the submission process. Declarations regarding conflicts of interest can also be collected via the **MDPI disclosure form**. The corresponding author must include a summary statement in the manuscript in a separate section "Conflicts of Interest" placed just before the reference list. The statement should reflect all the collected potential conflicts of interest disclosures in the form.

See below for examples of disclosures:

Conflicts of Interest: Author A has received research grants from Company A. Author B has received a speaker honorarium from Company X and owns stocks in Company Y. Author C has been involved as a consultant and expert witness in Company Z. Author D is the inventor of patent X.

If no conflicts exist, the authors should state:

Conflicts of Interest: The authors declare no conflicts of interest.

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Editorial Procedures and Peer-Review

Pre-check

Immediately after submission, the journal's Managing Editor will perform the technical pre-check to assess:

- Overall suitability of the manuscript to the journal/section/Special Issue;
- Manuscript adherence to high-quality research and ethical standards;
- Standards of rigor to qualify for further review.

The academic editor (i.e., the Editor-in-Chief in the case of regular submissions, the Guest Editor in the case of Special Issue submissions, or an Editorial Board member in the case of a conflict of interest and of regular submissions if the Editor-in-Chief allows) will be notified of the submission and invited to perform an editorial pre-check. During the editorial pre-check phase, the academic editor will assess the suitability of the submission with respect to the scope of the journal, as well as the overall scientific soundness of the manuscript, including the relevance of the references and the correctness of the applied methodology. Academic editors can decide to reject the manuscript, request revisions before peer-review, or continue with the peer-review process and recommend suitable reviewers.

Peer-Review

Once a manuscript passes the initial checks, it will be assigned to at least two independent experts for peer-review. A single-blind review is applied, where authors' identities are known to reviewers. Peer review comments are confidential and will only be disclosed with the express agreement of the reviewer.

In the case of regular submissions, in-house assistant editors will invite experts, including recommendations by an academic editor. These experts may also include *Editorial Board Members* and Guest Editors of the journal. Potential reviewers suggested by the authors may also be considered. Reviewers should not have published with any of the co-authors during the past three years and should not currently work or collaborate with any of the institutions of the co-authors of the submitted manuscript.

Optional Open Peer-Review

The journal operates optional open peer-review: *Authors are given the option for all review reports and editorial decisions to be published alongside their manuscript. In addition, reviewers can sign their review, i.e., identify themselves in the published review reports.* Authors can alter their choice for open review at any time before publication, but once the paper has been published changes will only be made at the discretion of the *Publisher* and *Editor-in-Chief*. We encourage authors to take advantage of this opportunity as proof of the rigorous process employed in publishing their research. To guarantee impartial refereeing, the names of referees will be revealed only if the referees agree to do so, and after a paper has been accepted for publication.

Editorial Decision and Revision

All the articles, reviews and communications published in MDPI journals go through the peer-review process and receive at least two reviews. The in-house editor will communicate the decision of the academic editor, which will be one of the following:

- *Accept after Minor Revisions*:
  The paper is in principle accepted after revision based on the reviewer's comments. Authors are given five days for minor revisions.

- *Reconsider after Major Revisions*:
  The acceptance of the manuscript would depend on the revisions. The author needs to provide a point by point response or provide a rebuttal if some of the reviewer's comments cannot be revised. A maximum of two rounds of major revision per manuscript is normally provided. Authors will be asked to resubmit the revised paper within a suitable time frame, and the revised version will be returned to the reviewer for further comments. If the required revision time is estimated to be longer than 2 months, we will recommend that authors withdraw their manuscript before resubmitting so as to avoid unnecessary time pressure and to ensure that all manuscripts are sufficiently revised.
- *Reject and Encourage Resubmission*:
  If additional experiments are needed to support the conclusions, the manuscript will be rejected and the authors will be encouraged to re-submit the paper once further experiments have been conducted.
- *Reject*:
  The article has serious flaws, and/or makes no original significant contribution. No offer of resubmission to the journal is provided.

All reviewer comments should be responded to in a point-by-point fashion. Where the authors disagree with a reviewer, they must provide a clear response.

