Veterinary Parasitology 194 (2013) 84–97

Contents lists available at SciVerse ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) second edition: Guidelines for evaluating the efficacy of parasiticides for the treatment, prevention and control of flea and tick infestations on dogs and cats

A.A. Marchiondo[a,*], P.A. Holdsworth[b], L.J. Fourie[c], D. Rugg[a], K. Hellmann[d], D.E. Snyder[e], M.W. Dryden[f]

[a] Zoetis Inc., (formerly Pfizer Animal Health), 333 Portage Road, Kalamazoo, MI 49007, USA
[b] Animal Health Alliance (Australia) Ltd., Locked Bag 916, Canberra, ACT 2601, Australia
[c] ClinVet International (Pty) Ltd., P.O. Box 11186, Universitas 9321, Bloemfontein, South Africa
[d] KLIFOVET AG, Geyerspergerstr. 27, D-80689 München, Germany
[e] Elanco Animal Health, 2500 Innovation Way, Greenfield, IN 46140, USA
[f] Department of Diagnostic Medicine/Pathobiology, Kansas State University, Manhattan, KS 66506, USA

ARTICLE INFO

Keywords:
Fleas
Ticks
Ectoparasiticides
Insect growth regulators
Dog
Cat
Therapeutic efficacy
Persistent efficacy
Updated guidelines
Tick categorization
Repellency
Systemic compounds: Topical compounds

ABSTRACT

These second edition guidelines, updated from the 2007 version (Marchiondo et al., 2007), are intended to assist the planning and conduct of laboratory and clinical studies to assess the efficacy of ectoparasiticides applied to dogs or cats for the purpose of treating, preventing and controlling flea and tick infestations. Major revisions to this second edition include guidelines on the assessment of systemic flea and tick products, an update of the geographical distribution of the common fleas and ticks species on dogs and cats, determination of flea and tick efficacy based on geometric versus arithmetic means with respect to geographic regulatory agencies, modification of tick categorization in the assessment of efficacy, expanded guidelines on repellency and anti-feeding effects, enhanced practical field study guidance, and considerations on the ranges of flea and ticks for infestations in laboratory studies. The term ectoparasiticide includes insecticidal and acaricidal compounds, as well as insect growth regulators. The range of biological activities from animal treatment that are considered include: repellency and anti-feeding effects, knockdown, speed of kill, immediate and persistent lethal effects, and interference with egg fertility and subsequent development of off-host life cycle stages. Information is provided on the selection of animals, dose determination, dose confirmation and field studies, record keeping, interpretation of results and animal welfare. These guidelines are also intended to assist regulatory authorities involved in the approval and registration of new topical or systemic ectoparasiticides, and to facilitate the worldwide adoption of harmonized procedures.

1. Introduction

These second edition guidelines updated from the 2007 version (Marchiondo et al., 2007), for evaluating the effectiveness of parasiticides against flea and tick infestations on dogs and cats follows similar publications from the World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) for evaluating anthelmintics in a variety of species (Powers et al., 1982; Düwel et al., 1986; Jacobs et al., 1994; Wood et al., 1995; Duncan et al., 2002; Yazwinski et al., 2003), detection of anthelmintic resistance in nematodes of veterinary importance (Coles et al., 1992),

* Corresponding author.
E-mail address: alan.marchiondo@zoetis.com (A.A. Marchiondo).

0304-4017/$ – see front matter
http://dx.doi.org/10.1016/j.vetpar.2013.02.003

anticoccidials in chickens and turkeys (Holdsworth et al., 2004) and ectoparasiticides on ruminants (Holdsworth et al., 2006a, 2006b, 2006c, 2006d; Vercruysse et al., 2006).

The 2007 guidelines (Marchiondo et al., 2007) aimed to address the most current rationale and methodologies in evaluating the efficacy of flea and tick parasiticides leading to verbatim adoption, in some cases, by many regulatory agencies globally. But, with the passage of time, advancements in topical and systemic pulicidal and acaricidal chemistry, the use of combinations of active ingredients to expand claims, and the progress in methodologies have necessitated this revision. The major revisions to this second edition include guidelines on the assessment of systemic flea and tick products, an update of the geographical distribution of the common fleas and ticks species on dogs and cats, determination of flea and tick efficacy based on geometric versus arithmetic means with respect to geographic regulatory agencies, modification of tick categorization in the assessment of efficacy, expanded guidelines on repellency and anti-feeding effects, enhanced practical field study guidance, and considerations on the ranges of flea and tick infestations in laboratory studies. As emphasized in the original guideline (Marchiondo et al., 2007), if in a particular circumstance an alternative approach to those listed herein is deemed more fitting, a reasoned argument for its employment should be prepared and discussed with appropriate regulatory authorities before initiation of the study program.

