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Guideline

World Association for the Advancement of Veterinary Parasitology (WAAVP) second edition: Guideline for evaluating the efficacy of parasiticides against ectoparasites of ruminants

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ABSTRACT

This second edition guideline was prepared to assist in the planning, conduct and interpretation of studies to assess the efficacy of parasiticides against ectoparasites of ruminants. It provides updated information on the selection of animals, dosage determination, dosage confirmation and field studies, record keeping and result interpretation. This guideline is intended to assist investigators on how to conduct specific studies, to provide specific information for registration authorities involved in the decision-making process, to assist in the approval and registration of new ectoparasiticides, and to facilitate the worldwide adoption of standard procedures.

1. Introduction

This second edition guideline offers a different layout to the original suite of guidelines (Holdsworth et al., 2006a,b,c,d; Vercruysse et al., 2006) in that here they are consolidated into a single updated document. The first part of this guideline captures, among other things, the fundamental principles involved in dosage determination and confirmation of a candidate ectoparasiticide product plus the follow up confirmatory field studies for demonstrating therapeutic and/or persistent efficacy. This part is then supported by five appendices – each one detailing data requirements particular to target ectoparasite groups, viz: Appendix A; (Page 4) – ticks (Ixodidae); Appendix B; (Page 10) – biting and nuisance flies; Appendix C; (Page 14) – myiasis causing flies; Appendix D; (Page 19) – biting, sucking lice and sheep ked; and Appendix E; (Page 23) – mange and itch mites.

The original 2006 WAAVP guidelines aimed to establish consensus on international standards for the efficacy of new ectoparasiticides, be they insecticidal or acaricidal in activity. Vaccine type products were not considered in these guidelines. They attempted to recognise and reflect principles recommended by the scientific community as appropriate and necessary for the collection of scientific data. Consideration was given to regulatory requirements for the generation of efficacy data as published by key regulatory agencies. This approach ultimately led to the adoption of WAAVP guidelines, in some cases, by regulatory agencies without elaboration or clarification. Other regulatory agencies however recognised these guidelines but accompanied them with agency specific supporting guidance.

As 15 years has now passed since the initial publication of the original five guidelines, advances in parasiticide chemistry, formulation, and delivery mechanisms along with development of methodologies necessitate this update.

Since no International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH)

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guidelines exist to offer direction in supporting the conduct of efficacy studies relating to investigational ectoparasiticide products, this WAAVP document offers guidance that may in parts parallel data requirements that support product regulatory authorisation. All studies should be conducted to an international scientifically recognised quality standard.

While the WAAVP guidelines aim to establish consensus on standards for studies to establish the efficacy of ectoparasiticides (actual ectoparasiticidal effect), they do not provide guidance on the evaluation of the effectiveness of ectoparasiticides. The evaluation of the effectiveness of an ectoparasiticide comprises the assessment of its efficacy and additionally considers a range of other factors that may influence the efficacy such as adverse effects/side effects, physiological status of the animals, class of animals, sex, breed or strain and the general safety profile in the target animals of the ectoparasiticide.

Detailed statistical guidance is not given in this guideline and as such it should be sought for each protocol. However, where statistical comparisons are made regarding the means of parasite counts from treated and untreated animals, the geometric mean should be used in preference to the arithmetic mean.

Since ectoparasiticide studies cannot be undertaken without the use of host animals, care must be taken to ensure the welfare of all animals used in the studies. Adequate husbandry such as appropriate nutrition and housing, should be in place. Animals should be selected that are appropriate for the purpose including consideration of breed type, body condition and temperament. Facilities to handle the animals should be present and in good condition. Parasite levels must not become so high on the animals that they cause undue stress. Studies should not continue for unnecessarily long periods of time. Where the investigational ectoparasiticide product is clearly not working and satisfactory parasite management/control is not achieved, the study should be terminated.

All results of each study conducted with the investigational ectoparasiticide product should be documented. Extenuating circumstances, which could explain anomalous results should be detailed.

Therapeutic and persistent efficacy field studies should be conducted at times consistent with the usual peak season for the target parasite in a region. Dosage determination and dosage confirmation studies, which utilise experimental parasite infestations may be conducted out of the peak season for the target parasite so that the study data would be available to initiate field studies at the following peak target parasite season. This approach can be a useful time saver.

Ruminant hosts covered in this guideline encompass cattle, sheep and goats. It is acknowledged that both sheep and goats are perceived as minor species in some countries, and as such regulatory leeway is often given in relation to ectoparasiticide product development under those circumstances. This guideline however treats all named ruminants equally unless stated otherwise in the accompanying appendices.

2. Evaluating efficacy

2.1. Introduction

Controlled studies utilising host animals traditionally focus, if applicable, initially on establishing therapeutic efficacy using three study types: dosage determination, dosage confirmation and field efficacy. Persistent efficacy may be supported by dosage confirmation and field study results.

Dosage determination and confirmation studies are conducted preferably using pen facilities and experimental parasite infestations on the host animals. Techniques to facilitate experimental parasite infestations are detailed in the appendices. For field studies, natural parasite infestations are advocated although in some circumstances, with justification and where permitted, experimental infestations may supplement field exposure. Efficacy must be generated for each parasite species and all the parasite life cycle stages to be pursued on the proposed label of the investigational ectoparasiticide product. Advice should be sought from regulatory authorities to the required number of dosage determination, confirmation and field studies including the number of geographical regions that will be included in such field studies.

Sponsors may deviate from undertaking dosage determination studies as detailed here and use alternative methods to establish an effective dose. In such cases, detailed justification for the technique used and all results obtained, should be documented. However, it is recommended that the studies detailed here are followed.

2.2. Animal studies

2.2.1. Efficacy

Controlled studies are necessary to assess the efficacy of an investigational ectoparasiticide product. The study design will evaluate therapeutic and any perceived persistent efficacy. Efficacy of the investigational product is usually determined by the comparison of parasite numbers on groups of treated and negative (or in some cases, if adequately justified, positive) control animals. Parasitised animals are randomly allocated into treated and negative control groups from rankings of pre-study parasite counts or other declared clinical signs (see the appendices for specifics). An exception to this last statement would be with biting and nuisance flies (see Appendix B for an alternative approach). Efficacy is determined by comparing the numbers of target parasites on the treated animals with those of the negative or positive used control group, subject to the study protocol adopted. See specific appendices for further details.

The efficacy of the investigational ectoparasiticide product can be calculated using formulae detailed in the respective appendices.

With therapeutic efficacy, studies will be required to demonstrate a percentage efficacy for the target parasite. See the specific appendices for stated efficacy levels. Ectoparasiticide products based on chemical groups such as macrocyclic lactones (ML) or arthropod growth regulators have differing modes of action to older ectoparasiticides, which may necessitate a different approach to therapeutic and persistent efficacy assessment. See the specific appendices for elaboration.

With persistent efficacy, studies will be required to demonstrate a percentage efficacy against target parasites on treated groups of animals during the period for which persistent activity is to be investigated. See the specific appendices for details.

2.2.2. Animals

2.2.2.1. Selection. In general, animals should be from the same parasitological background (exposure or non-exposure to parasite infestation) whether the parasite infestation has been acquired naturally or experimentally induced. Where possible, animals should be from the same source and should be of similar weight, age and breed/type. For most dosage determination and dosage confirmation studies, young animals (e.g. approximately 6–12 months old in pen facilities and 18 months–2 years in field studies) are preferred. Age exceptions occur where paralysis tick is the target organism (see Appendix A for specifics). Any prior drug and chemical treatment of the animals should be known to avoid effects on the study results from residual activity.

Naturally infested animals (with the exception of studies designed for biting and nuisance flies) should be sourced from areas where a high prevalence of the target parasite has been recorded and a profile on the parasite population may be evaluated (chemical susceptibility, etc.) if of interest.

During a study, the animals should be identified by uniquely numbered ear tags or other suitable means. To accurately calculate individual doses of the investigational ectoparasiticide product to be administered, all animals should be weighed prior to the study beginning (=treatment). It is good practice to weigh the animals again at the end of the study as this can provide data on the welfare of the animals during the study and may demonstrate benefits of the treatment.

2.2.2.2. Allocation. In general, randomised block design studies should

be conducted. Animals should be randomly allocated to the treated and negative control groups. Effects of treatment on performance among groups should be reduced by stratified random allocation to treatment groups according to characteristics that have the potential to influence the results of the study (e.g. parasite counts, body weight, age, sex). For example, if the study has 24 animals in four groups of six animals, then the animals should be ranked on parasite counts, the four animals with the lowest counts should be allocated at random, one to each group. Then the four with the next lowest counts should be randomly allocated to the four groups and so on. This gives four starting groups with approximately equal parasite counts before treatment.

To prevent cross-contamination with treatment and cross-infestation with parasites, the animal or where appropriate groups of animals, should be maintained in isolation for the duration of each study so that there is no contact with animals from any other group. Facilities used for handling animals can be a source of cross-contamination and appropriate measures should be taken to reduce this risk between the examinations of treatment groups. Personnel involved in the collection of efficacy data or ranking assessments should be masked to the treatment assignment of the animals.

2.2.2.3. Management. Animals should be managed similarly and with due regard to their wellbeing according to applicable regulations. All study animals should be confined under similar conditions according to local practice. Animals should be offered food according to local practice and be provided with a ration suitable for their age and condition. Fresh water should be available throughout the study.

In general, animals should be acclimatised to the facilities and the feeding type/regime for at least 7 days prior to the study start (=treatment).

The health of the animals should be observed at regular intervals during the study and recorded. Any routine husbandry measures that might be performed after animals are obtained should be documented. All medications should be administered by properly trained personnel with usage based upon a diagnosis that indicates a medication is required following proper documentation procedures. Medications to animals should not be administered in the proximity of the site where the investigational ectoparasiticide product was administered. No parasiticide other than the investigational product should be administered at any time during the acclimatisation period or during the study without justification and documentation.

2.2.2.4. Numbers. It is advocated that at least six animals per group in each dosage determination and in each dosage confirmation study group must be infested with the target parasite at the start of the study (modification required for biting and nuisance flies – see Appendix B). This is justified, in part, by considerable animal variation in efficacy that may occur with a particular ectoparasiticide or formulation. For example, considerable animal-to-animal variation has been found in the efficacy of ML pour-on formulations and certain parasite development inhibitor compounds. A larger number of animals should be included in field studies as noted in the relevant appendices.

2.2.3. Study types

2.2.3.1. Dosage determination studies. Usually pen facilities are utilised for dosage determination studies to establish the effective dose to manage the target parasite with the investigational ectoparasiticide product. Such studies are also used to determine the least susceptible (least sensitive, dose limiting) target parasite species and parasite life cycle stage, that is likely to be present in the field. Studies are recommended to be carried out with the final or near final formulation and the range of doses selected based on preliminary studies that are likely to indicate the approximate effective dose. For a conventional dosage determination study, four uniformly, parasite infested groups (induced infestations) of animals are preferred: negative/vehicle/placebo control, 0.5, 1 and 2 times the anticipated effective dose.

2.2.3.2. Dosage confirmation studies. Usually pen facilities are utilised for studies to confirm the effective dose established in the dosage determination studies, against the target parasites and parasite life cycle stages, as calculated in dosage determination studies, as well as the length of protection (persistent efficacy) that is provided against parasite re-infestation. The results of dosage determination studies can be used to confirm the dosage, if the final formulation was used, each target parasite species and parasite life cycle stage were used, and the formulation administration occurred under the proposed investigational product label recommendations.

For a dosage confirmation study, two adequately and uniformly parasite infested groups of animals are used: a negative or vehicle treated control group and a group treated with the selected dose.

Where artificial rainfall is used to test the efficacy of a topically applied investigational product before or after heavy rain, the method of wetting the animals used and the equivalent in terms of natural rainfall should be stated (e.g. artificial rain applied by inverted sprinklers, equivalent to a rainfall of 20 mm in a storm lasting 30 min). The time of animal wetting before or after investigational product application should be recorded (e.g. 0, 2 h, etc.).

These studies can also identify peculiarities and side effects when the final, i.e. commercial formulation of the investigational product is used.

Combinations of ectoparasiticides can be valuable for managing different parasites with one application to the animal. It is not possible to set precise guidance because several factors influence the choice of compounds and dosages. Where relevant, data on the efficacy and lack of interference of individual chemical compounds in a combination formulation, as described in earlier sections, must be provided.

2.2.3.3. Field studies. Field studies are utilised to generate data to confirm efficacy demonstrated in dosage determination and dosage confirmation studies with the investigational ectoparasiticide product under field conditions.

For a field efficacy study, two groups of animals are used: one group treated with the investigational product at the effective dose and the second group being the negative (or in some cases, if adequately justified, the positive) control. Appropriate consideration needs to be given to vagility of the target parasite species.

2.2.4. Investigational product application methods

An investigational ectoparasiticide product could be applied to an animal as a topical preparation (e.g. dip, pour-on, spot-on, ear tag or spray) or by a systemic route (e.g. oral or injectable formulations). For backline application (e.g. pour-on, spot-on), volumes to be delivered must be calculated according to body weights indicating whether animals were weighed before or after feeding. The investigational product should be applied according to intended label directions. An oral or injectable preparation dose must be calculated according to body weights. For an injectable preparation, the route of administration (e.g. subcutaneous or intramuscular) must be stated.

Animals in pen and field studies of plunge and shower dip applications, topical sprays, or jetting/spray races should be treated in the manner recommended on the investigational product label. After charging the dip at the recommended rate, samples should be taken before, during, and at the end of dipping. Dip samples must be stored appropriately prior to dispatch to test the concentration of active ingredients and other chemical contents in the dip was according to validated analytical methods.

The method of application should be clearly detailed. For plunge dips the length, depth, number of times the animals were submerged after entering the dip, the swim length of the dip and average time in the dip should be recorded.

For shower dips, jetting/spray races, the brand and model of dip used should be stated together with the operating pressure and output of the pump. The length of time that animals are sprayed, and the combination

of upper and lower nozzles used should be stated. For plunge and shower dips as well as jetting/spray races, the method of mixing the chemical in the sump, the method of addition of fresh chemical and amounts added should be recorded. If the dip equipment collects dip run-off and recirculates dip fluid, then this information should also be recorded.

For automatic jetting races, the operating pressure and output can be estimated by subtracting the volume of dip wash left after dipping from the volume at the start and dividing by the number of animals dipped. For hand-jetting, the hand jetter can be calibrated for low volume versus time, and a standard application time used for each animal.

CRediT authorship contribution statement

Peter Holdsworth developed the initial draft of this paper and then invited fellow authors to critique and build on this. The aim and focus was to update, where necessary, guidances that were initially published in 2006. All authors contributed equally to develop the final draft of the guideline.

Declaration of Competing Interest

The authors report no declarations of interest.

Appendix A. Guidance for efficacy studies involving infestation with Ixodidae

A.1 Introduction

There are three families of Ixodida, of which the Ixodidae (hard ticks) are the largest group, followed by the Argasidae (soft ticks). The family Nuttallillidae consists of only one species *Nutalliella namaqua*. Approximately 80 % of all the ticks of veterinary importance belong to the Ixodidae (Table A1). Their life cycles are varied but are generally of three basic types comprising the three-host life cycle (e.g. *Amblyomma* spp., most *Rhipicephalus* spp., some *Hyalomma* spp. and *Ixodes* spp.), the two-host life cycle (e.g. some *Rhipicephalus* spp. and most *Hyalomma* spp.) and the single-host life cycle (e.g. some *Rhipicephalus* spp.).

Table A1
Examples of important Ixodidae ticks on domestic ruminants.

Family Ixodidae	Number of different hosts to complete life cycle	Common name
Ixodes spp.	3	
I. scapularis		Deer tick, Black legged tick
I. persulcatus		Taiga tick
I. ricinus		Sheep tick
I. rubicundus		South African paralysis tick
I. holocyclus		Paralysis tick
Dermacentor spp.		
D. variabilis	3	American dog tick
D. albipictus	1	Winter tick, Moose tick
D. andersoni	3	Rocky Mountain wood tick
D. marginatus	3	Ornate sheep tick
D. reticulatus	3	Ornate cow tick, Ornate dog tick, Meadow tick and Marsh tick
D. silvarum		
Haemaphysalis spp.	3	
H. longicornis		*Bush tick*
H. punctata		*Red sheep tick*
H. sulcata		
H. parmata		*Winter tick*
H. aciculifer		
H. inermis		
H. rugosa		
Rhipicephalus spp.		
R. bursa	2	
R. evertsi	2	Red legged tick
R. appendiculatus	3	Brown ear tick
R. microplus	1	Tropical fever tick Cattle tick
R. australis	1	Cattle tick, Southern cattle fever tick
R. decoloratus	1	Blue tick
R. annulatus	1	Cattle fever tick, Blue tick
Amblyomma spp.	3	
A. americanum		Lone-Star tick
A. maculatum		Gulf Coast tick
A. cajennense		Cayenne tick
A. hebraeum		South African bont tick
A. variegatum		Tropical African bont tick
Hyalomma spp.	2	
H. anatolicum anatolicum		Camel tick
H. dromedarii		
H. marginatum rufipes		

Ticks can harm their hosts directly or indirectly. Direct harm results in blood loss, tick burden as well as toxicoses. Tick bites can be injurious and cause severe hide damage, including abscessation providing a route for secondary infection. Crumpled ear pinnae, sloughed tears, missing tail tips, lameness and foot rot can all result from tick infestation. Ticks causing toxicosis (particularly paralysis) include approximately 60 species in the genera *Ixodes*, *Dermacentor*, *Rhipicephalus* and *Argas*. Ticks also cause metabolic disturbances in the host, for example, inappetence caused by *Rhipicephalus* spp. Indirectly, several tick species cause economic loss because as vectors they transmit a diverse suit of pathogenic organisms. Some tick vectors that parasitise domestic ruminants are of veterinary public health importance because the pathogens they transmit are zoonotic and affect humans. The diversity of economically important pathogens transmitted by tick vectors to animals include arboviruses, rickettsias, anaplasmas, tularaemia, babesias, and theilerias.