Author Appeals

Authors may appeal a rejection by sending an e-mail to the Editorial Office of the journal. The appeal must provide a detailed justification, including point-by-point responses to the reviewers' and/or Editor's comments using an **appeal form**. Appeals can only be submitted following a "reject and decline resubmission" decision and should be submitted within three months from the decision date. Failure to meet these criteria will result in the appeal not being considered further. The *Managing Editor* will forward the manuscript and related information (including the identities of the referees) to a designated *Editorial Board Member*. The Academic Editor being consulted will be asked to provide an advisory recommendation on the manuscript and may recommend acceptance, further peer-review, or uphold the original rejection decision. This decision will then be validated by the *Editor-in-Chief*. A reject decision at this stage is final and cannot be reversed.

Production and Publication

Once accepted, the manuscript will undergo professional copy-editing, English editing, proofreading by the authors, final corrections, pagination, and, publication on the **www.mdpi.com** website.

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Promoting Equity, Diversity and Inclusiveness within MDPI Journals

Our Managing Editors encourage the Editors-in-Chief and Associate Editors to appoint diverse expert Editorial Boards. This is also reflective in our multi-national and inclusive workplace. We are proud to create equal opportunities without regard to gender, ethnicity, sexual orientation, age, religion, or socio-economic status. There is

no place for discrimination in our workplace and editors of MDPI journals are to uphold these principles in high regard.

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Resource Identification Initiative

To improve the reproducibility of scientific research, the **Resource Identification Initiative** aims to provide unique persistent identifiers for key biological resources, including antibodies, cell lines, model organisms and tools.

We encourage authors to include unique identifiers - RRIDs- provided by the **Resource Identification Portal** in the dedicated section of the manuscript.

To help authors quickly find the correct identifiers for their materials, there is a single **website** where all resource types can be found and a 'cite this' button next to each resource, that contains a proper citation text that should be included in the methods section of the manuscript.

ANEXO B - Normas da Revista Parasites & Vectors - Artigo II e III

General formatting and standard English usage

Unless indicated otherwise below, all text should be in Times New Roman, 12-point, with double-line spacing and left justified. Include line and page numbering. Headings and subheadings should be in bold, with 14-point font for main sections (Background, Methods, Results, Discussion and Conclusions) and 12-point font for other sections. Second level subheadings should be indicated in italics (see below). Examples of formatting below are highlighted in blue. The text should be presented in standard English. British and American usage are accepted, but not a mixture of these.

Parts of a manuscript

Title

Title should be concise and focus on the main point of the article. Abbreviations should be avoided. Use the following format (14-point font, bold): The complete genome of the sand fly Lutzomyia longipalpis **Author names**

Indicate the given name(s) (in full) and family name(s) of each author and check that all names are accurately spelled and in the correct order. Use the following format (12-point font, bold): John Smith1†, Alex Silva2† and João de Barro2 *

If two or more authors have contributed equally to the study, please indicate as follow:

†John Smith and Alex Silva contributed equally to this work.

IMPORTANT: There is an increasing number of requests for corrections of misspelled names, order of authors, and wrong affiliations after publication online. This can be

easily prevented if all authors carefully double-check their names and institutions before approving the manuscript.

Affiliations

Present the authors' affiliation addresses (where the actual work was done). Indicate all affiliations with a superscript number immediately after the author's name and in front of the appropriate address. Use the following format:

1 University of California San Francisco, San Francisco, California, USA

2 Tarpon Fishing University, Olinda, Brazil

Correspondence

The corresponding author indicated in the submission system should be the same indicated in the manuscript text. Use the following format:

*Correspondence: john.smith@tarpon.uk

Email addresses Include initials and e-mail address for all authors, as follows:

JS: john.smith@univida.uk

AS: alex.silva@univida.uk

JB: joão.barro@univida.uk

Abstract and keywords

Abstracts should not exceed 350 words and should be divided into sections. Provide 3-10 keywords, which should preferentially be available at the Medical Subject Headings (MeSH) database. Use the following format:

Abstract

Background: Text.

Methods: Text.

Results: Text.

Conclusions: Text.