The present guidelines collate methodologies from numerous sources with the objective of providing a basis for the rationalization, simplification and harmonization of studies required for the evaluation and licensing of small animal ectoparasiticides in different countries. These methods reflect standards and principles commonly recognized by the scientific community as appropriate and necessary for collecting robust scientific data for this purpose. It is hoped that they will (1) serve as models for regulatory officials responsible for developing meaningful efficacy registration requirements within their countries, (2) assist investigators in preparing basic plans for effective definition of the efficacy of a new investigational material, and (3) reduce the number of study animals used in testing for cost saving and animal welfare considerations. The last point is of particular significance because, with a few exceptions such as *in vitro* tick repellency studies, the evaluation of ectoparasite treatment and control cannot be realistically achieved without the use of host animals.

2. Overview

Fleas and ticks are obligate blood-feeding ectoparasites with complex life-histories involving on- and off-host life-cycle stages. Depending upon circumstances, infestations can result in blood-loss, irritation, hypersensitivity and transmission of pathogens (viruses, bacteria or parasites). The purpose of individual treatments may be to provide short-term relief or longer term control. For example, ectoparasiticides may be used therapeutically to alleviate discomfort and/or halt blood-loss, or prophylactically to prevent or reduce bites thereby averting associated clinical manifestations and transmission of pathogens. To achieve these objectives, animal treatments can be employed to: (1) kill the parasites already on the animal at the time of treatment; (2) repel new host-seeking arthropods and/or prevent/reduce biting; (3) kill parasites newly-acquired by the host for a period of time after treatment (persistent (or residual) activity); and (4) progressively reduce or eliminate off-host life-cycle stages in the environment. The latter can be achieved by treatment programs that (1) stop parasite-infested animals disseminating eggs into the environment, (2) render infertile eggs that are produced, or (3) interfere with the development of off-host life-cycle stages by the transfer of biologically significant amounts of active material from the treated animal to its immediate surroundings.

Active substances that kill adult fleas are known as insecticides, pulicides or adulticides, while those killing ticks are termed acaricides. The biological activity of a pulicide or acaricide cannot be described fully by a single 'efficacy' value. Commonly used criteria for different aspects of pulicidal activity include: 'knockdown', 'immediate efficacy', 'therapeutic efficacy' and 'speed of kill or action' to define the effect of treatment against pre-existing or challenge infestations; 'short-term persistent efficacy (or prophylactic efficacy)', which refers to the period over which the establishment of new infestations is prevented (typically up to 4–6 weeks); and 'long-term persistent efficacy' which is usually applied to sustained release devices or systemic formulations providing control for extended periods (typically $\geq$ 3 months).

Pulicidal or acaricidal activity *per se* does not necessarily prevent flea bites or tick attachment before they succumb to the lethal effect of the active material. Some compounds, however, have repellent and/or anti-feeding effects. These may be manifested by fleas or ticks avoiding or leaving their host, or by failing to bite, attach or feed. These attributes contribute obvious benefits in the management of flea allergic dermatitis and in preventing the transmission of tick-borne pathogens.

Molecules that exert their action by interfering with the development of the egg or other off-host life-cycle stages are called insect growth regulators (IGRs) or insect development inhibitors (IDIs). The efficacy of each molecule is judged by its effect on the hatchability of eggs and/or subsequent survival of off-host life-cycle stages, and by the duration of those effects.

Definitions of these terms are given in the Glossary at the end of these revised guidelines and further discussion of these terms and their significance in the design of control programs may be found, for example, in Marchiondo et al. (1990), Marchiondo (1993), Dryden and Rust (1994), Carlotti and Jacobs (2000), Rust (2005), and Halos et al. (2012).

3. Fleas and ticks on dogs and cats

The cat flea, *Ctenocephalides felis felis*, is a near ubiquitous problem globally in both cats and dogs, whereas *C. canis* is largely confined to dogs and more restricted in its distribution (Table 1). Cats and dogs may occasionally harbor other flea species that normally parasitize other hosts such as rabbits, hedgehogs, and poultry. Cat fleas are

Table 1
Main flea species on cats and/or dogs.