A.2 Animal studies

Studies utilising host animals focus initially on establishing a therapeutic efficacy using three study types: dosage determination, dosage confirmation and field efficacy. Persistent efficacy may be supported by dosage determination, dosage confirmation and field study results. Establishment of therapeutic and persistent efficacy may be combined in one and the same study.

Dosage determination and dosage confirmation studies are conducted preferably using pen facilities and experimentally induced tick infestations on host animals. Experimental tick infestation can be induced using manual transfer from a laboratory colony of ticks to the animal (see Annex A for details). Ticks used in experimental infestations should be from so-called recent field isolates kept, where possible, for less than 10 years in the laboratory, and preferably with an established acaricide resistance profile. For field studies, natural tick infestations are advocated. Efficacy data must be generated for each tick species and all targeted parasitic tick stages for the investigational product. All parasitic tick life cycle stages may be used on animals in the same study. For two-host and three-host ticks, only adult ticks are usually used in the animal studies and *in vitro* comparison between immature and adult stages of each tick species should be provided to demonstrate that immature stages are as susceptible or more so than the adults if appropriate.

A.3 Study types

In vitro studies: Although not necessarily allowing for conclusions as

to the efficacy of investigational products against ticks on infested animals, *in vitro* evaluations involving one or a combination of assays can provide useful information concerning the potential acaricidal activity of the experimental compound against ticks. These assay results may be used as preliminary guidance prior to dosage determination or confirmation studies. Methods are available to determine LC50 values (Finney, 1971) for any potential contact acaricide. Such methods rely on exposure of ticks to impregnated filter papers (Stone and Haydock 1962; Shaw, 1966; Tatchell, 1974), immersion of ticks in solutions (containing either aqueous or solvent/detergent systems if the acaricide does not have a high solubility in water) (Drummond et al., 1973), or incorporating the acaricide in a blood meal fed to the ticks (Trentelman et al., 2017; Kroeber et al., 2007). While *in vitro* evaluations are not obligatory data for product registration, one or a combination of these assays can provide useful information concerning the potential acaricidal activity of the experimental compound against ticks. These assay results should not be used as a basis for determining the likely field use dose for a particular acaricide; however, they may be used as preliminary guidance prior to dosage determination or confirmation studies.

Dosage determination studies: Pen facilities are utilised for dosage determination studies to calculate the effective dose to manage the target ticks with the investigational product. These studies are also used to determine the least susceptible (least sensitive, dose limiting) tick species that is likely to be present in the field.

Dosage confirmation studies: Pen facilities are utilised for dosage confirmation studies to establish the effective dose against target ticks, as well as the length of protection (persistent efficacy) against tick re-infestation due to treatment with the investigational product.

Controlled studies using animals are advocated to assess the efficacy of an investigational product against ticks. The study design will evaluate therapeutic and/or persistent efficacy. Efficacy of the investigational product is usually determined by the comparison of tick numbers on groups of treated and negative control animals. Parasitised animals are randomly allocated into treated and negative control groups from a ranking of pre-study tick counts (as described in section 2.2.2.2 of main text and Annex A). For single-host ticks, efficacy is determined by comparing the numbers of engorging or fully engorged female ticks from treated animals with those on the negative control animals [tracer cattle in field studies (see *Field studies* section and Annex B)]. Numbers of fully engorged ticks are determined in pen facilities. Counts of semi-engorged female ticks are usually done only on one side of the animal in field studies. Such counts normally provide an accurate estimate of the number of ticks that should engorge within 24 h.

The efficacy of the investigational product for single-host ticks can be estimated using the formulae detailed in Annex A (pen facilities) and Annex B (field studies).

For multi-host ticks, including paralysis ticks, efficacy is determined by calculating the percent reduction in adult tick numbers in pen facilities. The formula shown in Annex A should be used. In field studies, negative control animals should be used where possible, and the efficacy calculations should follow those described in Annex C. Where there is information that ticks in immature stages are less affected by the investigational product than adult ticks (e.g. in pen studies), then a dosage confirmation or field study should be conducted with these tick stages. Where ticks cause severe paralysis, animal welfare concerns need to be addressed in the first instance.

The emergence of parasite populations resistant to chemicals used to treat them, or the adverse effect of established resistance mechanisms on new chemical classes, continues to pose a serious threat to stable chemical parasite management strategies. It is therefore essential to establish the resistance profile of both laboratory and field strains of the ticks used in dosage confirmation and field studies.

Therapeutic efficacy: For single-host ticks, pen studies will be required to demonstrate a percentage efficacy for the parasite. For therapeutic efficacy, at least 90 % efficacy is preferred and the protocol detailed in Annex A should be used to determine efficacy over a 22-day period post-treatment. It is important to determine daily percentage efficacy because 1) it can demonstrate that the test acaricide is working quickly; 2) information is gained on what stages of the tick life cycle are being killed on the host; and 3) informed decisions based on average percentage efficacy can be made to extend the study over the 22-day period.

Onset of efficacy: How quickly acaricides work is important for the transport of cattle across international borders or other boundaries where tick status is regulated. Onset of efficacy can be evaluated in pen and field studies. The protocol outlined in Annex A should be used for pen studies and Annex B for field studies. If the test product has a slow onset of acaricidal action then the percentage efficacy may be calculated over any 22 days that it is most effective, e.g. between Days 4 and 30 for MLs or Days 15 and 50 for acarine growth regulators (AGRs) formulated products containing the active fluazuron, or other actives with similar mode of action.

Any acaricides that do not have a rapid knockdown effect on ticks may necessitate a different approach to therapeutic (and persistent) efficacy assessment. These acaricides can be assessed over the expected period of time (days or weeks) to deliver at least 90 % efficacy, thereby allowing for some initial delay for these compounds to reach full acaricidal efficacy once applied to the host animal. Efficacy and use recommendations for the investigational product should include detailed recommendations on the frequency and seasonal timing of treatments required to achieve a satisfactory tick management program. These recommendations should be based on the results of field studies.

For multi-host ticks at least 90 % efficacy is preferred. This level of therapeutic efficacy should be achieved preferably within 4 days after administration of the investigational product. Due to the biology of some tick species where male ticks first attach, start feeding and then produce attraction-aggregation-attachment pheromones that stimulate the female ticks to attach, the onset of activity of systemically acting products may be delayed because of the delay in attachment/engorgement of female ticks and thus exposure of the ticks to the active. This biology must be considered in the timing of the challenge of animals to generate 'established' tick infestations. For calculation of efficacy, methods cited in Annex C should be used. In the case of paralysis ticks, a higher efficacy level (e.g. for *Ixodes holocyclus* a minimum of 95 % efficacy against adult female ticks) is required due to animal welfare considerations. Furthermore, no individual calf should sustain less than 90 % reduction of *I. holocyclus* burden after treatment. For animal welfare reasons, efficacy data may not be generated using existing semi-engorged or engorging female *I. holocyclus*. Therefore, if necessary, product use instructions should contain a statement indicating that protection may not be afforded against paralysis if engorging ticks are already present on cattle at the time of treatment or if ticks engorged recently and dropped from the animals. It is known that *I. holocyclus* toxin is only slowly removed from the host. For other paralysis ticks in which larger numbers are needed to potentially paralyse a host, the minimum acceptable efficacy may be less than 95 % but sponsors should demonstrate adequate protection against paralysis.

Persistent efficacy: For single-host ticks, the residual protection (persistent efficacy) period is from Day 22 post-treatment until the average efficacy calculated over a weekly period drops below the preferred 90 % threshold. The method described in Annex A should be used to determine protection in pen facilities by continuing infestations beyond the treatment day and continuing tick counts beyond Day 22. The method described in Annex B should be used for field studies by continuing counts beyond 22 days ensuring that adequate exposure to the parasite has occurred during this time, given the seasonality of the exposure.

For multi-host ticks, data demonstrating at least 90 % efficacy against ticks on treated groups of animals should be achieved during the period for which persistent activity is to be claimed. The methods cited in Annex C should be used to make the calculations to support such a claim. Both dosage confirmation and field studies can be used for these studies.

For the paralysis tick *I. holocyclus*, the suggested, allowable protection period is the interval from Day 0 (day of treatment) until the last assessment when no attached ticks survived for longer than 3 days on any individually treated calf. For other paralysis ticks with lower toxicity, the same requirement is preferred unless the sponsor can demonstrate that a longer protection period is justified. Both dosage confirmation and field studies can be used for this purpose.

Naturally infested animals should be sourced from areas where a high prevalence of tick infestation has been recorded to ensure the animals have reached a stable level of immunity to ticks. Experimental tick infestations should preferably be established on parasite naïve animals to increase the likelihood of achieving a similar tick population on each animal. Animals that are debilitated, suffering from disease or injury, fractious or otherwise unsuitable should not be included in a study.

It may be necessary to protect study animals against diseases transmitted by the ticks. Animals should be immune to possible tick transmitted diseases or be vaccinated (e.g. *Babesia*, *Anaplasma*) prior to the study. This may require a longer acclimatisation period.

It is recommended that data also be generated on the reproductive capacity of targeted single-host ticks surviving treatment, as such effects can influence the overall impact of the treatment. For example, some synthetic pyrethroids (SPs) and subsequently developed systemically active compounds can cause a reduction in tick egg laying (when testing MLs) and can possess ovicidal effects (when testing AGRs), which adds considerably to overall tick efficacy.

With single-host ticks, the traditional parameter for efficacy is derived from the tick kill, achieved during the period of 3 weeks following treatment of cattle infested with all parasitic stages of the cattle tick in pen studies (Wharton et al., 1970). Determination of this parameter is based on a comparison between the numbers of adult female cattle ticks engorging on treated versus negative control animals. These laboratory studies may utilise nominated resistant strains. In some cases, for example where *in vitro* generated data have indicated a potential problem with either SP or amidine resistant tick strains, the limited availability of such strains, and quarantine precautions in the field, may mean that a pen facility becomes the only practical method of generating therapeutic efficacy data.

The period of persistent efficacy is assessed in pen facilities by continuing the therapeutic efficacy study so to infest treated and the negative control animals twice weekly, beginning on Day 2 post-treatment. The study should be continued until tick re-infestation of treated cattle is established, as determined by a comparison of adult tick collections or counts, between the treated and negative control groups. Where long persistent efficacy is expected, re-infestation after treatment can be delayed. However, it must be shown that, following the first post-treatment infestation, no or few ticks are collected. The few ticks collected should not result in overall study efficacies being lower than suggested in this guideline. It is essential that cattle topically treated with the investigational acaricide should be exposed to normal, outdoor, weather conditions during studies to establish a persistent efficacy period.

For multi-host ticks, studies should be conducted with adult tick infestations following the procedure and calculations recommended in Annex C. The duration of the observations post-treatment of established infestations or after challenge with ticks will be determined by the life cycle of the tick being studied.

With paralysis ticks, study calves should be challenged with adult female ticks. Care should be taken to minimise stress, discomfort or any sign of paralysis in calves challenged by adult ticks. Ticks that measure 4 mm in length or more should be removed from calves and replaced with new unfed ticks, to minimise the chances of paralysis in the study animals. Any calves showing signs of paralysis should immediately be removed from the study and treated with a registered/approved paralysis tick acaricide. An anti-serum should be administered by a veterinarian. It should be noted that most cases of paralysis in calves are observed 5–13 days after tick attachment, and paralysis often occurs after ticks have dropped off the animal. For this reason, close observation of very young calves is required.

For less toxigenic paralysis ticks and species for which no antitoxin is available, the study animals should be closely observed; and if live ticks are noted on the animals, they should be counted and manually removed.

Field studies: Groups of animals used include: one group treated with the investigational product at the intended label recommended dose and the second group being the negative controls but removed at particular time intervals to avoid tick problems through the study period (see Annex B for specific study design recommendations). These negative controls are tracer cattle used to monitor the tick populations in the field or the tick populations on the cattle where experimental infestations are needed in the field studies (see Annex B). Care needs to be taken with pour-on preparations where the acaricide may be transferred by group/herd grooming. Separation of treated and control groups is critical. The number of negative control (tracer) animals should be at least 25 % of the number of investigational product treated animals. The aim in all field studies is to achieve a significant tick challenge. Tracer cattle should be used to monitor tick challenge. The inclusion of a minimum five tracer cattle in the same paddock with the investigational product treated cattle is recommended.

The use of tracer cattle gives a true indication of tick populations in the field and reduces the ethical issues relating to adverse effects of ticks on cattle health. Per study site, at least 20 investigational product treated animals per study group should be identified and assessed throughout the study.

In selecting suitable study sites and co-operators, it should be emphasized that treatments and observations should preferably be continued over at least one tick season. In therapeutic efficacy studies where candidate acaricides demonstrate less than 90 % tick efficacy in dosage confirmation, then field efficacy studies should be conducted over two tick seasons at every study site to ensure that an adequate level of tick activity can be achieved.

Where single and multi-host ticks occur together in the field on the same hosts, re-treatment intervals will be determined by the shorter feeding period of the multi-host ticks.

Investigational acaricides with greater than 90 % efficacy targeting single-host ticks: The purpose of these field efficacy studies is to evaluate the investigational product when used according to the manufacturer's instructions under practical conditions in the field and to obtain additional experience regarding acceptability and safety. The studies should be carried out with animals of a tick susceptible breed.

Where an investigational product's performance may be influenced by environmental factors, records should be kept of rainfall and temperature on the farm during the 24 h preceding and the 24 h after treatment and monthly rainfall on the farm should be recorded for the duration of the study.

Therapeutic and persistent efficacy as demonstrated in pen facilities should be confirmed under field conditions. Within the location, during time periods of defined significant tick challenge in the field, an efficacy assessment similar to that outlined in Annex B should be carried out using a minimum of 20 animals. In addition to pre-treatment tick counts, at least three counts on study cattle should be conducted on separate days during the 22-day period following treatment. Preferably these should be done to encompass maturing ticks resulting from adult, nymph and larval infestations at the time of treatment, e.g. counts on Days 1 and 3, 7 and 9, 16 and 21, respectively would suffice as a minimum requirement. This defines efficacy against immature ticks in the field, which is an important consideration for establishing quarantine requirements for the movement of livestock. Extension of the counting period beyond Day 22 should be used to verify persistent efficacy.

In any geographical location where a seasonal incidence of tick populations occurs and where a number of distinct generations per year may be

observed, persistent efficacy studies may be conducted as part of strategic tick management programs. In order to maximise the efficacy of a treatment regime and minimise the number of treatments required in such studies, recommendations should be developed to ensure that a management program is carried out at a time of year when the tick population is most vulnerable. Studies should preferably be conducted over a full tick season; but in tropical areas with year-round tick reproduction, studies should be carried out over 6 months and timing is less critical.

In accordance with these recommendations, the persistent efficacy studies in nominated geographical locations should be selected to assess the performance of the investigational product in any recognised strategic management program, i.e. control achieved during a complete tick season. Tick populations on a minimum 20 selected animals at each study site should be monitored throughout this period, preferably with counts conducted immediately prior to each treatment. It may not be reasonable to expect that negative control animals should be retained on site, during this prolonged period. However, evidence should be provided, from local observations in the area where a significant tick challenge existed for the study animals during the selected period (e.g. use of tracer animals). Treatment intervals should be planned on this basis of results obtained in previous therapeutic and persistent efficacy studies.

Investigational acaricides with greater than 90 *% efficacy targeting multi-host ticks*: Studies should be conducted with adult tick infestations, and the same procedures should be followed as detailed earlier for investigational acaricides with greater than 90 % efficacy targeting single-host ticks. Formulae cited in Annex C should be used for all efficacy calculations.

For paralysis ticks, it is essential that treated calves are pastured and exposed to normal weather conditions during these studies. At 1, 7, 14, 21 and 28 days after treatment (and other times as required in order to confirm the draft label information), all calves should be challenged with adult female ticks. At least 10 adult female ticks per calf should be included at each challenge. Negative control calves should be used to demonstrate the capability of the ticks to infest the animals. To obtain these numbers of ticks, additional field collected ticks may be used to infest study calves.

Animals should be inspected each day post challenge for tick attachment. Negative control calves should be examined at least once a day, and any engorging adult ticks should be identified and removed manually.

At the end of the last challenge period (no later than 5 days after the final challenge) all attached ticks should be removed manually and all calves should be treated with a registered/approved acaricide capable of killing any persisting ticks.

Investigational acaricides with less than 90 % efficacy targeting single-host ticks: If an investigational product does not provide at least 90 % therapeutic efficacy or greater than 90 % persistent efficacy (e.g. an investigational product with slower onset of action) for a continuous period of 3 weeks, the investigational product may be further assessed in studies run over two full tick seasons. These studies should be under different climatic conditions with defined treatment intervals throughout the tick seasons. These longer-term field studies are undertaken to develop and demonstrate the potential of such an investigational product to provide satisfactory stable levels of tick management. It is reasonable to expect that the results of such studies should be accorded particular significance in the product registration/approval process. Where at least 90 % efficacy is not achieved in therapeutic efficacy studies, it is particularly important to demonstrate that this longer-term approach can provide a tick management strategy equal to, or better than, that likely to be achieved with a traditional acaricide under long-term field use. This longer-term approach may integrate other management methods such as tick vaccine treatments, resistance management strategies, etc.

Investigational acaricides with less than 90 % efficacy targeting multi-host ticks: The same procedures as detailed for investigational acaricides with less than 90 % efficacy targeting single-host ticks can be followed. Efficacy should be calculated using the recommendations listed in Annex C.