Keywords: Word, Word, Word

Text sections

Brief reports need not be divided in sections. Reviews can be arranged at the author's discretion, but they should include a Background and a Conclusion. For Primers, see URL https://parasitesandvectors.biomedcentral.com/articles/10.1186/s13071-015-1185-7. Research articles should be divided into sections. Background should not be divided in subsections, but Methods and Results can be subdivided (first, second and third level subheadings) at the author's discretion. Please avoid the use of third and fourth level subheadings, but if really needed use a different formatting style to differentiate from previous subheadings. Results and Discussion can be separated or combined ("Results and discussion"). Discussion should not be divided into subsections, but if needed, use

the same format as for Methods and Results. Conclusions should be a single paragraph. Use the following format: Background First paragraph (no indent). Second, third etc. paragraphs (indent 1.27 cm). Methods First level subheadings First paragraph. Second, third, etc. paragraphs. Second level subheadings First paragraph. Second, third, etc. paragraphs. Third level subheading First paragraph. Second, third, etc. paragraphs. Results First paragraph. Second, third etc. paragraphs. Discussion First paragraph, Second, third, etc. paragraphs. Conclusions First (single) paragraph.

Supplementary information

Additional file captions should not exceed 300 words; any detailed information should be included as an introduction within the additional file itself. Clearly label the files indicating "Additional file 1, 2" etc. in the file names. All additional files (and their elements) should be numbered after the order of citation in the manuscript. All elements of the additional files should be cited at least once in the main manuscript as e.g. "Additional file 1: Table S1", "Additional file 2: Fig. S1", "Additional file 3: Text S1" etc. Use the following format:

Supplementary information

Additional file 1: Text S1. Capture methods. Fig. S1. Epsilon-trap as used for sampling tsetse species of the Morsitans group found in southern Africa. Table S1. Multivariable analysis of the effects of ovarian age, month, year and method of capture, and interactions between age and the three other variables, on the mean wing length of female tsetse captured in the field at Rekomitjie Research Station.

Additional file 2: Dataset S1. Digestion of sheep host hemoglobin in tick midguts.
Additional file 3: Dataset S2. Proteomics results for sheep host proteins in tick tissues.

Abbreviations

All genus names should be fully spelled out at first mention and then abbreviated. When presented in a list, the genus name may be abbreviated (e.g., “Different species of the genus Phlebotomus are implicated in the transmission of Mediterranean Leishmania infantum, including Phlebotomus ariasi, P. balcanicus, P. kandelakii, P. langeroni, P. neglectus, P. perfiliewi, P. perniciosus, P. sergenti and P. tobbi”; from https://parasitesandvectors.biomedcentral.com/articles/10.1186/s13071-021-04652-2).
When presented at the beginning of a sentence, the genus name should be spelled out again (even if already abbreviated). To avoid ambiguity in the text, abbreviations for mosquito genera and 6 subgenera can be made using two and three letters, respectively (see http://www.mosquitocatalog.org/abbreviations.aspx). The most relevant

abbreviations used in the manuscript should be listed in the Abbreviation section, as follows:

Abbreviations

SDS- PAGE: Sodium dodecyl sulphate–polyacrylamide gel electrophoresis; TBST: Trisbufered saline with Tween-20; WT: Wild type.

IMPORTANT: Abbreviations used only in figures and tables should not need to be included in the Abbreviation section. Abbreviations used in a figure should be defined in the figure caption. Abbreviations used in a table should be defined in the table caption or as a footnote. See specific instructions below.

Declarations

All manuscripts must contain the following sections (in the showed order):

• Acknowledgements
• Funding
• Availability of data and materials
• Authors' contributions
• Ethics approval and consent to participate
• Consent for publication • Competing interests
• Author details (optional)

Please refer to the BMC Editorial Policies in the hyperlinked headings above for more information on each declaration type.

If any of the sections are not relevant to your manuscript, please include the heading and write 'Not applicable' for that section.

IMPORTANT: There is an increasing number of requests for corrections on funding declarations, usually for funding sources that were mistakenly omitted. Please double check funding information with all authors before submission.

References

Please use the Vancouver reference style. All references must be numbered in ascending numerical order; references in figures and tables are numbered last. Reference numbers should be provided in the main manuscript text in square brackets, e.g. Woo et al. [1] or

[1–5] or [1, 7, 9, 13–15] or (see [2] for a review). Please follow strictly the format of the examples, including the spacing and punctuation. Note that: (i) abbreviated journal names should be provided; (ii) all organism Latin names should be in italics; (iii) capital letters should be used only for the first word of the article title and proper nouns; (iv) author lists of more than six authors should be presented with ", et al." after the sixth author and (v) non-breaking spaces between words for references copied directly from Internet sources should be removed. Use the following format:
Article within a journal Smith JJ. The world of science. Am J Sci. 1999;36:234-5.