Flea species	Australia	Europe	Japan	USA	South Africa	South America
Ctenocephalides felis felis	X	X	X	X	X	X
Ctenocephalides canis	–	X	X	–	X	X

usually troublesome because of the intense irritation and allergy they can cause, but they also transmit the tapeworm *Dipylidium*, and the filarial parasite *Acanthocheilonema (Dipetalonema)*, as well as many microbial agents (*Bartonella*, *Rickettsia*, *Mycoplasma*). Eggs laid in the pelage fall to the ground where the flea larvae develop and pupate. The life-cycle is completed when the adult emerges from the cocoon and jumps onto a host. If this happens to be a human, consequences include annoyance, papular urticaria and the possibility of zoonotic disease hazards such as tapeworm infection, cat scratch fever (caused by *B. hensellae*) or flea-borne spotted fever (caused by *R. felis*).

Ticks generally have specific environmental requirements that limit their geographical distribution and local impact (Table 2). For example, *H. elliptica* is of particular importance in South Africa and *I. holocyclus* is restricted to the east coast of Australia. *Rhipicephalus turanicus* is another important tick species infesting cats and dogs in South Africa and various countries in Southern Europe (Walker et al., 2000; Estrada-Peña et al., 2004). *Rhipicephalus sanguineus* collected from cats in Mediterranean parts of Europe may actually be *R. turanicus*. *Rhipicephalus sanguineus* is the one tick that has adapted well to indoor conditions, especially kennels, and is widely distributed throughout the world. Typically, each life-cycle stage (larva, nymph, adult) takes a single protracted blood-meal and drops to the ground to molt. An exception is *R. bursa* whose larva molts and stays on its first host. Some species, particularly *Amblyomma* spp., produce deep painful bites that can become secondarily infected. Several tick species transmit serious microbial and protozoan diseases of animals and humans such as Lyme disease, *Rickettsia* spotted fever, tularemia, Q fever, piroplasmosis, hepatozoonosis, and cytauxzoonosis. Some others, such as *A. americanum* and *D. variabilis* in North America and *I. holocyclus* in Australia, are of considerable clinical importance because toxins injected with their saliva induce ascending paralysis.

In laboratory studies, dogs are routinely infested with selected tick species, for example *R. sanguineus* and *D. variabilis*, under controlled conditions (Estrada-Pena and Ascher, 1999). In contrast, laboratory infestations of cats can be problematic. A naturally lower rate of parasite attachment and more meticulous host grooming habits lead to high variability in establishment. Cats can, however, be naturally parasitized with *A. americanum* (larvae, nymphs and adults), *D. variabilis* (nymphs and adults), *D. andersoni* (nymphs), and *I. scapularis* (nymphs) in the USA (Dryden and Payne, 2004). For South Africa, *H. elliptica* and *R. turanicus* infest both cats and dogs.

4. Evaluating efficacy against fleas and ticks

4.1. Introduction

In vitro techniques are often used in the discovery and preclinical phases of the development of potential insecticides and/or acaricides. Determination of the "least sensitive" tick species for use in dose determination studies also can be conducted using *in vitro* techniques. Methods are available to determine LC_{50} values (Finney, 1971) by

Table 2
The main ixodid tick species that feed on dogs and cats.

Tick species	Australia	Europe	Japan	USA	South Africa	South America
Amblyomma americanum	–	–	–	X	–	X
Amblyomma cajennense	–	–	–	–	–	X
Amblyomma aureolatum	–	–	–	–	–	X
Amblyomma ovale	–	–	–	–	–	X
Amblyomma triguttatum	X	–	–	–	–	–
Dermacentor variabilis	–	–	–	X	–	–
Dermacentor reticulatus	–	X	–	–	–	–
Haemaphysalis elliptica[a]	–	–	–	–	X	–
Haemaphysalis longicornis	X	–	X	–	–	–
Haemaphysalis flava	–	–	X	–	–	–
Ixodes hexagonus	–	X	–	–	–	–
Ixodes holocyclus	X	–	–	–	–	–
Ixodes ovatus	–	–	X	–	–	–
Ixodes ricinus	–	X	–	–	–	–
Ixodes scapularis	–	–	–	X	–	–
Ixodes pacificus	–	–	–	X	–	–
Rhipicephalus sanguineus	X	X	X	X	X	X
Rhipicephalus bursa	–	X	–	–	–	–
Rhipicephalus turanicus[b,c]	–	X	–	–	X	–

[a] Apanaskevich et al. (2007). Until recently, *H. elliptica* was reported in South Africa as *H. leachi.*
[b] Walker et al. (2000).
[c] Estrada-Peña et al. (2004).

exposure, for example, of appropriate flea or tick life-cycle stages to a range of concentrations of the active chemical in or on liquid or solid media such as whole blood or impregnated filter papers (Stone and Haydock, 1962; Anon, 1971; Drummond et al., 1973; Tatchell, 1974; Rust and Reierson, 1989). Such studies can provide useful data, for example, on the spectrum of potential insecticidal/acaricidal activity, on synergistic activities (Prullage et al., 2011a), or on resistance profiles (by exposing defined flea or tick strains), but they rarely give more than a broad indication of the dosage required for topical or systemic application to an animal.