For an investigational product with a slower onset of action, the draft product label must state the period of time required to kill the nominated percentage of ticks on treated ruminants.

A.3.1 Annex A

Basic short-term efficacy studies—methods and analysis of results using single-host tick infested cattle held in pens (Roulston et al., 1968).

A.3.1.1 Materials and methods. Tick naïve steers approximately 6–12 months old are preferred for the pen studies and preferably of a tick-susceptible breed. Cattle should be acclimatised to the facilities for an appropriate period after which they can be prepared for randomisation to groups and the subsequent assessment of therapeutic efficacy by conducting multiple infestations over a period of approximately 24 days. During this period, each animal should be experimentally infested with 2000–5000 larvae of a selected strain of the target cattle tick species, on approximately 10 separate occasions. The number of larvae used and infestation events over this period needs to be carefully balanced based on the size of the animals used to prevent too high or low tick burdens. In general, at least 10 % of the total number of larvae used for infestations should be collected as engorged females from control animals indicating that the larvae used were viable and the infestation on the cattle adequate. When using tick resistant cattle breeds, less than 10 %, but always more than 1 % of ticks collected should be adequate.

Cattle may be allowed to graze during this infestation period in an open paddock, with supplementary feeding, as required, if it can be demonstrated that the pastures are tick-free, and it poses no biosafety risk to the environment.

Approximately 4 days prior to treatment, cattle should be individually penned to facilitate collection and counting of detached engorged female ticks. For the duration of this period and after treatment, penned animals must be fed an adequate diet to maintain good health and weight gains and must be provided water *ad libitum*. Adult engorged female ticks dropping from each animal must be collected daily prior to cleaning the pens and counted. Various types of pen designs can be used to facilitate tick collection but in general pens with grid flooring through which detached ticks can drop to a collection pan or surface are preferred compared to pens with solid flooring on which detached ticks can be mechanically damaged by the animal.

For studies requiring further assessment on tick fertility, collected ticks must be cleaned, dried, counted and weighed. In order to assess tick fertility, a random sample of 50 adult ticks, or maximum number available where less than 50 are collected per week, can be weighed and incubated at 27^{0}C and 85 % RH for at least 7 days to determine weight of eggs produced. Viability of eggs produced may be rated visually according to pre-defined criteria or alternatively may be assessed by estimating the egg hatching success. Prior to treatment all animals should be weighed and ranked according to tick burden assessed on the basis of ticks collected over a 3-day period. Where there are insufficient pens to accommodate one study animal per pen, then ranking can be based on 3 days side counts of 4.5–8 mm female ticks (*Rhipicephalus* spp.).

Animals must be randomised to study groups using the individual total tick count over the 3-day period as the primary ranking criterium, ensuring that the study groups are as best as possible balanced with regard to pre-treatment tick counts. On Day 0, animals are treated immediately after the daily collection of ticks is completed and these should be discarded safely and not considered for any efficacy calculations. For sprays, treatment may be carried out by manual spraying (where only limited quantities of formulated test product are available) or by whatever application method is

planned for the final commercial product. Particular care should be given when spraying animals not to contaminate control animals through spray drift. Daily collection of adult engorged female ticks dropping from each animal or treated group and incubation of a sample of the ticks collected (if required) must be continued, as described above, until Day 22, to assess treatment efficacy for control of the different parasitic stages present on infested cattle at the time of treatment.

To determine the protective period (persistent efficacy), infestations can be continued post-treatment day. To reduce any adverse effects on negative control animals, the infestations may be reduced to twice per week and for long-term studies reduced even more by infesting animals twice every other week or even over a 1-week period once a month. Ticks collected from Day 23 onwards should be used to calculate persistent efficacy on a weekly basis. To determine the onset of efficacy, the percentage efficacy may be calculated over any 22 days that it is most effective (e.g. Days 4–26).

A.3.1.2 Analysis of results. Various methods for calculating efficacy can be used but the appropriate method should be based on the specific study design and objectives of the study. Percentage efficacy of treatment on parasitic stages based on tick survival can be calculated for each day post-treatment (DayN) as follows.

EC is the expected count of ticks in treated group if left untreated.

$$\textit{Percentage tick survival on DayN} = \frac{\textit{Mean tick count treated group on DayN}}{EC} \times 100$$

Where DayN is any given day post-treatment and,

$$EC = \frac{\textit{Mean tickcount on controls on DayN} \times \textit{Total pretreatment tickcount treated group}}{\textit{Total pretreatment counts control group}}$$

Percentage efficacy based on egg production is calculated as follows on a daily basis.

Firstly, A, the egg production from ticks surviving in the treated group is determined.

$$A = \frac{\textit{Total egg weight from treated incubated ticks} \times \textit{Number of ticks collected}}{\textit{Number of ticks incubated}}$$

Secondly, B, the egg production that would be expected from the treated group if it had not been treated is determined.

$$B = \frac{\textit{Total egg weight from control incubated ticks} \times EC}{\textit{Number of ticks incubated}}$$

The usual number of ticks incubated is 50.

Therefore, $\textit{Percentage efficacy} = 100 \times (1 - \frac{A}{B})$ (Abbott's formula applied on each day)

For studies where stable tick burdens on control animals are expected over time the same method might be applied but using mean counts in place of daily estimates:

$$\textit{Percentage efficacy} = 100 \times (1 - \frac{Mt}{Mc})$$

(Abbott's formula applied to mean counts)

Where,

Mc = Mean number of live attached ticks in the untreated (control) group.

Mt = Mean number of live attached ticks in the treated group.

The same calculation can be used to calculate the reduction in egg production in ticks collected from treated animals compared to ticks collected from control animals based on the average daily egg production weight per tick.

Some acaricides such as MLs can also reduce the weight of ticks surviving treatment. To obtain a more meaningful percentage efficacy, the weights of ticks rather than numbers can be used in the calculations.

Where an acaricide also reduces the percentage egg hatch (e.g. AGRs like fluazuron), this can also be included in efficacy calculations. The percentage egg hatch is difficult to determine accurately therefore an estimate is acceptable provided details of the determination method are documented.

Percentage efficacy based on egg production and egg hatch is calculated using a variant of Abbott's formula as follows on a daily basis:

$$\textit{Percentage efficacy} = 100 \times \left(1 - \frac{A \times \%\ \textit{egg hatch of treated group}}{B \times \%\ \textit{egg hatch of control group}}\right)$$

A.3.2 Annex B

Basic short-term efficacy studies—methods and analysis of results using single-host tick infested cattle held under field (paddock) conditions (Wharton et al., 1970).

A.3.2.1 Materials and methods. Naïve steers approximately 18 months to 2 years old are preferred for the study. Where field infestations of tick larvae are low, cattle may be prepared for the study over a period of 24 days, during which time each animal will be experimentally infested with 5000 larvae of an appropriate resistant strain of the cattle tick on 10 separate occasions. Under conditions favouring heavy field populations of the cattle tick, naturally infested animals should be used. Tick burdens on selected animals in this case should exceed 20 standard 4.5–8 mm adult female ticks counted on one side of each animal on Day 0 (treatment day) of the study but prior to the actual treatment. Infestations should indicate that this level will be maintained on negative control animals for 21 days following Day 0, treatment day. In the 4 days prior to treatment, counts of 4.5–8 mm ticks should be carried out on at least three occasions. On Day 0, treatment day, all animals should be weighed just prior to actual treatment, if the treatment dose is to be based on this criterion and ranked according to tick burden. Animals should be randomly allocated from this ranking to the treated and negative control groups to ensure an even distribution between the groups as described in Annex A. A minimum of 20 animals is preferred per treated group, with a minimum of five negative controls.

These controls will be used as tracers for the tick population in the field or in experimental infestations. The tracer cattle must be removed from the study paddock no later that Day -7 (7 days prior to treatment) and kept in a separate paddock, and ticks should be counted from the sides of the tracer cattle on Day 0 (treatment day) and counts again should occur on Days 1, 3, 7 and 9 after treatment. This avoids introduction of ticks from tracer

animals onto the pasture, which could interfere with longer-term tick management studies. The tracer animals can be returned to the study paddocks after all the ticks have dropped, for further assessment of the single-host tick field population size. Alternatively, to avoid health problems caused by ticks on tracer control animals, they can be treated with a suitable acaricide, and fresh tracer animals introduced. The next group of tracer animals should be introduced to the paddock on Day -7 and removed on the treatment day (Day 0). The ticks should be counted on them on Days 16 and 21. For longer-term studies, tracer animals should be introduced every 2 weeks for a 1-week period and ticks on these animals should be counted 21 days after being introduced into the paddock. This should also be the same day as the treated cattle are counted for ticks. Where a persistent efficacy period is to be assessed, counts and possibly experimental infestations will need to be continued.

A.3.2.2 Analysis of results. Percentage efficacy of treatment on parasitic stages, based on tick survival, will be calculated on a side count basis as follows:

$$\textit{Daily percentage tick survival} = \frac{AD}{BC} \times 100$$

Where,

A = Number of ticks counted on the negative control group prior to treatment.

B = Number of ticks counted on the negative control group on the specified day after treatment.

C = Number of ticks counted on the treated group prior to treatment.

D = Number of ticks counted on the treated group on the specified day after treatment.

Percentage tick survival for the 1–21 days period can be calculated similarly. Counts refer to standard 4.5–8 mm adult female ticks.

A.3.3 Annex C

A.3.3.1 Therapeutic efficacy for multi-host ticks. A target therapeutic efficacy of at least 90 % should be achieved preferably within 4 days after the administration of the investigational product. Due to the biology of some tick species where male ticks first attach, start feeding and then produce attraction-aggregation-attachment pheromones that stimulate the female ticks to attach, the onset of activity of systemically acting products may be delayed because of the delay in attachment/engorgement of female ticks and thus exposure of the ticks to the active. This biology has to be considered in the timing of the challenge of animals to generate 'established' tick infestations.

For the assessment of efficacy, the number of ticks counted on the treated group is compared to the number of ticks counted on the negative control group. Following investigational product administration on Day 0, ticks may be counted *in situ* daily on the animals followed by a removal tick count at Day 4. Removal tick counts are used to calculate efficacy of the treatment using Abbott's formula:

$$\textit{Percentage efficacy} = 100 \times \left(1 - \frac{Mt}{Mc}\right)$$

Where,

M_c = Mean number of live attached ticks in the negative control group.

M_t = Mean number of live attached ticks in the treated group.

A.3.3.2 Persistent efficacy for multi-host ticks. A target persistent efficacy of at least 90 % should be demonstrated through repeated challenge of animals with ticks (e.g. weekly/7-day interval or bi-weekly/14-day interval) after the administration of the investigational product. For the assessment of efficacy, tick counts of treated animals are compared to tick counts of untreated (control) animals. Following challenge at a given time point (Day) after administration of the investigational product (Day 0), ticks may be counted *in situ* daily on the animals followed by a removal tick count at Day 4. Due to the biology of some tick species where male ticks first attach, start feeding and then produce attraction-aggregation-attachment pheromones that stimulate the female ticks to attach, the onset of activity of systemically acting products may be delayed because of the delay in attachment/engorgement of female ticks and thus exposure of the ticks to the active. This biology has to be considered to postpone the removal tick count if needed. Removal tick counts are used to calculate efficacy of the treatment using Abbott's formula:

$$\textit{Percentage efficacy} = 100 \times \left(1 - \frac{Mt}{Mc}\right)$$

Where,

M_c = Mean number of live attached ticks in the negative control group at a specific time point.

M_t = Mean number of live attached ticks in the treated group at a specific time point.

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Appendix B. Guidance for efficacy studies involving infestation with biting and nuisance flies

B.1 Introduction

Many Diptera (true flies) are parasitic on livestock. Certain adult flies cause severe irritation by biting and blood sucking and in the process transmit the causal agent of many ruminant infectious diseases. A short overview of such parasitic flies is published by de Leon et al. (2020).

Management of biting and nuisance flies is challenging, even when based on sound biological knowledge because of their complex ecology. It is possible with some species to destroy immature stages at their breeding sites provided that these sites are easily accessible and not widely dispersed. Management measures can be aimed at the adults during host contacts or at their resting places. Fly management is generally best achieved with chemicals when both the immature and adult stages of the fly are targeted.

For clarity and simplicity in this appendix, two groups are used to describe biting and nuisance flies. Biting flies are listed as buffalo/horn flies (*Haematobia* spp.), stable flies (*Stomoxys calcitrans*) and tsetse flies (*Glossina* spp.), while nuisance flies are listed as *Musca* spp., *Fannia* spp. and *Muscina* spp., etc. Flies such as tabanids and hippoboscids, etc., are not listed here, as management of these and related flies can be covered by study designs for the aforementioned focus groups. Efficacy guidance on *Melophagus ovinus* is given in the lice guidelines as this fly, in contrast to others, spends its entire life on sheep.

B.1.1 Biting flies

B.1.1.1 Buffalo/horn flies (Haematobia exigua, Haematobia irritans, Haematobia stimulans). These flies are small dark obligate parasites of cattle with a typical muscid life cycle. Both sexes are haematophagous and feed mainly on cattle or water buffalo. On cattle they rest particularly on the withers, shoulders and flanks but also the neck, ribs and back. They will fly in order to change position. They take frequent small blood meals. Densities of a minimum of 50–200 are considered to lower milk yield in dairy herds and weight gains in cattle (Kunz et al., 1991). Although in North America, *H. irritans* is the intermediate host of *Stephanofilaria stilesi* that can cause dermatitis in cattle, horn flies are not considered to be important mechanical vectors of pathogens because of their sedentary nature.

Differences in ecology and population dynamics of different buffalo/horn fly species on cattle are likely. Variations in susceptibility to several insecticidal classes also exist (Farnsworth, 1997; Farnsworth et al., 1997). Resistance to current and proposed chemical treatments should be clearly defined if the intent is to demonstrate high efficacy against relevant pest populations.

To provide optimal fly management, the number and timing of chemical treatments can vary significantly. It is, therefore, critical that basic efficacy in support of fly treatments should be generated under local conditions.

Where investigations into periods of protection relating to the use of the investigational product are to be considered it is advisable to use long-term studies, which demonstrate strategies for the seasonal management of buffalo/horn fly populations. Studies should also assess knockdown and persistent efficacy periods for single investigational product treatments. Recommendations as to the number, seasonal timing and intervals between treatments must be detailed. These recommendations should be based on results obtained from field studies.

B.1.1.2 Stable flies (Stomoxys calcitrans). Stomoxys calcitrans irritates stock particularly around stables and feedlots and can cause reductions in milk yield and weight gain. Stable flies act as both biological and mechanical vectors of disease. They are the intermediate host of nematodes including *Setaria cervi*. Stable flies are persistent biters, often engaging in interrupted feeding. This makes them excellent mechanical vectors of blood dwelling pathogens including *Trypanosoma evansi* in a number of hosts. It is believed that stable fly salivary secretions cause toxic reactions, which can reduce the immunological response of the hosts to disease.

Management involves destruction of their breeding sites. Adults respond to visual stimuli but are not attracted to baits. Residual chemical spraying of their resting places, including hosts, helps manage adult fly populations. Cook (2020) offers a review of stable fly management options.

B.1.1.3 Tsetse flies (Glossina spp.). Cattle are the primary domesticated ruminant host of tsetse flies. The flies are active during the day, hunt by sight and smell and will feed every 3 days. Both sexes suck blood and are capable of transmitting trypanosomes, which cause sleeping sickness in humans and nagana in cattle.

One tsetse species occurs on the Arabian Peninsula. All others are confined to the African continent and some nearby islands. The flies infest an estimated 10 million square kilometres of land from the semiarid margins of the Sahel through the tropical rain forests, to the sub-tropical savannahs of Kwa-Zulu Natal in South Africa. Three groups of tsetse fly are recognised: the forest or fusca group, which inhabit the forests of west, central and east Africa; the savannah or morsitans group inhabiting the savannahs of west, east and southern Africa; and the riverine or palpalis group whose five species are found mainly in the forests and riparian vegetation of west and central Africa. While tsetse fly is acknowledged as a serious biting fly with major economic effects in Africa, its unique and restricted geographic situation are such that guidance will not be detailed further here other than to highlight the FAO (1998) reference and Uilenberg (1998) that detail how to conduct studies to manage this fly.

Tsetse management is most often achieved by using stationary (cloth) targets or moving (cattle) targets, aerial spraying, and in the case of severe outbreaks of human African trypanosomiasis, fogging. In certain areas where the risk of fly reinvasion is low (e.g. Zanzibar), sterile insect technology has been used to good effect. In the case of cattle, insecticides are applied to the body of the host either by means of a pour-on, or by hand spraying or plunge dipping.

Insecticidal activity should be tested over an entire season if any management statements are to be made.

B.1.2 Nuisance flies (Musca spp., etc.)

These flies may also carry pathogens externally on their body and transmit anthrax and glanders in this way. *Musca autumnalis*, the face fly,

transmits the nematode *Thelazia* spp. to the eyes of cattle. *Parafilaria* spp., a nematode parasitising the subcutis is also transmitted by *Musca* spp.

Nuisance (fly worry) caused by these flies is considerable, in particular by the face fly *M. autumnalis*. *Musca vetustissima* sucks at the skin causing superficial wounds and much distress in Australia.

Movement of *Musca* flies between faeces and food makes them ideal transmitters of human and animal disease. House flies (*Musca domestica*) harbour up to 100 different pathogens and generally propagate disease by mechanically transmitting pathogenic organisms that cause cholera, typhoid, bacillary dysentery, bovine mastitis and conjunctivitis (*Moraxella bovis*) (Gerhardt et al., 1982; Kopecky et al., 1986; Iwasa et al., 1994).