Article within a journal (no page numbers) Rohrmann S, Overvad K, Bueno-de-Mesquita HB, Jakobsen MU, Egeberg R, Tjønneland A, et al. Meat consumption and mortality - results from the European Prospective Investigation into Cancer and Nutrition. BMC Medicine. 2013;11:63.

Article within a journal by DOI Slifka MK, Whitton JL. Clinical implications of dysregulated cytokine production. Dig J Mol Med. 2000; doi:10.1007/s801090000086.
Article within a journal supplement Frumin AM, Nussbaum J, Esposito M. Functional asplenia: demonstration of splenic activity by bone marrow scan. Blood 1979;59 Suppl 1:26-32.

Book chapter, or an article within a book Wyllie AH, Kerr JFR, Currie AR. Cell death: the significance of apoptosis. In: Bourne GH, Danielli JF, Jeon KW, editors. International review of cytology. London: Academic; 1980. p. 251-306.

OnlineFirst chapter in a series (without a volume designation but with a DOI) Saito Y, Hyuga H. Rate equation approaches to amplification of enantiomeric excess and chiral symmetry breaking. Top Curr Chem. 2007. doi:10.1007/128_2006_108.

Complete book, authored Blenkinsopp A, Paxton P. Symptoms in the pharmacy: a guide to the management of common illness. 3rd ed. Oxford: Blackwell Science; 1998.

Online document
Doe J. Title of subordinate document. In: The dictionary of substances and their effects. Royal Society of Chemistry. 1999. http://www.rsc.org/dose/title of subordinate document. Accessed 15 Jan 1999.

Online database

Healthwise Knowledgebase. US Pharmacopeia, Rockville. 1998. http://www.healthwise.org. Accessed 21 Sept 1998.

Supplementary material/private homepage
Doe J. Title of supplementary material. 2000. http://www.privatehomepage.com. Accessed 22 Feb 2000.

University site
Doe, J: Title of preprint. http://www.uni-heidelberg.de/mydata.html (1999). Accessed 25 Dec 1999.

FTP site Doe, J: Trivial HTTP, RFC2169. ftp://ftp.isi.edu/in-notes/rfc2169.txt (1999). Accessed 12 Nov 1999.

Organization site
ISSN International Centre: The ISSN register. http://www.issn.org (2006). Accessed 20 Feb 2007.

Dataset with persistent identifier Zheng L-Y, Guo X-S, He B, Sun L-J, Peng Y, Dong S-S, et al. Genome data from sweet and grain sorghum (Sorghum bicolor). GigaScience Database. 2011. http://dx.doi.org/10.5524/100012.

Figure formatting and requirements

• Do not submit figures in Word or PowerPoint files. • Figure captions (max 300 words) should be provided in the main manuscript (after References), not in the graphic file. For figures with multiple panels (a, b, c, etc), all panels should be mentioned in the figure caption. Only high-resolution figure files will be accepted: - EPS (or PDF): Vector drawings. Embed the font or save the text as ‘graphics‘. 8 - TIFF: Colour or grayscale photographs (halftones), use a minimum of 600 dpi.
- TIFF: Bitmapped line drawings, use a minimum of 1000 dpi.
- TIFF (or JPG): Combinations bitmapped line/halftone (colour or grayscale), use a minimum of 500 dpi.

• Each figure of a manuscript should be submitted as a single file that fits on a single page in portrait format (with figure number indicated in the file name). Do not include figures inside the manuscript text or in a separate word document. • Multi-panel figures (those with parts a, b, c, d etc.) should be submitted as a single composite file that

contains all parts of the figure. Panels should be labelled with lowercase letters (a, b, c, etc) or capital letters (A, B, C, etc), but not a mixture of them. (e.g. https://parasitesandvectors.biomedcentral.com/articles/10.1186/s13071-019-3714-2/figures/1 and https://parasitesandvectors.biomedcentral.com/articles/10.1186/s13071-019-3714-2/figures/2).