Laboratory studies using experimentally induced animal infestations are still required to establish the optimum dose-rate of an investigational formulation, and to confirm its immediate and persistent efficacy profile. Clinical field studies are subsequently needed to confirm activity against natural infestations under a wide range of conditions. There are many potential interactions in actual usage that laboratory testing cannot always anticipate or adequately replicate. For example, the degree or duration of efficacy of a particular investigational product could be influenced by infestation pressure and the natural specific susceptibility of the parasite population used, by the host breed, coat type, behavior and husbandry of treated animals; by exposure to sunlight, rain, swimming, bathing, or even water pH; as well as by other climatic and geographical considerations.

It cannot be assumed that investigational formulations will give the same results on dogs and cats (or other pet animals such as rabbits and ferrets) even if they harbor the same parasite species. Separate studies are therefore necessary for each host species.

The number, and nature, of laboratory and clinical studies is dependent on the required label claims, the availability and accessibility of infested animals, and aspects of the biology of the target ectoparasite. Four factors determine the strength and nature of the efficacy label claim: (1) the parasite species and life cycle stages that are controlled, (2) the degree of control, (3) the anatomical coverage of the investigational compound, and (4) the duration of the control afforded.

Regarding the parasites themselves, various factors such as the specific laboratory population used, physiological condition, and age may result in intraspecific variations in susceptibility to insecticides and acaricides.

Pulicides, acaricides, IGRs, and IDIs may exert their action topically or systemically and can be administered in a variety of ways, for example: orally, by injection, or as spot-ons, line-ons, sprays, shampoos or collars. In the case of topically applied non-systemic products, study designs should allow a period of time, which may be up to or longer than 24 h depending on formulation, for spreading and distribution of the active ingredient from the high concentration at the site of application to lesser concentrations at distal regions on the body of the host animal. In the case of systemic products, applied topically or given orally, the time to reach the plasma EC_{90} or more and the duration of the concentration (area under the curve) over the threshold will define the potential curative and the persistent efficacies.

4.2. Animal studies

4.2.1. Controlled study design

Controlled studies are advocated for the laboratory assessment of efficacy against both fleas and ticks. In this type of study, the efficacy of the test material is determined by comparing parasite numbers on groups of treated and untreated (negative control) animals under carefully standardized conditions at specific time points during the study. Acclimatization of the animals to the conditions (environmental) and factors (physical housing conditions, social housing conditions, dietary, experimental and handling procedures) they are likely to experience while in the study is important for optimization of animal welfare as well as to minimize interanimal variation, thereby strengthening the quality of data for *in vivo* studies. No set of firm guidelines are available on the period of acclimatization, but flexibility and professional judgment of the investigator, IACUC, and sponsor are needed in determining the duration and how the animals will be prepared for research (Schapiro and Everitt, 2006). After an acclimatization period of at least 7 days (in case animals are transferred to new facilities, otherwise a shorter period may be appropriate), animals are ranked according to the number of parasites that establish on each individual after a pre-allocation infestation (Section 5.4), and are then randomly allocated to study groups. The pre-allocation infestation is eliminated preferably by combing or alternatively by chemical treatment (for example, nitenpyram) before the study commences. Animals are observed at appropriate intervals during and after treatment to record adverse reactions and clinical side effects. Parasite counts following subsequent infestation(s) (as detailed in Sections 4.2.2 and 4.2.3) are used for efficacy calculations.

If the distribution of parasite counts within each group is skewed the geometric mean is generally considered to be a better estimate of central tendency for comparison than the arithmetic mean. There is, however, an alternative view that the arithmetic mean is the only appropriate tool for estimating ectoparasiticidal efficacy against ticks and fleas on dogs and cats based on the calculations done by Dobson et al. (2009) and applied to tick and flea study evaluations (EMEA/CVMP/005/00-Rev 2, 2011). Therefore both geometric and arithmetic means should be recorded to be aligned with regional regulatory requirements. For example, a global development program will require efficacy studies determined on geometric means for the USA Environmental Protection Agency (USA-EPA) and The Center of Veterinary Medicine (CVM) of the United States Food and Drug Administration (FDA) as opposed to arithmetic means for The European Medicines Agency (EMA).