Hydrotaea (sweat flies) closely resemble *Musca* species and feed on exudates of animal's eyes, nose and mouth. *Hydrotaea irritans* (head fly) is the most important species. Management of this fly is similar to that for *Musca* species.

Insecticides can be used to manage adult flies and larvae and are used as baits, residual sprays, feedthroughs and for topical application to animals.

B.2 Efficacy

The controlled study is advocated to assess the efficacy of insecticides against adult or larval flies. Proper study design can evaluate applicable therapeutic and persistent efficacy. Efficacy of the investigational product is determined by the comparison of fly numbers on groups of treated and negative control animals.

Study animals are randomly allocated into treated and negative groups, and a positive control group should be included when indicated. Laboratory-reared flies are released around or onto animals when undertaking dosage determination and dosage confirmation studies. However, for field studies, natural fly populations are used. The formula of choice to calculate efficacy depends on the ability to obtain pre-treatment parasite counts and whether parasite numbers on the negative control animals are likely to vary over the study duration.

For most studies Abbott's formula[1] (Abbott, 1925) is advocated.

The Henderson–Tilton formula[2] (Henderson and Tilton, 1955) is advocated if the parasite counts in the control animals vary significantly over the study duration.

In field studies where qualitative assessment of investigational product performance may occur, different calculations to assess efficacy may be used. Such calculations must be documented and justified.

The emergence of fly populations resistant to chemicals used to manage them, or the adverse effect of established resistance mechanisms on new insecticide classes, continues to pose a serious threat to the stability of chemical parasite management strategies. It is, therefore, essential to establish the resistance status of both laboratory and field fly strains used in pen facilities and field studies. Any indication of cross-resistance to the investigational product in existing resistant fly strains requires that these populations be used in studies.

Ectoparasiticides in modern day production systems are only one of a number of management tools used in combination to target fly populations. It is difficult to rely on a specific percentage efficacy (therapeutic and persistent) as an indicator of how products will perform in the field under high fly pressure. Based on controlled studies (fly exposure houses), a level of confidence can be generated in performance of the investigational product with a percentage efficacy of at least 90 % being considered an indicator of high performance.

Investigational products with insect growth regulator (IGR) properties targeted for fly management require special consideration. In studies with such types of investigational products, fly larvae should be collected from the dung of at least six control animals at each evaluation time point in a study (Steelman et al., 2003).

B.2.1 Animal numbers

Depending on the fly species and life cycle stage being targeted, at least six animals should be used per group in each of the dosage determination and dosage confirmation study groups. A larger number of animals should be included in field studies (see section 2.3.3 for guidance).

B.2.2 Adequate fly infestation

Adequate infestation in treated and control groups should be evaluated on Day 0 (day of treatment) and in the control group at the termination of the study. This is recommended to validate that the animals were exposed to a sufficient number of flies. Additionally, it permits the comparison between study groups, conclusions based on a statistically significant difference and that the observations will be reproducible. Adequate infestation should be established prior in the study protocol. In general, the term 'adequate' is equated to a mild to heavy fly infestation. Table B1 summarises the number of parasites recommended for induced and natural infestations.

B.2.3 Study types

Studies utilising host animals may focus initially on establishing a therapeutic efficacy. In general, dosage determination and confirmation studies

Table B1
Number of parasites recommended for induced and natural infestations in dosage determination and dosage confirmation studies.

Parasite	Recommended adult fly numbers per animal
Buffalo/horn flies (*Haematobia* spp.)	200
Stable flies (*Stomoxys* spp.)	10
Nuisance flies (*Musca* spp.)	150

[1] Abbott's formula: 100 x (1- T/C) where geometric parasite counts of the treated (T) and control (C) animals occur.

[2] Henderson–Tilton formula: 100 x [1 – (Ta x Cb)/(Ca x Tb)] where parasite counts on treated (T) and control (C) animals occur. Tb is the number of parasites counted per treatment group before treatment, Ta the number counted after treatment, Cb the number counted from the control group before treatment and Ca is the number counted from the control group after treatment.

are conducted using pen facilities to hold control and treated animals. Persistent efficacy may also be demonstrated from pen and field study results. Dosage determination and dosage confirmation studies are conducted using laboratory-reared flies released or in containers, depending on the fly species, with a mesh positioned to allow feeding, on host animals. For field studies, natural fly challenges are usually used. Efficacy data must be generated for each targeted fly species. The fly counting technique employed should be adequately documented.

B.2.3.1 Dosage determination studies. Dosage determination studies are used to establish the optimal concentration required for satisfactory fly knockdown and for the persistent efficacy period. These results are useful in the assessment of any suspected resistance developed in field use.

Alternative methods other than dosage determination studies detailed here may be used to establish an effective dose (e.g. *in vitro* studies). In such cases, details of the technique used and all results obtained should be documented.

B.2.3.2 Dosage confirmation studies. These studies are used to demonstrate what, if any, length of protection against re-challenge is provided as well as identifying peculiarities, contraindications, side effects, etc., when the commercial formulation of the product is used on animals. Pen studies are also used to generate data on whether heavy rainfall immediately before or after treatment affects efficacy of the investigational product.

Animals exposed to laboratory-reared flies are used in the studies. Information on the history and insecticide susceptibility of laboratory strains needs to be recorded. However, laboratory strains of flies used in studies should be from a field isolate less than 10 years old. Animals of the age likely to be most commonly treated with the investigational product should be used.

Cattle treated with the investigational product may be held individually in temperature and humidity-controlled rooms. Details of the fly breeding regimen along with the fly release techniques should be recorded.

While no procedures were identified for the species, it is proposed to refer to section 2.3.2.1 for guidance in combination with information supplied in the publications of Campbell et al. (1971) and Clements et al. (1977).

Two study procedures are advocated. In the first, caged flies are applied to areas of treated skin at various intervals after treatment to measure persistent efficacy. During these ‘patch studies’ animals should be restrained in stocks while in the study area.

Detail should be recorded of the fly (*M. domestica* or other *Musca* spp.) breeding techniques as well as the design and application method for the fly cage used to attach flies to the animals. Details should be documented of the number of fly cages applied to each animal, the body sites for application and the duration of exposure. Knockdown should be recorded after 30 min and mortality after 24 h. These results are averaged for each animal and an assessment made of the period of residual activity of the compound. Negative (untreated/vehicle treated) control groups need to be incorporated in these studies to account for natural mortality in the fly population.

The second procedure is an in-feed chemical control study. Details should be recorded of the weight of animals used, animal pairing (treated and control) technique and feed adaptation procedure for the animals. Procedures for mixing the investigational chemical in the feed should be thoroughly detailed. Allocation methods of paired animals to the treated and negative control groups should be described as well as the feeding rates and regimen per animal. Technique and timing of manure collection should be documented along with the bioassay procedures of seeded first stage *M. domestic*a (WHO insecticide susceptible strain or specify the strain used, e.g. by insecticide susceptibility spectrum) larvae, incubation techniques and scoring method for emerging adult flies. Negative (untreated/vehicle treated) control groups need to be incorporated in these studies to account for natural mortality in the fly population.

B.2.3.3 Field studies. The purpose of field efficacy studies is to evaluate the investigational product when used under practical conditions in the field and to obtain additional experience as to acceptance and safety. The studies should be carried out with a number of breeds or types of animals of different ages and sexes in several geographic locations to cover variations in fly populations, variable environmental conditions, and different feeding and management practices. Studies should be carried out in areas where the flies are endemic. Records should be kept of rainfall and temperature on the farm for 24 h pre- and post-treatment, and daily rainfall on the farm should be recorded for the duration of the study.

For a field efficacy study, the number of negative control animals should be at least 25 % of the number of the investigational product treated animals. Details on the number and sites of such studies should be recorded. At each study site, at least 20 monitor animals should be identified and assessed throughout the study to show improvement of signs associated with the fly challenge.

Group size is critical to ensuring that statistically significant results are obtained.

Cattle should be ranked and randomly allocated to treated and control groups in pairs to ensure an even distribution of buffalo/horn fly challenges between the two groups. A minimum of 20 animals should be kept in each group. Other animals treated with the investigational product on the treatment day can be run with the experimental group. Care must be taken to avoid any contact between the treated and control groups during the study.

Studies must be designed to generate data demonstrating that the investigational product provides sustainable long-term control under a variety of climatic and seasonal conditions as well as in a number of different locations to account for strain differences within the fly population.

Study sites selected must have a significant fly challenge. Due consideration needs to be taken in developing these final studies to involve flies that represent an issue in the region where the investigational product is intended for ultimate commercial use.

At least 50 animals, each with in excess of 200 flies per animal, should be available at the start of the study. Two holding paddocks, with similar pasture qualities and stocking rates should be available for the duration of the study. These paddocks should be capable of holding a minimum of 25 animals and they should be no less than 500 m and no more than 3 km apart. To ensure that there is a continuous and stable buffalo/ horn fly population, the animals should have been grazed on the pasture for at least 3 weeks prior to treatment. On three occasions, in the 10 days before the start of treatment, fly burdens on individually identified animals should be assessed by the same observer using binoculars (Bean et al., 1987). In the case of heavy challenges (greater than 200 flies per side of each animal), counting in blocks of 50 should minimise errors. Counts should be carried out at the same time every day. Weather conditions should be recorded. Care should be taken to avoid weather conditions like strong winds, which may influence the levels of apparent infestation and the likely predilection sites of the flies on the animals.

Using similar studies to those described above, strategic management recommendations for buffalo/horn fly control can be established. These data will be useful, among other things, in contributing to proper long-term management of the investigational product by the end user.

Where an investigational product is designed for seasonal fly management and the method of application does not result in immediate reduction in fly numbers, the results of strategic studies may offer supportive data on such use of the investigational product. Field studies in support of integrated fly management plans should be scheduled to start at the beginning of the buffalo/horn fly season in each area.

Prior to the initial treatments, fly counts should be carried out on at least 25 animals in order to establish the level of challenge. The time periods in weeks post-treatment that animals are monitored should be detailed based on the investigational product's performance in earlier pen/insectary studies. The animals should then be monitored at defined intervals for 3 months. The maintenance of a control herd in heavily challenged areas may not be feasible for the extended period. Evidence must, however, show that significant buffalo/horn fly pressure was maintained during the study period. Monthly counts on local cattle in the surrounding areas may provide supporting evidence.

An investigational product in this category could involve several delivery systems including self-treatment of animals with back rubbers, ear tag applications, other sustained release devices or a strategic series of treatments during a critical period in the buffalo/horn fly season. These approaches could also effectively test integrated management methods based on the combined use of traps, lures or biological control agents that are designed to gradually reduce buffalo/horn fly populations. Integrated fly management studies should ensure that firm recommendations will be made with respect to seasonal timing, duration and where applicable the number of treatments required to produce a satisfactory level of fly control. These recommendations may well differ from region to region.

The adequacy of the fly challenge needs to be demonstrated. It is best to have three groups of a minimum of 20 animals each (investigational product treated group, negative control group and a vehicle treated group). The groups should be held in holding areas of not less than 500 m apart but not exceeding 3 km separation. The fly counting technique and regimen for each animal should be adequately detailed (Lima et al., 2002; Lysyk and Steelman, 2004).

Fly numbers at the facility can be assessed daily using sticky flypapers. Trapped flies should be identified to confirm the nuisance species present.

Flies are counted on three body sites namely the head, the body above the belly line and the legs below the belly line by taking a spot count of flies at the site at the beginning of a 30 sec counting period plus all fly landings on the body site within the counting period.

Prior to the day of treatment (Day 0), three pre-treatment counts should be conducted on the animals. The counts will be used to rank the study animals for treatment group allocations after which they will be blocked into the necessary groups.

Time periods (in days) after treatment when fly counts are to be conducted should be detailed and continued until fly counts for the treated and negative control groups become similar. Fly worry will be assessed through the same time periods, by observing the animals for 1 min and counting the following movements: head, tail, legs and skin (skin irritation assessment +1 to +5) for the group as a whole.

Meteorological data such as rainfall, ambient temperatures, humidity and wind speed at the time of the counts will be recorded for the duration of the study.

If it is envisaged that the investigational product is to be used for seasonal fly management and there is no immediate reduction in the fly counts then the study should be conducted for the entire season at a number of study sites at different geographic locations. Evidence of significant fly pressure for the period of the study must be given.

Refer to section 2.3.3.1.2 for guidance.

The purpose of the studies is to establish the period of persistent efficacy (expressed in 7-day intervals) afforded by the investigational product against re-challenge of treated animals by fly larvae or adult fly waves (depending on product label statements) using a controlled study design.

A persistent efficacy period established through dosage determination and dosage confirmation studies (see section 2.3) is confirmed in field studies. Field studies to establish a persistency period require study group allocation to be completed prior to treatment on Day 0. The control animals are to be separated and returned to their grazing paddock before the investigational product is applied to the treated group. Once treated, all the animals should be observed in order to determine the 'knockdown period' (reduction of flies to an insignificant level). Single side fly counts should be carried out in the paddocks on Days 3 and 7, and then at weekly intervals until the level of re-challenge has reached that of the pre-treatment levels.

Animals are challenged immediately following treatment, and then weekly until persistence is no longer evident. The period of persistent efficacy against fly re-challenge is considered to have ceased when the mean fly number in the treated group has reached 10 % of the mean figure derived from similar counts on the control animals.

Flies moving in from outside the study site can cause variations in fly numbers. These increases tend to be both aberrant and temporary. It is, therefore, important to continue assessing past the initial apparent re-infestation to ensure that there is a continuous progression of re-challenge towards the control baseline.

Refer to section 2.3.3.1.2 for guidance.

Refer to section 2.3.3.1.2 for guidance.

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Appendix C. Specific guidance offered in relation to efficacy studies involving myiasis causing flies on ruminants

C.1 Myiasis causing flies

C.1.1 Introduction

Myiasis flies are found in two groups namely warble and 'strike' (blow and screwworm) both of which have parasitic larvae that damage internal and external host tissues. Therapeutic efficacy can be aimed at the adult and/or their larvae during contact with the host or at their resting places. This appendix gives guidance on investigational products to treat ruminants targeting larval stages of myiasis flies.

C.1.1.1 Warble flies (Hypoderma bovis, Hypoderma lineatum, Przhevalskiana silenus, Dermatobia hominis). Warble flies belong to the Oestridae, obligatory parasites whose larvae migrate through the host tissue and live as subcutaneous parasites developing into boil-like swellings (warbles, nodules) under the skin of mammals. The adult flies of these species do not feed and live only for a short time. Four species are of major veterinary importance with *H. bovis* and *H. lineatum* parasitising cattle, *P. silenus* parasitising goats and *D. hominis* parasitising domestic animals with significant economic impact in cattle (Zumpt, 1965).

Differences existing in the life cycle of warble fly species warrant special consideration when designing studies for the investigational product development.

Warble treatment generally involves the application of non-systemic or systemic insecticides to the body of the host. Non-systemic insecticides are only effective when warbles have already developed under the host's skin and the swellings are perforated so that the insecticide can come in contact with the parasite. It is preferable to treat warbles while they are in their early stages of development.

Products that provide insecticidal activity over extended periods (e.g. long acting injectables or boli) should be evaluated in studies conducted throughout the period for which the efficacy is to be investigated (e.g. protection from infestation for several weeks or for an entire grazing/fly season).

C.1.1.2 Nasal bots (Oestrus ovis). Nasal bot flies (*O. ovis*) are included here since contemporary broad spectrum ectoparasiticides targeting myiasis causing flies have the potential to target nasal bot larvae. Nasal bot flies deposit live larvae in the nostrils of the host (sheep and goats). Larvae migrate to the head sinuses (the turbinates and septum) and after several months of development depending upon the season of the year, drop to the ground to complete development to the adult form.

C.1.1.3 Strike flies (e.g. Lucilia spp., Chrysomya spp., Sarcophaga spp., Cochliomyia hominivorax,. Wohlfartia magnifica, Calliphora spp.)

Blow flies belong to two calliphorid subfamilies, the Calliphorinae and Chrysomyinae, while the flesh flies that cause myiasis are found in the sarcophagid subfamily, Sarcophaginae. The calliphorids are metallic flies; green, blue or purplish in colour. The sarcophagid flies are non-metallic and dull grey, yellow, black or brown in colour. Most species in these groups develop in carrion or faeces, but some may deposit their eggs onto living animals. Developing larvae, which feed on the host tissues cause myiasis.

Blow fly strike is caused by flies belonging to the genera *Lucilia*, *Chrysomya* and *Calliphora*. It can be a devastating disease of sheep with hundreds of eggs laid depending on the-fly species. Under favourable climatic conditions these hatch within 12–18 h. *Lucilia cuprina* and *Lucilia sericata* are the most important sheep blow flies. *Chrysomya chloropyga* and *Chrysomya rufifacies* will also attack live sheep. The adaptation of carrion flies to breeding on live sheep is a major problem. Control of sheep blow flies depends largely on prophylactic measures using insecticides, management practices (crutching) and breeding (less wrinkled breech on wool sheep). Clinical blow fly strike is normally associated with underlying tissue damage.

Table C1
Details of myiasis causing flies covered in this appendix.