• Figures should be numbered in the order they are first mentioned in the text (e.g. "Fig. 1", “Fig. 2”, and so on) and uploaded in this order.

• Each figure should be closely cropped to minimize the white space surrounding the illustration. This improves accuracy when placing the figure in combination with other elements when the manuscript is prepared for publication on our site.

• Make sure you use uniform font size and type (preferred font: Arial 12-20-point) in all figures. All figure elements, including letters, numbers, and symbols, must be in high-definition and legible at their final size.

• Make sure all images are free of copyright restrictions. This includes shapefiles use for maps. Open sources are available (e.g. https://qgis.org/en/site/) and should be used. Maps must contain a scale and a north arrow (compass rose).

• Abbreviations used in figures should be defined in the figure caption (within parentheses or as a list of abbreviations used; see example below).

• Figure captions should follow the journal style, which can be found in previously published papers (e.g., https://parasitesandvectors.biomedcentral.com/articles/10.1186/s13071-019-3714-2/figures/1 and https://parasitesandvectors.biomedcentral.com/articles/10.1186/s13071-019-3714-2/figures/2). Here are also other examples:

Fig. 1 Mean number (± standard error, SE) of Anopheles arabiensis eggs (a) and Culex quinquefasciatus egg rafts (b, c) laid in different agrochemical treatments (b and c are results 9 for laboratory and field experiments, respectively). Different lowercase letters indicate significant differences between treatments. AS, ammonium sulfate; DAP, diammonium phosphate

Fig. 2 Location of four eastern equine encephalitis virus foci study sites in Connecticut, 2010–2011. C, Chester; K, Killingworth; M, Madison

Table formatting and requirements

• All tables should be provided at the end of the manuscript after figure captions or in separate files and not embedded in the manuscript text.

• Tables should not be embedded as figures or spreadsheet files, but should be formatted using ‘Table object’ function in your word processing program. Do NOT create tables by using the space bar and/or tab keys. Do not submit tables in Microsoft Excel.

• Do NOT use the enter key within the body of the table. Instead, separate data horizontally with a new row. Do not insert blank columns or rows.
• Colour, shading or bold should not be used. Parts of the table can be highlighted using superscript, numbering, lettering, or symbols, the meaning of which should be explained in a table caption. Use asterisks (not bold) to indicate statistical significance.
• Tables should be numbered in the order they are first mentioned in the text (e.g. "Table 1", "Table 2", and so on).
• Abbreviations used in tables should be defined in the table caption (within parentheses or as a table footnote).

Borders should be used in a simple manner to help the typesetter identify correctly column headings. Use borders for top/bottom of the table and column headings only (see below). Asterisk (*) is used ONLY to indicate statistical significance, this and other superscripts should be converted to superscript letters with explanations below the table, as in the example below:

Graphical abstracts

A graphical abstract must be uploaded during submission. The graphical abstract should be an eye-catching image in landscape orientation. Do not submit a screenshot of the written abstract or a simple table as a graphical abstract; it must be an eye-catching image. It should be relevant to the topic covered and serve to attract readers' attention to the article. The graphical abstract image may be one of the images included in the article or any other image the authors feel to be appropriate. The image should be uploaded as a high-resolution file (e.g., TIFF 300-600 dpi, LZW compression) and not as Word or PowerPoint files. Please note that graphical abstract images must comply with BMC's copyright policy.

Reporting statistical analysis results

Complete results (not only a P-value) from statistical analyses should be reported in the text each the time you are referring to statistical results. You should include the test statistic values (t, H, F, etc.), the degrees of freedom and exact P-value. If the exact P-value is very small, e.g. 10-9 indicate as $P < 0.0001$.

Writing numbers

Spell out numbers one through nine; 10 and above, use numerals. Use numerals to write numbers immediately before a unit of measure, time, dates, and points on a scale (even

if they are lower than 10). Use one decimal place for percentages (e.g., 99.78% should be rounded to 99.8%).