Parasite name	Geographic distribution	Host range (hosts)	Other common names	Development time (host)	Type of myiasis
Lucilia sericata	Temperate zones of northern hemisphere	Broad	Common green bottle	3–6 days	Facultative
Lucilia cuprina	Cosmopolitan (warmer parts)	Broad	Sheep green bottle, Sheep blow fly	3–6 days	Facultative
Chrysomya spp. (*C. bezziana*)	Tropical–subtropical	Bovine, other mammals including humans	Old world screwworm	5–10 days	Obligatory
Sarcophaga spp.	Cosmopolitan	Broad		Variable	Facultative
Cochliomyia hominivorax	Warmer regions of the Americas	Broad	New world screwworm	5–7 days	Obligatory
Wohlfartia magnifica	Cosmopolitan	Broad	Wound myiasis fly	3–5 days	Obligatory
Calliphora spp. (*C. vomitosa, C. vivina, C. augur, C. stygia*)	Cosmopolitan	Broad	Blue blow fly	Variable	Facultative

Screwworm myiasis is produced by larvae of *Cochliomyia* (*Callitroga*) species including *C. hominivorax* and *C. macellaria* and *Chrysomya* species including *C. bezziana*. They occur in tropical areas of the world where they lay eggs on fresh wounds with the larvae penetrating the host tissues creating an open, growing lesion. They parasitise livestock, warm blooded wildlife and occasionally man (Zumpt, 1965). Table C1 summarises relevant details of the myiasis causing flies covered in this appendix.

C.1.2 Study types

Studies utilising host animals focus initially on establishing a therapeutic efficacy. Persistent efficacy may also be demonstrated using pen facilities and field study results. Dosage determination and dosage confirmation studies are conducted using pen facilities with either naturally infested animals or laboratory-reared flies released on, or caged, depending on the fly species, to infest host animals. For field studies, natural fly infestations are usually used. Efficacy data must be generated for each targeted fly species with the investigational product.

C.1.2.1 Dosage determination studies. For certain myiasis causing flies with a wide host range, dosage determination studies conducted in one host species (e.g. cattle) could be extrapolated to other animal species (e.g. sheep or goats). This approach may produce substantial savings in the data-generating process. Under this condition, dosage confirmation and field studies would still be recommended for the investigational product.

Standardised serological tests (e.g. ELISA) may be used to identify animals when natural parasite infestations are considered for studies (e.g. *Hypoderma* spp.).

C.1.2.2 Dosage confirmation studies. Dosage confirmation studies ascertain whether the effective dose is capable of eliminating fly larvae from all ruminants that are treated under the proposed conditions for use. This could involve studies to show efficacy of the investigational product under a variety of conditions of use (breed, length of wool, type of equipment, quality of water used as diluent, etc.). Care needs to be taken when evaluating investigational products such as pour-on preparations where the parasiticide may be transferred by self-grooming or it contains relatively persistent active chemicals. Separation of treated and control groups is critical.

Pen studies demonstrate length of persistent efficacy (if any) against re-infestation/strike. These studies also identify peculiarities, side effects, etc. when the commercial formulation of the investigational product is used. Pen facilities are also used to demonstrate that heavy rain falls immediately before or after treatment does not alter the efficacy of the investigational product.

Animals are exposed to flies in the studies. Laboratory strains of fly should be from a field isolate less than 10 years old and ideally of known resistance status. Animals of the age likely to be most commonly treated with the investigational product should be used.

C.1.2.3 Field studies. The purpose of field efficacy studies is to evaluate the investigational product when used under practical conditions in the field and to obtain additional experience as to acceptance and safety. The studies should be carried out in a number of breeds or types of animals of different ages and sexes in several geographic locations to cover variations in the parasite populations, variable environmental conditions, as well as different feeding and management practices. Studies should be carried out in areas where the parasites are endemic. For studies undertaken outdoors, records should be kept of rainfall and temperature for 24 h pre- and post-treatment, and daily rainfall should be recorded for the duration of the study.

For field efficacy studies, a group treated with the investigational product at the effective dose and a second group (the remainder of the herd/flock) being the negative control group is advocated. Animals treated with the investigational product should be identified and assessed throughout the study to show a reduction in the signs associated with the infestation by the target fly species. At least 10 % of the animals treated with the investigational product should be examined for parasite presence.

The purpose of the studies is to establish the period of protection afforded by the investigational product against re-infestation of treated animals by fly larvae. The persistent efficacy of an investigational product will determine the length of time that animals are protected from fly larvae re-infestation (= protective period).

C.1.3 Animals

C.1.3.1 Selection. Where possible, animals should be from the same parasitological background (exposure or non-exposure to parasite infestation) and should be of similar weight, age and breed/type.

Naturally infested animals should be obtained from areas where a high prevalence of the parasite infestation has been recorded. For studies to demonstrate efficacy of an investigational product against first instars of *Hypoderma* spp. and *P. silenus*, animals, preferably less than 1 year old, should be selected on the basis of positive serology (e.g. ELISA) results to confirm previous exposure to infestation. For studies with *O. ovis*, some animals may be necropsied pre-study to confirm the presence of the parasites. In addition, induced infestations may be used.

C.1.3.2 Numbers. For dosage determination and dosage confirmation studies, all animals used must be seropositive (*Hypoderma*, *Przhevalskiana*) or adequately infested with larvae at every evaluation time point when warble flies are being assessed. In studies to assess the efficacy of the investigational product against warbles or *O. ovis*, a study will be considered valid if at least six animals in the control group are infested.

C.1.4 Efficacy

The controlled study is advocated to assess the larvicidal efficacy of investigational products. The study design will evaluate therapeutic and/or persistent efficacy. Efficacy of the investigational product is determined by the comparison of larvae numbers on groups of treated and negative control animals. Study animals are randomly allocated into treated and negative control groups. A positive control group should be included if needed. The formula of choice to calculate efficacy depends on the ability to obtain pre-treatment parasite counts and whether parasite numbers on the

negative control animals are likely to vary over the study duration.

For most studies Abbott's formula[3] (Abbott, 1925) is advocated.

The Henderson–Tilton formula[4] (Henderson and Tilton, 1955) is advocated if the parasite counts in the control animals vary significantly over the study duration.

Efficacy of at least 90 %, supported by appropriate statistical analysis is recommended to demonstrate acceptable efficacy of the investigational product. Parametric and/or non-parametric statistical methods, as appropriate, should be used to determine the significance of efficacy.

The emergence of myiasis causing fly population resistant to chemicals used to manage them, or the adverse effect of established resistance mechanisms on new insecticide classes, continues to pose a serious threat to the sustainability of chemical-based parasite management strategies. It is, therefore, essential to establish the resistance status of both laboratory and field fly strains used in pen facilities and field studies where possible. Any indication of cross-resistance to the investigational product in existing resistant myiasis causing fly strains requires that these populations be used in studies. The resistant isolate used should be characterised, indicating the name of the insecticides to which the parasite is showing reduced efficacy.

For a therapeutic efficacy, a reduction of larval counts should be at least 90 %, calculated using Abbott's formula to compare treated and negative control animal groups. The evaluation is conducted by observing the infested animals until the parasitic phase of the fly life cycle is completed. Animals in the negative control group should have an adequate infestation of the targeted warble fly species stated on the investigational product label. Larvae may be cultured to determine the fly species. For the assessment of persistent efficacy, infestation of animals should be assessed by counting the larvae periodically through to the end of the larval stages of the life cycle. On each occasion, some larvae should be collected to determine viability and confirm species. Depending on the parasite species, persistency of treatment may be evaluated following pen or field study design. At least 90 % efficacy is also required if targeting nasal bot flies.

C.1.5 Types of studies

C.1.5.1 Dosage confirmation. Animals of 1 year old are recommended for use. Control animals should receive a vehicle treatment. Natural or induced infestation should be used. Induced infestation can be used in *D. hominis* studies. Approximately 25, first instar larvae should be applied on the dorsal midline of the animals. Induced infestations are recommended for persistent efficacy studies and animals should be examined at weekly intervals. In the case of *Hypoderma* spp., the animals in the study should be evaluated until the completion of the larval development in the host. The efficacy of the investigational product is evaluated by counting the number of larvae on both treated and control animals.

As it remains impossible to experimentally rear *O. ovis* in laboratory facilities, first stage larval harvest is undertaken in a slaughterhouse according to a method described previously (Yilma and Dorchies, 1993). Briefly, after sectioning of the animals' head, larvae are recovered with a small brush, identified and rinsed in saline. Examination under a stereoscopic microscope is used to confirm larvae viability. Recovered larvae in saline are placed in the nasal passage of the animal. The infective dose should be approximately 50 larvae per animal. Larvae from goats may be used for infestation of sheep and goats, but sheep-generated larvae cannot be used in goats.

It is preferable to select lambs or kids born and raised indoors to avoid natural infestations. In countries where winter (or dry season) hypobiosis occurs, studies should commence at the beginning of the hypobiotic period so that high numbers of first stage larvae in naturally infested animals are available for induced infestations. Studies should be undertaken in facilities that are insect proofed. No insecticide should be used in the pen facilities for at least 2 weeks before the study commences.

A negative control group is used along with other groups treated with the investigational product 2–3 days after induced nasal bot infestation. Animals are euthanised 7 days post-treatment, the larvae are harvested, identified and counted. In control animals, 30 % of infective larvae should survive for the challenge to be valid.

In order to evaluate possible preventative efficacy of an investigational product, treatment should be administered to separate groups of study animals each on 6, 5, 4, 3, 2 and 1 week before larval infestation. A negative control group should be included. All animal groups are slaughtered 7 days post larval infestation. The efficacy of the investigational product is determined by comparing the number of larvae in the control animals with the number remaining in the treated animals.

C.1.5.2 Field therapeutic efficacy. Generally, efficacy of treatments is assessed by counting the live larvae that developed in the warbles under the host skin.

Hypoderma spp. and *P. silenus* infestations are assessed by examining, animals from January onwards in the northern hemisphere. Assessment of infestation is by inspection and palpation on the back and sides for the presence of warbles. The examination should be conducted at intervals not greater than 2 weeks. The hair on the backs and sides of the animals may be clipped to facilitate the counting of warbles. In addition, it may be helpful to chart the position of each warble and to record the presence or absence at each subsequent examination.

Larvae that emerge or are sufficiently mature enough to be identified to species should be expressed from the host. These larvae should be collected in a manner, which allows a determination of their viability (live larvae are collected from firm swellings with a well-developed breathing hole, show movement and respond to mechanical stimulation; dead/resorbing larvae are collected from soft swellings with a healing/healed breathing hole, larvae are flaccid and do not respond to mechanical stimulation). Emerging larvae may be collected with special devices fitted on the animals (Barrett, 1981) or by removal using hydrogen peroxide infused in the furuncle (Scholl and Barrett, 1986). All emerging larvae should be collected, identified to species, stage and development (James, 1947; Grunin, 1965; Zumpt, 1965) unless identification is prevented by decomposition prior to collection.

For *D. hominis* infestation, animals should be examined by inspection and palpation on the back and sides for the presence of warbles. Any larvae that emerge or are sufficiently mature to be identified should be expressed. The expressed larvae should be collected in a manner that allows a determination to be made as to their viability at the time of collection. In studies to assess the therapeutic efficacy, warbles may be counted daily and emerging larvae collected for up to 10 days post-treatment, when all larvae should be expressed to determine viability and larval stage. In studies to assess therapeutic and prophylactic efficacy, numbers of live larvae should be recorded at about 10 days post-treatment and re-infestation should be

[3] Abbott's formula: 100 x (1- T/C) where geometric parasite counts of the treated (T) and control (C) animals occur.

[4] Henderson–Tilton formula: 100 x [1 – (Ta x Cb)/(Ca x Tb)] where parasite counts on treated (T) and control (C) animals occur. Tb is the number of parasites counted per treatment group before treatment, Ta the number counted after treatment, Cb the number counted from the control group before treatment and Ca is the number counted from the control group after treatment.

assessed by counting the larvae periodically throughout the study.

Although it is probably simpler to use naturally infested animals for testing products against warble fly larval infestations, techniques to establish experimental (induced) infestations have been described for *Hypoderma* spp. (Weintraub, 1961; Weintraub et al., 1961; Boulard and Weintraub, 1973; Colwell, 2001) and *D. hominis* (Chaia et al., 1975; Moya-Borja et al., 1997). The history (e.g. source, previous exposure to endectocides/insecticides, number of laboratory passages, method of storage and maintenance) of the parasites used in the induced infestation studies should be determined.

Studies with *O. ovis* have to be undertaken in endemic areas. Before commencing studies two or three animals should be killed in order to measure the level of natural fly infestation and the ratio between the three larval stages. After selection, animals should be housed indoors in insect proofed facilities or they should be transferred to areas where the infestation is absent. Each group should include 10 animals. Animals are slaughtered a minimum of 7 days post-treatment with larvae harvested, identified and counted.

C.1.5.3 Field persistent efficacy. Persistent activity against warble flies can be assessed by treatment with the investigational product at selected intervals prior to the establishment of experimental infestations (Colwell and Jacobsen, 2002). Groups of animals are treated with the investigational product at weekly intervals prior to infestation. In addition, one group should be treated coincident with infestation and one group should remain untreated as the control. Infestations should be carried out by blocks, e.g. larvae from a single batch should be used to infest one animal from each treated group. This is to account for the variations in larval viability. Evaluations of efficacy should be carried out by palpation for detection of larvae, enumeration of larvae, and collection of mature third instars.

Studies with nasal bot flies are undertaken during the period of adult fly activity. As it is difficult to ensure universal natural infestation of all study animals, it is compulsory to conduct these studies with the same number of control and treated animals. Half the animals in the control group are slaughtered on the day of treatment to evaluate the level of natural infestation. Treated animals are kept outdoors exposed to natural infestation. The treated group is slaughtered after the longest period of preventative effectiveness determined by the experimental infestation studies conducted in the dosage confirmation section. The same day, animals of the control group are slaughtered.

C.2 Strike flies

C.2.1 Animals

C.2.1.1 Selection. See section 1.3.1.

C.2.1.2 Numbers. All animals to be used must be adequately infested with larvae at every evaluation time point when strike-causing flies are being assessed. When conducting dosage confirmation studies on sheep exposed to strike-causing flies (natural infestation); flocks of at least 100 animals may be used in each study group.

C.2.2 Efficacy

The controlled study is advocated to assess the larvicidal efficacy of investigational products. The study design will evaluate therapeutic and/or persistent efficacy. Efficacy of the investigational product is determined by the comparison of larval fly numbers on groups of treated and negative control animals. Study animals are randomly allocated into treated and negative control groups, plus in some cases a positive control group. The formula of choice to calculate efficacy depends on the ability to obtain pre-treatment parasite counts and whether parasite numbers on the negative control animals are likely to vary over the study duration.

For most studies Abbott's formula[5] (Abbott, 1925) is advocated.

The Henderson–Tilton formula[6] (Henderson and Tilton, 1955) is advocated if the parasite counts in the control animals vary significantly over the study duration.

Efficacy of at least 90 % supported by appropriate statistical analysis is recommended to demonstrate acceptable efficacy of the investigational product. Parametric and/or non-parametric statistical methods, as appropriate, should be used to determine the significance of the efficacy.

For screwworm flies, efficacy against myiases is calculated by the formula:

$$Percentage\ efficacy = 100 \times \left(1 - \frac{Tb}{Ta}\right)$$

Where,

T_a equals mean number of myiases in the treated group on Day 0.

T_b equals mean number of myiases in the treated group on any particular day post-treatment.

The emergence of strike fly populations resistant to chemicals used to manage them, or the adverse effect of established resistance mechanisms on new insecticide classes, continues to pose a serious threat to the sustainability of chemical parasite management strategies. It is therefore essential to establish the resistance status of both laboratory and field strains of the strike flies used in pen and field studies. The method of resistance identification and quantification should be documented. Any indication of cross-resistance to the investigational product in existing resistant strike fly strains, requires that these strains be used in studies. The resistant isolate used should be characterised indicating the name of the insecticides to which the parasite is showing reduced efficacy.

Therapeutic efficacy may be calculated based on the comparison of the percentage of investigational product treated animals cleared of fly strike (cure rate). Persistent (prophylactic) efficacy can be evaluated in myiasis strike-free animals with efficacy of the investigational product calculated as

[5] Abbott's formula: 100 x (1- T/C), where geometric parasite counts of the treated (T) and control (C) animals occurs.

[6] Henderson–Tilton formula: 100 x [1 – (T_ax C_b) /(C_a x T_b)], where parasite counts on treated (T) and control (C) animals occurs. T_b is the number of parasites counted per treatment group before treatment, T_a the number counted after treatment, C_b the number counted from the control group before treatment, and C_a is the number counted from the control group after treatment.

the percentage of investigational product treated animals, which remained myiasis strike free.

For both scenarios, groups of negative control animals should be evaluated for percentage of myiasis strikes to validate the study design.

For therapeutic efficacy against fly strike/myiasis, data generated must demonstrate that the investigational product will kill all targeted larval stages of fly species present in an established strike.

For persistent efficacy (protection) from fly strike/myiasis, data generated must demonstrate that the investigational product will prevent the establishment of new strikes or re-strikes of all targeted species of flies in treated animals. The protection period (days) for an investigational product is usually calculated based on at least 90 % persistent efficacy.

C.2.3 Types of studies

C.2.3.1 Dosage confirmation. The size of the treated group will be determined by statistical consideration. A negative control group should be included. A pool of sheep should be retained so that any sheep that are lost to the study for any reason can be replaced.

Treated sheep of a specified wool length and quality are then challenged with known strains of the fly species.

Techniques and procedures for conducting larval implant studies are well documented (Hughes and Shanahan, 1978). In the case of insect growth regulators, implants should be checked 48–72 h after study commencement. Larval implants onto control sheep or sheep treated with fast-acting chemicals should be checked after 24 h.

When assessing fly strike dressings as treatments against existing strikes, the investigational product should preferably demonstrate efficacy in killing active, advanced third instar field derived larvae on sheep, as this is the stage most likely to be detected by farmers.

When a study is conducted on a fly strike preventative whose efficacy is known or suspected of being affected by resistance, it is recommended that the resistance level be determined by laboratory bioassay.

For studies to demonstrate efficacy of an investigational product in/on cattle against *C. hominivorax* or other screwworms, cattle need to be allocated to treated or negative control (vehicle or saline treatment) groups. Experimental protocols have been described for infestation of hosts with screwworm larvae (Moya-Borja et al., 1993a,b). For example, on the day of treatment (Day 0), 12 animals should be allocated in pairs to a treated or control group. Two hours after treatment, two patches of skin approximately 8 cm in diameter are shaved on the right side of each animal. One patch is located on the shoulder and the other on the rump. Under local anaesthetic, a longitudinal or circular incision measuring approximately 4 cm in length is produced in the middle of each patch. Each incision is infested with 50 recently hatched first instar *C. hominivorax* larvae. From Days 0 to 8 post-treatment, animals are examined twice daily for the presence of myiasis caused by the fly larvae. The healing process of the incisions is evaluated and scored using the following criteria:

- 1—Incision is healed;
- 2—Healing process is in progress;
- 3—Incision is infested.

Infested wounds are defined by bleeding and the presence of serohaemorrhagic or purulent exudates and later by the presence of live fly larvae. Third instars that complete larval development and exit the wounds are collected to verify their viability.

Cochliomyia hominivorax larvae used for the induced challenge should be recent field isolates obtained from grazing paddocks with a previous history of naturally occurring myiases. Isolates can be obtained using modified screwworm wind-orientated traps baited with fresh liver. First instars used for challenge are hatched from within the laboratory and should be no more than 4–6 h old at the time of infestation. Those instars that complete development in the wounds and exit are collected and incubated in physiologically preferred conditions with these conditions monitored through the study.

Efficacy is calculated by comparing the number of infested wounds in the treated cattle at Day 0 (day of treatment) with the number of infested wounds at Days 1 through to 8 post-treatment. The number of myiases and number of larvae recovered from control calves are reported only to verify the progression of the infestation in the absence of treatment.

C.2.3.2 Field therapeutic efficacy. Study group size is critical to ensuring that statistically significant results are obtained. In field studies focusing on fly strike for group sizes of 100 animals, there would need to be 0 % strike in a treated group, if only 6 % control strike occurred, for the result to be significant. Similarly, to be 95 % sure of proving that 5 % strike incidence in a treated group was significantly different to 25 % strike in a negative control group, the treated groups would have to contain at least 90 animals each. Information on critical group sizes for significant differences between treatments is available in Fleiss (1981). In studies with negative controls, 15 % of control animals (excluding re-strikes) should sustain strikes throughout the observation period of the study. Depending on group size, the number of strikes in the treated group may have to be zero for the result to be significant.

Study data must demonstrate that the investigational product when used, as directed, will be effective in a range of geographic environments as well as in animals of variable age, breed, sex, wool diameter, wool length, etc.

Ideally, but not necessarily, field studies should be conducted under a range of climatic conditions. The goal is to conduct studies when flies are active and when weather conditions are conducive to fly strike. Studies should enable useful information to be obtained on the ability of the investigational product to prevent fly strike for a specified period; the persistent efficacy period; the ability to treat existing strikes and the ability to prevent re-strike.

It is recommended that weaners or unclassed hoggets be used where possible to increase the likelihood of strike. Records of whether sheep have been shorn previously should be included.

Where applicable all treated groups should be segregated after treatment until dry, and thereafter managed as a single herd/mob. A similarly sized negative control group (and/or a known standard treatment group) may be included but this is at the investigator's discretion.

Fly strike studies of sheep off shears (with 24 h of shearing) or in short wool (1–42 days after shearing) should compare the investigational product with a positive control product with similar claims for fly strike protection for off shears or short wool respectively. A negative control group should be included to demonstrate adequate fly pressure.

Details of daily rainfall, maximum temperature and wind velocity should be maintained from the time the sheep are treated until the completion of

the study. As well, it is recommended that flytrap catches over a 24 h period each week should be recorded if no negative control flock is included. Weekly maximum/ minimum temperature and records of days with wind speed exceeding 30 km/h would be adequate. These points are strongly recommended to avoid controversy regarding the adequacy of fly pressure during particular studies.

Animals should be inspected in the paddock daily during fly wave conditions and at least every 3 days under less severe conditions. Fly struck animals should be treated accordingly and removed from the study.

Records (pictorial, where possible) should be kept on the date at which any strikes are detected, including the position, size, severity and stage of maggot present. When a study is conducted on a fly strike investigational product whose efficacy is known, or suspected, of being affected by an existing fly resistance, it is recommended that the resistance level of the fly population be determined by laboratory bioassay. This is to ensure that the investigational product is adequately challenged by flies, whose resistance level is typical for most areas.

C.2.3.3 Field persistent efficacy. An investigational product pursuing a period of protection from re-strike should provide supportive data from studies with the target animal host. Sheep which have had strikes dressed in accordance with the investigational product label directions should be exposed to blow flies in the paddock and/or in a fly exposure house at intervals of not less than 1 week (e.g. 7, 14 and 21 days, etc.) after treatment and inspected daily for egg masses and re-strikes. A standard treatment should be included for comparison. Documentation on treatment (both investigational product and standard treatment) should include whether struck wool, etc. was removed prior to treatment. The standard treatment used in the study should be applied according to the specific investigational product label directions. Group size should take account of the need for statistically significant results. A negative control group is recommended. Records of egg masses laid onto dressed strikes and incidence of re-strike will provide information on blow fly repellency and protection from re-strike.

For *C. hominivorax* and similar screwworms, an investigational product pursuing protection should provide at least supportive data from pen studies. The number of treated and control groups will be dictated to by the anticipated persistent efficacy period. For example, if the anticipated persistent efficacy period is envisaged to be 14 days, then six groups would be the minimum requirement for a study. On Day 0 in such a study, three groups would be treated with the investigational product and the other three groups (controls) would be treated with a carrier vehicle or saline. At Days 3, 7 and 14 post-treatment, four incisions (two on each side) are made on each animal from each group. Each incision is infested with 30 recently hatched first instar larvae, for a total of 120 larvae per animal. Individual incisions are then evaluated from Days 0 to 8 post-infestation following the procedures detailed in section 2.3.1.

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Appendix D. Guidance for efficacy studies involving biting lice, sucking lice and sheep keds

D.1 Lice of ruminants

Lice (Phthiraptera) of the suborders Anoplura (sucking lice) and Ischnocera (biting or chewing lice) are commonly occurring ectoparasites of ruminants with their major economic effects being a function of lice density. Biting lice of concern are *Bovicola bovis* (cattle), *B. ovis* (sheep) and *B. caprae*, *B. limbatus* and *B. crassipes* (goats). Sucking lice of concern on cattle are *Haematopinus eurysternus*, *H. quadripertusus*, *H. tuberculatus*, *Linognathus vituli* and *Solenopotes capillatus*; on sheep are *L. pedalis*, *L. africanus* and *L. ovillus*; *L. stenopsis* infests goats.

The two groups of lice have different feeding strategies: all Anoplura are haematophagous ectoparasites, whereas the Mallophaga species of domestic ruminants ingest hair, skin and skin products. Lice are hemimetabolous insects, i.e. their life cycle includes an incomplete metamorphosis. The entire life cycle of the lice takes place on the host and comprises the egg, three nymph stages and the adult. Under optimal conditions the complete life cycle may be as short as 2 weeks, however, on average it may take about 3–5 weeks.

Melophagus ovinus, commonly known as the sheep ked, is a wingless, bloodsucking ectoparasitic fly in the family Hippoboscidae that spends its entire life on sheep. As larviparous flies, single eggs are ovulated and passed to the uterus of the female sheep ked and after 7–8 days of feeding and growing, the fully developed larva is deposited and forms a puparium within 12 h of parturition. Duration of the pupa stage is usually longer in winter (up to 5 weeks) than in summer (about 3 weeks). After adult emergence and mating, it takes the female fly about 2 weeks to produce its first offspring. The sheep ked can transmit *Trypanosoma melophagium*.

In the case of sheep keds, for the purpose of this appendix, efficacy evaluation study techniques, etc. may generally follow the techniques laid down for sucking lice on sheep.

Cattle lice cause skin irritation while sucking or feeding on epithelial debris and exudates. Cattle rub and scratch to relieve the irritation, resulting in large areas of skin becoming denuded of hair as well as skin abrasions. Sucking louse infested cattle may become anaemic and unthrifty. Both dairy and beef herds can be infested. Poorly nourished stock, young animals and calves suffer the most. The intensity of louse infestations appears to be influenced by the ruminant's skin and coat condition, skin surface temperature and intensity of light.

Sheep lice cause intense irritation whilst feeding which leads to restlessness, constant scratching and rubbing, interrupted feeding, stress and production loss. Heavy infestations of sucking lice can also cause anaemia with *L. pedalis* causing lameness.

Goat lice cause similar effects to sheep lice. Biting lice in particular, cause serious damage in those goats with specialised fibres (Angora and Cashmere). The effects of sucking lice may be severe and scabby bleeding areas can be seen on affected goats.

Sheep keds cause irritation, making sheep restless so they do not feed well. Pruritus leads to wool-biting, rubbing and wool loss; staining of the wool by the keds' faeces reduces its value.

Infestation by lice and sheep keds can cause damage in cattle-hides and in sheep-skins resulting in defects to the leather called light spot and fleck in cattle or cockle in sheep (caused by infestation with biting lice or keds) (Heath et al., 1995a,b).

D.2 Evaluating efficacy against lice

D.2.1 Application techniques for investigational product

D.2.1.1 Cattle. See section 2.2.4 in first part of the main guideline.

D.2.1.2 Sheep. Sheep can be treated off-shears, with short or long wool and product labels must indicate to which wool length the formulation is to be applied/administered. Off-shears relates to sheep treated within 24 h of shearing. Short wool relates to sheep treated >24 h and up to 6 weeks after shearing. Long wool relates to sheep treated >6 weeks after shearing.

For backline, plunge and shower applications see cattle advice.

D.2.1.3 Goats. In the case of goats, little information exists on the application of ectoparasiticides. However, application of insecticides to goats generally may follow the techniques laid down for cattle except for fibre producing goats where adherence to sheep application techniques should be followed.

D.2.2 Efficacy

Efficacy of the investigational product treatment is determined by the comparison of louse numbers on groups of treated and untreated animals. Parasitised animals are randomly allocated into treated and negative/vehicle/placebo treated control groups. The formula of choice to calculate efficacy depends on the ability to obtain pre-treatment parasite counts and whether parasite numbers on the negative control animals are likely to vary over the study duration.

For most studies Abbott's formula[7] (Abbott, 1925) is advocated.

The Henderson–Tilton formula[8] (Henderson and Tilton, 1955) is advocated if the parasite counts in the control animals vary significantly over the study duration.

In contrast to endoparasites, where an accurate measure of present populations can be made at necropsy, the assessment of louse burdens by examination of selected areas of skin surface is less precise. Therefore, a measurement of response to treatment requires the repeated examination of parasitised areas or previously parasitised areas of the skin surface. Examinations at about weekly or two weekly intervals for 56 days after treatment will assure an accurate estimation of louse burdens in therapeutic efficacy studies. This observation period covers at least two complete life cycles of the lice and allows monitoring the population dynamics of the lice, which multiply on the host.

For each louse species to be claimed the criteria of efficacy is to achieve statistically significant ($p \leq 0.05$) differences in louse counts between treated and negative control groups and at least 90 % efficacy over the 56-day observation period. Appropriate statistical methods should be used to determine the significance of efficacy. These should be fully defined prior to conducting a study so that the study design will be satisfactory for the analysis of the data and obtaining meaningful (significant) results.

In field studies where semi-quantitative parasite counts of investigational product performance may occur, different calculations to assess efficacy may be used. Such calculations must be documented and justified.

In addition to efficacy parameters, other clinical observations (e.g. lesion scoring, pruritus, wool derangement) can be considered.

[7] Abbott's formula: 100 x (1-T/C) where geometric parasite counts of the treated (T) and control (C) animals occur.

[8] Henderson–Tilton formula: 100 x [1 – (Ta x Cb)/ (Ca x Tb)] where parasite counts on treated (T) and control (C) animals occur. Tb is the number of parasites counted per treatment group before treatment, Ta the number counted after treatment, Cb the number counted from the control group before treatment and Ca is the number counted from the control group after treatment.

Especially with the biting louse of sheep in some regions of the world, the emergence of louse populations resistant to chemicals used to manage them, or the adverse effect of established resistance mechanisms on new insecticide classes, continues to pose a serious threat to the sustainability of chemical-based parasite management strategies. In these regions, it is therefore essential to establish the resistance status of both field and laboratory louse strains used in dosage determination, dosage confirmation and field studies. Any indication of cross-resistance to the investigational product in existing resistant louse strains, requires that these strains be used in studies.

D.2.3 Study types

Studies should initially focus on the establishment of a therapeutic efficacy. In addition, persistent efficacy may also be demonstrated. Dosage determination and confirmation studies are conducted under controlled conditions (pen facilities) with induced (experimental) or natural louse infestations of host animals being permitted. For field studies, natural louse infestations are advocated. Efficacy data should be generated for each targeted louse species for the investigational product. Studies undertaken to generate data to support a persistency claim should be supported with field studies where applicable.

In the case of goats, see section 2.1.3 for guidance.

D.2.3.1 Dosage determination studies. For a dosage determination study, four groups of adequately infested animals are used: negative control, 0.5, 1 and 2 times the anticipated effective dosage. For a study to be considered valid, live lice must be demonstrated on at least five control animals at each post-treatment observation. The effectiveness for each dosage is determined by comparison of the parasite burden in the treated and control animals.

D.2.3.2 Dosage confirmation studies. Natural or induced infestations may be used in these studies. Induced infestation can be obtained using seeder animals or with manual transfer of lice from infested animals (see section 2.4 for guidance). Animals of the age most likely to be treated with the investigational product should be used.

For a study to be considered valid, live lice must be demonstrated on at least five control animals each post-treatment observation.

D.2.3.3 Field studies. The purpose of field efficacy studies is to evaluate the investigational product when used as directed in the protocol under practical conditions in the field and to obtain additional experience as to acceptance and safety. The studies should be carried out in a number of breeds or types of animals of different ages and sexes in several geographic locations to cover variations in the parasite populations, variable environmental conditions and different feeding and management practices. Studies should be carried out in areas where the parasites are endemic and only with naturally acquired louse infestations. In case of outdoor studies, records should be kept of rainfall and temperature on the farm for 24 h pre- and post-treatment, and when topical formulations are being evaluated, daily rainfall on the farm should be recorded for the duration of the study.

The number of control animals should be at least 25 % of the number of investigational product treated animals. Separation of the treated and control groups here needs special consideration. Where authorities permit, the use of a negative control group is strongly recommended as lice populations can vary dramatically over time and especially as seasonal conditions vary.

At each study site, at least 20 monitor animals that have been treated with the investigational product should be individually identified and assessed throughout the study to show improvement of the signs associated with the louse infestation.

D.2.4 Persistent efficacy studies

Studies designed to demonstrate persistent efficacy need to include challenge of animals at regular intervals throughout the defined period after treatment. The first challenge should not be initiated less than 7 days post-treatment; subsequent challenges should be made at not less than weekly intervals. Animals can be challenged by manual transfer of lice directly to individual animals or by exposing groups of treated animals to infested negative control animals for defined periods. Minimum group size should be six animals.

In studies with manual louse transfer, groups of louse-negative animals have to be treated at intervals prior to a day of challenge (e.g. 21, 14 and 7 days prior to challenge). On that day (= single point challenge), all animals including a group of negative control animals will be infested with a predetermined number of lice (see section 2.4). In case of testing persistency efficacy of pour-ons and spot-ons, lice should not be placed directly at the site of investigational product application. Thereafter, the animals should be observed for at least 2 weeks and then examined for louse numbers at around weekly intervals for at least an additional 3 weeks.

In the case of sucking lice on cattle, persistent efficacy of a systemic injectable investigational product may be assessed by evaluating the survival of lice placed in containers glued on the back of the animals. For this technique, one container is glued on each side of an animal in an area where the hair was previously clipped. Twenty motile female lice are placed in each container.

The number of surviving lice is counted by visual inspection for the three ensuing days.

In studies where louse-negative (recipient) animals are exposed to infested animals they should have been treated at 7 days intervals prior to louse exposure so that exposure periods can be determined at 21–28, 14–21 and 7–14 days, respectively. In addition, a negative control group should be included. Seeder animals (rate of exposure: one seeder per recipient) have to be assigned to the groups at random after allocation based on louse counts conducted prior to start of exposure. After a 7-day exposure, seeders have to be removed. The animals should be observed for at least 2 weeks and then examined for clinical signs and louse numbers at weekly intervals for at least an additional 3 weeks. The study design attempts to mimic the natural transfer of lice, however, it is not exact as the pressure of challenge may vary as to size of the pen and the louse burden of the seeder animals.

The actual persistent efficacy (= length of prevention of establishment of lice infestation) is recorded as the last time point relative to treatment that failed to establish infestation. This means for studies with exposure to infested animals, that animals exposed through 21–28 days after treatment and found louse positive and animals exposed through 14–21 days after treatment found negative demonstrate a persistent efficacy of the investigational product of 21 days. Valid results will depend on live lice being found on the negative control sheep.

Infestations may be induced by exposing louse negative (recipients) to louse-infested animals (seeders) in the same pen or by hand transfer of lice from donor animals.

There are no strict recommended numbers of lice to be applied to induce infestations. In general, the higher the number of lice transferred the faster suitable infested animals should be available. It is important that the animals should be infested with the same number of lice per infestation. The size of the inoculum should be recorded. Lice population growth cannot be precisely predicted and is affected amongst other things by host variations and climate factors. Therefore, repeated examination (louse counts) is necessary to determine whether the recipients are adequately infested and suitable

for inclusion in the study.