ZooBank registration

ZooBank (http://zoobank.org/about) is the official registry of Zoological Nomenclature, according to the International Commission on Zoological Nomenclature (ICZN). All articles describing new species of animals to be published in Parasites & Vectors (online only journal) must be registered in ZooBank, as required by the ICZN Code. Registering your paper in ZooBank ensures that it is officially recorded as the first paper to describe this species. Registering the new species name is not a requirement, but it is recommended. The authors should update the ZooBank record after the online publication of the article. While failure to update a ZooBank record will not have any impact on availability of the new species name under the Code, it will keep the registered information hidden from the public.

Note on the use of italics

Use italics for genus and species names, symbols for genes, loci, alleles, and parts of chemical names as appropriate (including cis, trans, ortho, meta, and para). Symbols for proteins and gene names that are written in full should not be in italics. In zoology, scientific names of taxa above the genus level (families, orders, etc.) should not be in italics. For other organisms (i.e. algae, fungi, plants, viruses and prokaryotes), order, family, genus and species names should be in italics. Common Latin words and phrases that are part of standard English usage should 12 not be in italics. Several examples are listed below in “Additional spelling and formatting recommendations”.

Springer Nature author services

English language editing, academic translations, figure formatting, and other author services are available at Springer Nature (https://authorservices.springernature.com). The authors are advised to use these services, particularly when English editing and figure formatting changes are requested from reviewers and editors.

Additional spelling and formatting recommendations

Below are some additional spelling and formatting recommendations. We are flexible with these recommendations and alternative styles (e.g., sensu lato with or without italics) may be accepted. However, the style should be uniform in the entire manuscript.

IMPORTANT: Common names of winged insects belonging to the order Diptera should be written as two words (e.g., sand flies, house flies, louse flies, horse flies, black flies, and stable flies). A list of fly names may be found elsewhere (e.g., https://www.britannica.com/topic/list-of-flies-2073944).

ANEXO C - PARECER CONSUBSTANCIADO DO CEUA

UNIVERSIDADE ESTADUAL DE SANTA CRUZ
COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA)

CERTIFICADO

Certificamos que a proposta intitulada **"Avaliação do tratamento e da eficácia do princípio ativo fluralaner contra infestação por *Tunga penetrans* em cães"**, registrada com o nº **020/19** sob a responsabilidade de **Profa. Renata Santiago Alberto Carlos**, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela **COMISSÃO DE ÉTICA NO USO DE ANIMAIS da UNIVERSIDADE ESTADUAL DE SANTA CRUZ (CEUA/UESC)** em reunião de **06/11/2020.**

Finalidade: ( ) ensino (x) pesquisa
Vigência da autorização: **10/11/2020 a 31/10/2022**
Espécie/Linhagem/Raça: **Cães - *Canis familiaris***
Nº de animais: **90**
Peso / idade: **peso mínimo de 2 Kg / idade superior a 2 meses a 10 anos**
Sexo: **Machos e fêmeas**
Origem: **Domicílio - Vila Juerana - Ilhéus - BA**

Ilhéus, 10 de novembro de 2020

Rosana Maria de Oliveira Clark
Coordenadora da CEUA-UESC

UNIVERSIDADE **E**STADUAL DE **S**ANTA **C**RUZ
COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA)

CERTIFICADO

Certificamos que a proposta intitulada **"Estudo de campo para avaliar a eficácia de afoxolaner/milbemicina oxima contra *Tunga penetrans* em cães naturalmente infestados"**, registrada com o nº **017/21** sob a responsabilidade de **Profa. Renata Santiago Alberto Carlos** que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela **COMISSÃO DE ÉTICA NO USO DE ANIMAIS da UNIVERSIDADE ESTADUAL DE SANTA CRUZ (CEUA/UESC)** em reunião de **29/07/2021.**

Finalidade: ( ) ensino (**x**) pesquisa
Vigência da autorização: **01/09/2021 a 31/08/2024**
Espécie/Linhagem/Raça: **canina / *cão / canis lupus familiaris***
Nº de animais: **69**
Peso / idade: **não definido / idade acima de 2 meses**
Sexo: **Machos e fêmeas**
Origem: **Animais domiciliados, sob responsabilidade do seu tutor, na Vila Juerana Município de Ilhéus - BA.**

Ilhéus, 29 de julho de 2021

Rosana Maria de Oliveira Clark
Coordenadora da CEUA-UESC