One technique in cattle involves the manual transfer of lice from a donor animal directly onto the skin at the withers of a recipient animal. To prevent loss of lice from recipient animals, the animal's hair (length dependent) around the withers can be fastened using a rubber band after louse exposure. Alternatively, the recipient animals can be appropriately restrained. Lice collected from infested animals should preferably be transferred to non-infested animals within a few hours; this is especially important in the case of sucking lice. For the infestation of cattle, a minimum of 50 live lice is recommended.

For the infestation of sheep, approximately 50 live biting lice are placed at skin level on the shoulders and/or flanks. Lice for infestation may be collected in the following way: a sheep infested with lice is partially shorn and the wool containing live lice is placed tip uppermost on a mesh screen over a collecting tray and held overnight at about 30–37^{0}C and 70–80 % RH. A strong light is placed above the wool and live lice are collected the next morning. Lice could also be collected by a simple vacuum technique.

Lice used in the induced infestations should be from a recent field isolate less than 10 years old. Repeated infestations applied at 1-week intervals should increase the success of the infestation.

D.2.5 Lice counts

The animals should be examined visually for lice. Only live (motile) lice have to be considered and total individual louse counts (not distinguishing stages) are required. Live lice should be counted on the body surface by direct examination with the naked eye; in some cases, the use of a magnifying lens may be helpful. Counting is carried out after parting the hair coat/wool (coat opening). The number of sites, the number of partings per site as well as the location of the counting sites must be kept constant throughout a study. Any changes regarding these parameters during a study have to be justified and must apply to all study animals for consistency if appropriate. Total louse counts per animal are established by summation of the louse numbers at each site. Louse counts should be conducted on Day 1 or Day 0 prior to treatment, on Day 7 and at 7-day intervals thereafter until study termination on approximately Day 56. The sites of examination should be recorded on a silhouette. In the absence of lice from the selected sites, a thorough body search has to be carried out. Preferably, the same personnel should evaluate the animals throughout the study duration.

The larger the number of sites per animal examined, the more accurately louse burdens may be estimated.

For therapeutic efficacy studies, a minimum of six sites should be examined on each animal on each occasion in cattle. This also applies to sheep in the case of sucking lice, however in case of biting lice and ked infestations, 40 sites in total per animal should be examined.

For persistent efficacy studies, where manual louse transfer or exposure to infested animals is used, thorough body searches have to be carried out to count established lice.

However, adequately infested animals that are included in dosage determination or dosage confirmation studies should be demonstrated to be infested with a total of at least of 30 lice (counted from the selected sites) per animal. As louse numbers on negative control animals may decline over an 8-week observation period, animals where lice were counted on at least half of the sites examined at an occasion (e.g. lice are counted in three of six sites) can be considered to support the validity of a study.

D.3 Species specific considerations for lice studies

D.3.1 Specific considerations for cattle

Various louse genera have predilection sites on their hosts. In heavier infestations they spread from these sites over the entire body. *Bovicola bovis*, the biting louse, favours the top of the head, especially the curly hair of the poll and forehead, the neck, shoulders, back and rump. *Linognathus* and *Solenopotes* prefer the head, neck and dewlap. Each species of *Haematopinus* has its own preference; *H. eurysternus* occurs on the poll and at the base of the horns, in the ears, around the eyes and nostrils and even in mild infestations is found in the tail switch, while *H. quadripertusus* is limited to the tail region. Some species, notably *H. eurysternus* and *L. vituli*, are gregarious in habit, forming dense, isolated clusters. Except for *H. quadripertusus* all the species named are found throughout the world.

Lice are counted on at least six sites per animal. These sites are determined during thorough examination of the animal prior to treatment (Day 1 or Day 0 louse count). At each site (about 5–15 cm), the hair should be parted at least three times to expose the skin and the total louse counts are recorded at each site.

D.3.2 Specific considerations for sheep

In all studies with *B. ovis*, the level of infestation (total louse count) is determined at 40 sites in total per animal. These sites are examined by opening the fleece about 10 cm wide and counting all live lice detected. The total count from the 40 sites will constitute the body count for each animal. An alternative method is the inspection of 80 partings each about 5 cm in length, per sheep.

The sites examined should be spaced so that they are representative of the full area of the body covered by the fleece on each side of the sheep. On this basis, such sheep are thoroughly searched for 10–15 min, with particular attention being paid to predilection sites (neck, shoulder, withers, flank and rump) and areas with clumps of longer fleece.

Lice should be counted as late as possible before treatment. If the period between examination and treatment is more than 24 h then the interval should be stated.

For off-shears or short wool products, in addition to dosage determination and dosage confirmation studies, a minimum of three field studies should be conducted. Study designs should accommodate different wool thickness ('fibre diameter') of the breeds of sheep found in the region. As such the number of studies may need to be increased depending on regional sheep breed numbers. Each flock to be treated should have at least 200 sheep and studies will run for at least 20 weeks.

For long wool products, a minimum of six field studies is recommended, two each at fleece lengths equivalent to about 3, 6 and 9-months growth of wool. Three of the studies (one at each wool length) are to be conducted in fine wool sheep (<20 μm). Each flock to be treated should have at least 200 sheep. Where the investigational product aims to reduce lice to non-detectable levels, studies will run for at least 20 weeks or until the next shearing, whichever occurs first. Where the investigational product aims to reduce louse numbers by at least 90 % for at least 30, 60 or 90 days, studies will run for a minimum of 40, 70 or 100 days, respectively, or until the next shearing, whichever is the sooner.

In each field study, at least 25 tagged sheep from the flock should be examined before treatment and lice counted using the prescribed technique. All tagged sheep should have at least a moderate infestation of lice (30 lice per animal) at the pre-treatment examination. After treatment, the tagged

sheep are examined on at least two occasions. Where a product label statement for a protective period is being sought, one of these examinations should coincide with the protective period determined in earlier pen studies. The timing of the second inspection is determined by the label statement made for the investigational product.

At the final examination, if no live lice are found, another 25 treated sheep are selected and examined. Any sheep with signs of fleece derangement should be included among the extras. If there is no fleece derangement evident, the additional sheep should be randomly selected from the treated flock.

Negative control groups may be avoided in studies conducted on farms belonging to co-operators who are commercial woolgrowers.

After treatment with an investigational product that aims to eliminate lice, final inspections should be made in multiple dosage confirmation studies 12 months after treatment. In field studies, final inspections must be made at least 20 weeks after treatment.

D.4 Guidance for insect growth regulators

For an investigational product based on insect growth regulators or any product based on compounds that work over a protracted period, the investigational product label must nominate the period of time required to kill all lice on treated ruminants. The time nominated must be based on valid scientific data. Recommendations must be made on the time of application of the investigational product in relation to lambing/calving and time that must elapse before contact can be permitted with untreated, louse-free ruminants.

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Appendix E. Guidance for efficacy studies involving mange and itch mites

E.1 Mites

E.1.1 Mange mites

The parasitic mites of the families Sarcoptidae and Psoroptidae, known as ‘mange mites’, generally give rise to well-defined dermatoses. Mange mites live on the surface of the skin (*Chorioptes* and *Psoroptes*) or may be superficial burrowers (*Sarcoptes*). The entire life cycle takes place on the host and comprises the egg, the larval stage, two nymph stages (protonymph and tritonymph) and the adult mite. Under optimal conditions the whole life cycle may be as short as 11 days (*Psoroptes ovis*); however, on average it may take about 2–3 weeks.

Sarcoptes scabiei occurs commonly in cattle and small ruminants and causes sarcoptic mange. There is evidence that *S. scabiei* is the only species of the genus *Sarcoptes* (Zahler et al., 1998). Therefore, it is appropriate to declare in studies with *Sarcoptes* mites exactly the host species or use terms such as *S. scabiei* var. *bovis*, *S. scabiei* var. *ovis* and *S. scabiei* var. *caprae*. These mites burrow into the skin, preferring regions without wool or the less well-haired parts of the body such as head, axillae, groin and tail. Clinical signs are similar in all species and consist of a papillar eruption, the skin becomes thickened, and there is marked loss of hair and severe pruritus.

Psoroptic mange is a serious disease in cattle and sheep but less so in goats. The causative mites are species of *Psoroptes*. The species infesting ruminants are in cattle *P. ovis* and *Psoroptes natalensis* (less common), in sheep *P. ovis* and *Psoroptes cuniculi*, and in goats *P. cuniculi* (Bates, 1999). A possible conspecificity of *P. ovis* and *P. cuniculi* has been proposed by Zahler et al. (1998). Bates (1999) suggested that the *Psoroptes* infesting sheep (i.e. *P. ovis* and *P. cuniculi*) are not reproductively or ecologically isolated but are phenotypic variants of the same species. The presence of mites on the skin causes a local reaction with vesicle formation. The exudate from the vesicles coagulates and dries on the skin surface, resulting in the formation of a crust or scab of varying thickness. Lesions usually begin in areas thickly covered by hair or wool. There is marked pruritus, and the host’s scratching results in alopecia, erosions and crust development. Variation in susceptibility to infestation with, and variation in the predisposition to hypersensitivity to *Psoroptes* spp. has been documented in sheep and cattle. In goats, *P. cuniculi* is known as the ‘ear canker’ mite because of its predilection for the ear (causing otoacariasis).

Chorioptic mange occurs commonly in cattle, sheep and more rarely in goats. In cattle, two species have been described: *Chorioptes bovis* and *Chorioptes texanus*; *C. bovis* is the species present on sheep and goats (Heath et al., 1989). Generally, the condition is less severe than psoroptic or sarcoptic mange. These mites have a predilection for the perineum, udder, caudal areas of thigh, rump and feet. Lesions consist of alopecia, erythema, excoriations and (small) crusts associated with pruritus.

E.1.2 Itch mites

The genus *Psorobia*, formerly *Psorergates* (Baker et al., 1996), includes two species parasitising domestic ruminants: *Psorobia ovis* (sheep) and *Psorobia bos* (cattle). The life cycle of this parasite is detailed in Vercruysse et al. (2006).

Itch mites live in the superficial skin layers and can cause chronic irritation and skin thickening; however, the effects of this infestation may often only be very slight so that many cases are likely to remain undetected. In sheep, the earliest signs are small, pale areas of wool on the shoulders, body, thighs and flanks, which gradually extend over the rest of the fleece. These mites can cause severe irritation, with rubbing and biting at the fleece by the sheep. On close inspection there is wool derangement (Sinclair, 1976), which is either stringy or matted. Scurf accumulates on the skin and along the wool fibres. Fleece may be discoloured and yellow-orange in colour. This is often more intense at skin level than higher up on the fibres. In cattle, hair loss, slight skin thickening and some scaling were reported (Andrews et al., 1997). Although no studies have been reported on determining efficacy of acaricides against itch mite (*Ps. bos*) on cattle, methods to assess the efficacy *in vivo* of products in general have been collated (Fourie et al., 2019). Studies to test the efficacy of investigational products against *Ps. bos* may make use of study designs recommended/used for the assessment of efficacy of investigational products against *P. ovis*.

E.2 Evaluating the efficacy against mite

E.2.1 Cattle

With plunge or shower dip applications, after charging the dip at the recommended rate, the concentration of active ingredient in the dip fluid should be measured by appropriate analytical techniques before, during and at the end of dipping. The concentration of active ingredient in the dip fluid that is used to determine any efficacy period should be the minimum concentration measured under these conditions. The method of application should be clearly detailed. For plunge dips, the length, depth, number of times the animals are dunked after entering the dip, range of times in the dip and average time in the dip should be recorded. Management practices should ensure that the entire animal is adequately soaked with the investigational product. The duration of exposure time for cattle and the combination of upper and lower nozzles used should be stated. For plunge and shower dips, the method of mixing the chemical in the sump, the method of addition of fresh chemical and amounts added should be recorded.

E.2.2 Sheep

For sheep shower dips, the brand and model of dip used should be stated together with the operating pressure and output of the pump.

Sheep can be treated off-shears, in short wool or in long wool, and product labels must indicate which wool length the formulation is to be applied/ administered as well as generating efficacy data to substantiate such statements. Off-shears relates to sheep treated within 24 h of shearing. Short wool relates to sheep treated >24 h and up to 6 weeks after shearing. Long wool relates to sheep treated >6 weeks after shearing. For plunge and shower applications see cattle guidance.

E.2.3 Goats

In the case of goats, little information exists on how studies should be conducted. For the purposes of this appendix, all studies laid down for cattle should be followed.

E.2.4 Efficacy

In these studies, the effectiveness of an investigational product is determined by comparing the number of mites in groups of treated and negative/ vehicle/placebo control animals. Parasitised animals are randomly allocated into treated and negative control groups and the investigational product is administered as appropriate. Either induced infestations or naturally infested animals may be used. Formulae recommended to calculate efficacy vary and are subject to, amongst other things, whether there is likelihood for parasite numbers on the negative control animals to change over the study duration.

For most studies Abbott's formula[9] (Abbott, 1925) is advocated.

The Henderson–Tilton formula[10] (Henderson and Tilton, 1955) is advocated if the mite counts in the control animals vary significantly over the study duration.

In contrast to endoparasites, where an accurate measure of present populations could be made at necropsy, the assessment of mite burdens by examination of epidermal preparations will be less precise. Therefore, a measurement of response to treatment requires the repeated examination of epidermal preparations collected from affected or previously affected areas of the skin surface. Examinations at about weekly intervals for 56 days after treatment will assure an accurate estimation of mite burdens in therapeutic effectiveness studies. This observation period covers more than two complete life cycles of the mange mites and permits monitoring of the population dynamics of the mange mites, which multiply on the host.

For each targeted mite species, the criterion for efficacy is to achieve statistically significant ($p \leq 0.05$) differences in mite counts (all parasitic stages) between treated and negative control groups and at least 90 % efficacy (from Days 14 to 56 of the study). Appropriate statistical methods should be used to determine the significance of efficacy. These should be fully defined prior to conducting a study so that the study design will be satisfactory for the analysis of the data and obtaining meaningful (significant) results.

In field studies where semi-quantitative mite counts of investigational product performance may be used, different calculations to assess efficacy may be used. Such calculations must be documented and justified. Efficacy should be assessed at a minimum of three study time points (i.e. Days 14, 28 and 56).

In addition to efficacy parameters, other clinical observations (e.g. lesion scoring, pruritus) can be considered.

E.2.5 Numbers

The larger the number of animals and the more uniform the burden of mites, the more accurately acaricidal effectiveness may be demonstrated. However, based on pre-treatment mite counts, at least six adequately infested animals in each experimental group is the minimum number recommended.

E.2.6 Study types

For dosage determination studies, induced mite infestations are preferred, for dosage confirmation studies and field studies, natural infestations are advocated. Efficacy data for an investigational product must be obtained against each of the targeted species of mite.

To test persistent efficacy, controlled studies using induced mite infestations are advocated.

E.2.6.1 Dosage determination studies. See section 2.2.3.1 text of the main guideline.

E.2.6.2 Dosage confirmation studies. For a study to be considered valid, mites must be collected from at least five control animals at each post-treatment observation.

Studies should also demonstrate the investigational product efficacy under a variety of conditions of use (e.g. breed, length of wool, type of

[9] Abbott's formula: 100 x (1 - T/C), where geometric parasite counts of the treated (T) and control (C) animals occurs.

[10] Henderson–Tilton formula: $100 \times [1 - (T_a \times C_b)/(C_a \times T_b)]$, where parasite counts on treated (T) and control (C) animals occurs. T_b is the number of parasites counted per treatment group before treatment, T_a the number counted after treatment, C_b the number counted from the control group before treatment and C_a is the number counted from the control group after treatment.

equipment, quality of water used as diluent, etc.). Where experimental rainfall is used in dosage confirmation studies to test the efficacy of a topically applied investigational product before or after heavy rain, the method of wetting used and the equivalent in terms of natural rainfall should be stated.

E.2.6.3 Field efficacy studies. The purpose of field efficacy studies is to evaluate the investigational product when used as directed in the protocol under practical conditions in the field and to obtain additional experience as to acceptance and safety. The studies should be carried out in a number of breeds or types of animals of different ages and sexes in several locations to cover variations in the parasite populations, variable environmental conditions, and different feeding and management practices. Studies should be carried out in areas where the parasites are endemic and only with naturally acquired mite infestations.

The number of control animals should be at least 25 % of the number of investigational product treated animals. Separation of the treated and control groups here needs special consideration.

At each study site at least 20 of the investigational product treated animals should be identified and assessed throughout the study to show improvement of the signs associated with the mite infestation.

E.2.6.4 Persistent efficacy studies. Studies designed to demonstrate persistent efficacy need to include a challenge of animals at regular intervals throughout the defined period after treatment. The first challenge should not be initiated less than 7 days post-treatment; subsequently, challenges should be made at not less than weekly intervals. Challenge of animals can be done by manual transfer of mites directly to individual animals (cf. section 2.7) or by exposing treated groups of animals to infested, negative control animals for defined periods of time. Minimum group size should be six animals.

In studies with manual mite transfer, groups of mite-negative animals should be treated with the investigational product at intervals prior to the day of challenge (e.g. 21, 14 and 7 days prior to challenge). On the day of challenge (= single point challenge), all animals including a group of negative control animals should be infested with a predetermined number of mites. Thereafter, the animals should be observed for at least 2 weeks, and then examined for skin lesions and mite numbers in skin scrapings at about weekly intervals for an additional 3 weeks. In case of testing persistent efficacy of pour-ons and spot-ons, mites should not be placed directly at the site of application.

In studies with exposure to infested animals, groups of mite-negative animals have to be treated at intervals prior to the day of mite exposure at 7-day intervals (e.g. 28, 21 and 14 days prior to mite exposure so that exposure periods are 21–28, 14–21 and 7–14 days post-treatment, respectively). In addition, a negative control group should be included. Seeder animals (rate of exposure: one seeder per recipient) have to be assigned to the groups at random, after allocation based on mite counts conducted once prior to start of mite exposure. After exposure for 7 days, seeder animals should be removed. The animals should be observed for at least 2 weeks and then examined for skin lesions and mite numbers in skin scrapings at about weekly intervals for an additional 6 weeks. The study design mimics the natural transfer of mites; however, it is less exact as pressure of challenge may vary due to size of the pen and mite burden of the seeder animals.

The actual persistent efficacy (= length of prevention of establishment of mite infestation) is recorded as the last time point relative to investigational product treatment that failed to establish infestation. This means for studies with exposure to mite infested animals, animals exposed through 21–28 days after treatment and found mite-positive and animals exposed through 14–21 days after treatment found negative demonstrate a persistent efficacy of the investigational product of 21 days.

No studies have been identified on determining persistent efficacy against itch mites.

E.2.7 Induced infestations

Infestations may be induced by exposing mite negative (recipients) to mite-infested animals (seeders) in the same pen or by hand transfer of mites or skin scrapings from donor animals. Experience in the induction of mange is restricted to cattle and sheep.

E.2.7.1 Cattle. Both methods have been employed to induce mange in cattle; however, there is no reliable experience for inducing chorioptic mange in cattle.

Various techniques have been used to induce mite infestations. One technique involves the manual transfer of mites. Skin scrapings with a known number of mites from a donor animal are attached to the naïve host using, e.g. damp filter paper taped onto the study animals. Filter paper is attached for 2–3 days and the mite infestation is confirmed 2 or 3 weeks later examining each site on the study animals. Another technique employs the transfer of skin scraping material from a donor animal directly onto the skin at the withers of a recipient animal, where the material is held in place by fastening the long hairs using a rubber band. Mites collected from infested animals should preferably be transferred to non-infested animals on the same day or at least within 24 h. Mites used in the induced infestations should be from a recent field isolate less than 10 years old. Two infestations applied at least 1 week apart should increase the success of the infestation. The body region on which mites need to be applied will vary according to the mite species.

There are no strict recommended numbers of mites to be applied to induce infestations. In general, the higher the number of mites (*Sarcoptes* and *Psoroptes*) transferred the faster suitable mangy animals should be available. It is important that the animals should be infested with the same number of mites per infestation. The size of this inoculum should be recorded. Mite population/lesion growth cannot be precisely predicted and is affected amongst other things by host variations and climate factors. In case the mange lesions have a suitable size, the mite population can be estimated by counting the mites in a defined number of scrapings of definite size. Minimum mite numbers reported for induced infestations vary broadly:

for *Sarcoptes* at least 200–1500 mites;
for *Psoroptes* at least 50–100 mites;
for *Chorioptes* at least 500 mites.

E.2.7.2 Sheep. Both methods have been employed to induce psoroptic mange in sheep; however, there is no experience as to induce other types of mange in sheep.

Various techniques have been used to induce mite infestations. One technique involves the manual transfer of mites. Sheep should be experimentally challenged with *Psoroptes* mites by placing 25–100 live mites directly on to a small area over the withers or the backline at the shoulder. The wool needs to be fastened over the area with a rubber band to prevent scratching at the site of application. After 7 days, the rubber band needs to be removed. Challenge sites should be examined for presence of live mites and the development of scab lesions following challenge. Infestation should be allowed to progress for a minimum of 5 weeks before study initiation.

E.2.8 Variables

E.2.8.1 Mite counts and predilection sites. On each occasion, animals should be thoroughly examined for mange lesions, which should be recorded on a silhouette together with a description of the mange lesions. Preferably, the same personnel should evaluate the animals throughout the study duration.

Live (mange) mites should be counted in skin scrapings collected from the edges of active lesions or, if lesions regress during the study, from the area where active lesions were at study commencement. Scrapings should be made using a sharp spoon from a defined area (e.g. in cattle approximately 3 cm x 3 cm). The size should be adjusted according to the extent of the mange lesions on the animals and the size of the animals. The larger the number of scrapings per animal, the more accurately mite burdens may be estimated. For therapeutic efficacy studies with cattle, a minimum of six sites should be scraped on each animal on each occasion. According to the extent of lesions, these numbers may be reduced; however, the minimum number should be two scrapings per animal. For sheep and goats, the minimum number of sites to be scraped is two.

The number of scrapings per animal and size of scrapings have to be kept constant throughout the study. Any changes regarding number and/or size of scrapings during a study have to be justified and must apply to all study animals for consistency.

Samples should be evaluated within 8 h of collection. Scrapings should be taken on Day -1 or 0 prior to treatment, on Day 7 and at about 7-day intervals thereafter until study termination. The site of each scraping (and the total mange lesion size in relation to this) should be recorded on a silhouette. Only live (motile) mites have to be considered with total individual mite counts (not distinguishing stages) required.

A general definition of adequacy in mange mite infestation cannot be given because of the nature of this infestation:

- Mange mites are permanent ectoparasites that multiply on their hosts;
- The mite population on an animal can be subjected to important changes over time, e.g. in relation to season or body condition and nutrition of the host or development of effective defense mechanisms (immune responses) by the host;
- Mites are not evenly distributed within the mange lesions;
- Mite numbers in scrapings may be not correlated to mange lesion size;
- The mite burden of an animal can only readily be estimated by repeated examination of skin scrapings.

To give an indication, adequately infested cattle to be included in dosage determination or dosage confirmation studies should be infested with a minimum of 25 mites. These should be recovered from the majority of scrapings collected per animal (e.g. mites are counted in four of six scrapings). As mite numbers on negative control animals may decline over an 8-week observation period, an animal from which mites were recovered from at least half of the scrapings taken at an occasion (e.g. mites are counted in three of six scrapings) can be considered to support the validity of a study.

For sheep, a similar procedure should be applied, however, an adequately infested animal to be included in dosage determination or dosage confirmation studies should be demonstrated to be infested with in total at least five mites.

In sheep where itch mite is the target, the body should be examined by removing the fleece to skin level from an area measuring about 10 cm x 10 cm. The sites selected should be on the side of the sheep between a line joining the top of the shoulder and point of the hip and a line joining the bare areas of skin inside the fore limb and hind limb. When the site is prepared, a thin process oil is spread over the area and the scraping instrument is used to collect the sample. The oil containing the skin debris and itch mites is dropped into an appropriately marked 5 mL screw top container and stored.

Table E1
Predilection body sites for domestic ruminant hosts.

Parasite	Host	Body location
Sarcoptes spp.	Cattle	Neck and tail (sparsely haired areas)
	Sheep	Face, ears, axillae, groin
	Goats	Muzzle, around the eyes, inside the ears
Chorioptes spp.	Cattle	Base of tail, para-anal fold, hocks, inner aspects of hind legs, escutcheon
	Sheep	Legs, around the pasterns, scrotum
Psoroptes spp.	Cattle	Back, abdomen, tail-head, whole body
	Sheep	Axillae, groin, infra-orbital fossa, ear, whole body
Psorobia spp.	Cattle	Head, neck, shoulders, rump, ischial areas
	Sheep	Shoulders, body, flanks

After the required numbers of animals are scraped, the bottles containing the specimens should be examined either immediately or within 24 h. The oil is pipetted from the bottles onto microscope slides covered with a cover slip and examined with a microfiche reader using an appropriate lens magnification to differentiate each itch mite stage.

The results of the examinations are expressed as the number of living adults and nymphs per site. At each critical treatment examination during an efficacy evaluation, should the initial scraping be negative for live mites, further scrapings should be taken from sites adjacent to the initial site and from equivalent sites on the opposite side of the animal's body.

Each mite species has a predilection for certain host body sites (Table E1).

E.2.8.2 Clinical observations. To complement the mite count variable, changes of clinical signs associated with mange may be considered. Such evaluations are quantifiable by designing keys with values to reflect symptoms like pruritus and regression and/or healing of skin lesions. Lesion areas should be assessed both prior to treatment and at the end of the study period (e.g. by measuring the length and width of each skin lesion recorded on a silhouette). Scores to be used should be recorded in detail. In addition, measuring of changes in body weight is a useful variable.

E.3 Specific procedures to test efficacy against cattle mites

To test efficacy against cattle mites the procedures described under section 2 of this appendix are applicable.

E.4 Specific procedures to test efficacy against sheep mites

E.4.1 Mange mite (Psoroptes ovis)

Beside the examination of skin scrapings for live mites, mite counts may be based on counting the number of live mites (large motile mites) at a defined number of sites by parting the wool at the periphery of a lesion. However, skin scrapings must be examined at the end of a study from any unresolved lesion. Cryptic sites (i.e. the pinnae, the auditory canal, infra-orbital, infra-digital and inguinal fossae) should also be examined regularly at least three times (i.e. prior to treatment, after 4 weeks and at the end of the study).

Field efficacy studies are carried out on identified infested flocks, which should not have been treated with any acaricidal or insecticidal product for at least 3 months prior to the start of the study. Efficacy should be assessed utilising a critical study group (CSG), within the flock, comprising 20–50 sheep, each carrying an adequate infestation prior to treatment. The CSG should be treated with the remainder of the flocks at specific time points; however, during the course of the study, the CSG should be kept separated from the remainder of the flock.

For plunge dip formulations, sheep should be dipped for at least 1 min, with the head immersed twice. Pairs of CSG sheep should be dipped at the beginning after the initial dip charging and prior to replenishment of acaricide after approximately 25, 50 and 75 % of the remainder of the flock have been dipped. Remaining CSG sheep should be dipped last. Dip wash samples should be taken after the initial charging and before and after every replenishment for laboratory analysis.

Each sheep in the CSG should be examined prior to treatment and at intervals post-treatment until the end of the assessment period (nominally 8 weeks but this may be extended for up to 10 weeks) with scab lesion areas for each sheep calculated by measuring length and width of each lesion. Additionally, each animal should be examined for the presence of live mites to indicate an active infestation. An estimate should be made of mite numbers made along the periphery of each lesion by parting the wool at a defined number of sites. The cryptic sites of all sheep in the CSG should also be examined for the presence of live mites at the start and termination of the study. If appropriate, skin scrapings should be taken from all unresolved lesion areas and examined for live mites only.

Due to the serious welfare implications of this disease, untreated control groups are not recommended. Efficacy can be assessed by allowing a comparison of mite counts pre-treatment and post-treatment and having only the treated group.

E.4.2 Itch mite (Psorobia ovis)

A therapeutic efficacy should be supported by data that demonstrates a reduction of the mite population to non-detectable levels at all post-treatment examinations of treated sheep, in the presence of normal seasonal fluctuations in the population on negative control sheep kept under the same conditions.

Sheep included in studies should be subjected to skin scrapings and have a mite infestation of 10 mites/200 cm^2 of skin examined. At least four studies are recommended, two in late winter–spring and two in late summer–autumn. The purpose of the studies is to demonstrate that the minimum dosage rate or concentration of the investigational product that is recommended for commercial use, is capable of reliably controlling itch mites on all sheep that are treated under the proposed conditions for use. This could involve studies to show the investigational product efficacy under a variety of conditions of use (breed, length of wool, type of equipment, quality of water used as diluent, etc.). The groups of sheep should be kept in pens, which allow them normal exposure to prevailing weather.

Presence of fleece derangement is not always a good indicator of sheep with detectable infestations of mites. Presence of skin scurf is a fair indicator of mite infestation and sheep to be examined for suitability in studies should be selected in the first instance on the basis of moderate to heavy deposits of scurf on the skin.

In field studies, the most practical assessment of the impact of itch mite infestation on a flock is by visual assessment of the degree of fleece derangement present in the flock. If the animals are restrained in a small yard or treatment race for inspection, much of the affected body surface is obscured. Ideally, the sheep should be observed from both sides, as marked differences in the extent/degree of fleece derangement have been noted in individual sheep from one side to the other (Sinclair, 1976).

Presence of scurf on the skin is a useful indicator of changes in mite infestation after treatment. Scurf can be readily seen when the fleece is parted and is clearly revealed when the wool is removed prior to skin scraping. Presence of scurf should be scored on the basis of:

- 0—No visible deposit on the skin;
- 1—Sparse, scattered deposit;
- 2—A uniformly spread powdery deposit;
- 3—A thick deposit with obvious flakes.

Skin scrapings and skin scurf scores should be recorded within 24 h before treatment and at 1, 3, 6, 9 and 12 weeks after treatment.

Mites should be counted as late as possible before treatment. If the period between examination and treatment is more than 24 h then the interval should be documented.

Due to animal welfare implications of this parasite, negative control groups should not be used in field studies.

E.5 Specific procedures to test efficacy of goat mites

No standard protocols have been developed for goats and until more data become available, protocols similar to cattle are advocated.

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Glossary of terms in Appendices A–E

Adequate infestation: Adequately infested animals carry an ectoparasite burden, which is representative/typical for practical farm situations and enables a rigorous statistical evaluation of the effect of interventions. Indications of that burden may be derived from historical data or publications. To allow for a meaningful evaluation of the efficacy of veterinary (pharmaceutical/chemical) investigational products an adequately infested animal must carry a minimum ectoparasite burden (= minimum number of parasites per animal) to be included in a study. The minimum ectoparasite burden must be defined in the development of study protocols with respect to the duration of the study (repeated ectoparasite counts in the post-treatment observation period).

Biting flies: Flies that pierce the skin with their mouthparts and are normally blood feeders (stable flies, horn flies and tsetse flies). They induce pain caused by the mechanical insertion of their mouthparts, the salivary fluid may irritate and result in reactions (e.g. allergic responses) and they may transmit pathogens.

Blow fly strike: A common term for myiasis caused by flies of the Calliphoridae family. Blow is egg laying; and strike is the damage caused by development of the fly larvae.

Control (approved) product: Any approved product used according to label directions (in studies with a positive control group), or any placebo (vehicle/blank formulation – in studies with a negative control group), used as a reference in a study for comparison with the investigational veterinary product under evaluation.

Dosage determination study: A controlled study, utilising animals in pen facilities, to determine the effective dose required to kill the parasites that are targets of the investigational product. This study is also used to determine the least susceptible parasite species that is likely to be present in the field.

Dosage confirmation study: A controlled study, usually utilising animals in pen facilities, to confirm that the effective dose as determined in the dosage determination study, has acceptable efficacy in large groups of animals, as well as quantifying the persistent efficacy against re-infestation by the target parasites/parasite life cycle stages.

Efficacy: The quantum of therapeutic response that a veterinary (pharmaceutical/chemical) investigational product achieves against an ectoparasite, determined from several studies using infested animals.

Facultative myiasis: Infestation of live animals with dipteran larvae that are normally free-living on organic material, but that can also have a parasitic mode in the life cycle.

Investigational product: A veterinary (pharmaceutical/chemical) product administered via routes or exposure methods, with certain therapeutic and/or persistent activity to remove/kill ectoparasites after the treatment.

Itch mites: Two species parasitise domestic ruminants: *Psorobia ovis* (sheep) and *Psorobia bos* (cattle). Itch mites live in the superficial skin layers and can cause chronic irritation and skin thickening.

Knockdown effect: The immediate initial action of some chemicals on the nervous system of arthropods, which is characterized by inability to move (immobilisation). Knockdown may be followed by recovery or by death.

Mange mites: Parasitic mites of the families Sarcoptidae and Psoroptidae. They generally give rise to well-defined dermatoses. Mange mites live on the surface of the skin *(Chorioptes* and *Psoroptes*) or may be superficial burrowers *(Sarcoptes*).

Masked: The procedure used to reduce potential study bias in which designated personnel are kept uninformed of the treatment assignments in a study.

Myiasis: Infestation of live animals with dipteran larvae. The larvae may live in the skin, nasal cavities or be somatic in/on the host.

Negative control group: A group of animals included in a control study, which are not treated with the investigational product. This group usually receives no treatment at all but in certain circumstances may be treated with the vehicle carrier of the investigational product or with a placebo.

Nuisance flies: Flies possessing spongy mouthparts used for feeding on liquid films (house flies, blow flies and face flies). Some are facultative blood feeders and ingest blood at the edges of wounds. They cause annoyance to animals and may transmit pathogens both as a vector (e.g. *Thelazia*) and mechanically [e.g. transmitters of bacteria causing keratoconjunctivitis and mastitis].

Obligatory myiasis: Infestation of live animals with dipteran larvae that are dependent on an animal host and cannot complete their life cycle development without such a host.

One-host tick: Larval, nymph and adult stages of these ticks feed on a single animal until the mated, replete, or fully engorged females drop to the ground to oviposit.

Persistent efficacy period or period of protection: Extended therapeutic activity of the veterinary (pharmaceutical/chemical) investigational product measured in days after the treatment day. Sometimes referred to as prophylactic or protective effect.

Positive control group: A group of animals, included in a control study, that are not treated with the investigational product. Instead this group is treated with a registered/approved product that has a defined/recognised parasitic effect.

Prevention: The action of restricting an ectoparasite establishment on the host.

Semi-engorged (female) tick: Ticks with conspicuous filling of the alloscutum.

Three-host tick: The larva, nymph and adult stages of these ticks each feed on a separate animal host, the remainder of the life cycle is spent off the host in the vegetation of the surrounding environment.

Tracer cattle: Negative control animals introduced to an infested environment to assess the size of the tick population in a field study.

Two-host tick: The larva and nymph stages of these ticks feed on one host, and the adults on another host, the remainder of the life cycle is spent off the host in the vegetation of the surrounding environment.

Vagility: ability of an organism to roam freely about the environment.

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