Appropriate statistical methods should be used for assessing the significance of observed differences between groups. In general, the percentage efficacy is calculated from the following formula:

$$E = \frac{M_c - M_t}{M_c} \times 100$$

where E is percent efficacy, M_c and M_t refer to the number of live parasites on the control and treated animals, respectively. For ticks, the formula is applicable for topically applied contact acaricides but for systemically delivered acaricides, the use of live attached ticks may be more appropriate to assess efficacy. Efficacy will only occur if the tick attaches and ingests a toxic dose of the drug, thus counting only the number of live attached ticks provides the best method to assess activity for a systemic acaricide. If the ticks are live and free at 48 h post-infestation, they probably did not attach (which is rare after this amount of time) and, thus, these live free tick counts should not be used in the efficacy calculation. This is even more relevant for male *Ixodes* species that do not readily attach. Mode of action and tick biology should also be considered, especially with novel compounds and combinations, and the rationale for the tick category selection should be justified. A large number of live free ticks on treated animals could be due to an anti-feedant/detachment effect interfering with acaricidal efficacy.

4.2.2. *Efficacy against fleas*

The initial flea infestation (~100 fleas on dogs and ~50–100 fleas on cats) is usually applied ~24–72 h prior to treatment to assess immediate curative efficacy. The adequate retention rate of fleas for inclusion of animals in the study should be defined in the protocol, but generally, pre-treatment and non-treated animals should retain at least 25% of the applied flea infestation. The percentage of fleas remaining on a host after each experimental infestation can be highly variable and typically may be higher on dogs than cats because of more intense grooming in cats. Thus, cat infestations may require higher numbers of fleas or restriction of grooming.

The immediate therapeutic efficacy of the investigational material is evaluated at a pre-determined time post-treatment (typically ~24 up to ~72 h) by combing off and counting the surviving fleas (Appendix A.2). Groups of animals, each with its appropriate negative control group, can be flea counted at, for example, ~4, 8, 12 and 24 h post-treatment to provide additional data on speed of kill (Appendix A.2). The short-term persistent efficacy is measured by re-infesting the animals in each study group at ~7-day intervals and counting the fleas ~24–72 h later. Similar re-infestations at four-weekly intervals, reducing to two-weekly during the last month of expected protection, are used to monitor long-term persistent efficacy.

For specific label claims such as aids in or prevents the transmission of flea-borne pathogens, studies designed specifically to determine such a claim should be performed.

Flea species. The cat flea, *C. felis felis*, is cosmopolitan and the predominant species on both dogs and cats in most countries (Dryden and Rust, 1994) and may therefore be the only species necessary to be tested. It cannot be assumed, however, that other species such as *C. canis* are equally susceptible and, consequently, supporting data may be required for a label claim. Mixed flea populations may be used for testing provided that an adequate flea infestation is established on the test animal and that the fleas of different species are identified, counted and recorded.

Table 3
Categorization of ticks for thumb or comb counting.

Survival status	Attachment status	Interpretation
Live	Free or attached[a]	Acaricidal effect NOT demonstrated
Dead	Free or attached	Acaricidal effect demonstrated

[a] If justified, only live attached tick counts may be used to assess efficacy for systemically acting acaricides.

4.2.3. *Efficacy against ticks*

The design of controlled studies for measuring acaricidal efficacy against ticks is similar to that described for fleas (Section 4.2.2) except that the unfed tick numbers used for infestation (~50) is generally fewer for dogs or cats due to animal welfare issues defined by skin irritation and damage of repeated infestations by a specific tick species, e.g., *A. americanum*, and that parasite numbers are assessed by palpation, combing and visual inspection (Appendix A.3). Mixed tick populations (species) may be used for testing, in particular when trying to determine the least susceptible tick species, provided that an adequate tick infestation of each species is established on the test animals and that the ticks of different species are identified, counted and recorded. The retention rate of experimental tick infestations on the animal are generally ~20–50% of the infestation number but interspecific variations may occur. A tick retention rate of at least 20% on non-treated animals is generally acceptable for inclusion of animals in a study. However, lower retention rates can be used as justified in the protocol and with regional regulatory concurrence. Ticks placed on an animal previously treated with an effective compound usually detach from and then may die off the host within ~24–48 h without having taken a complete blood meal, but some may remain attached. Thus, the ticks on the animal can be alive (recommend assessment of ticks as alive by confirmation of movement after gentle touching with a probe, CO_2 exposure, and/or warming) or dead, attached or non-attached (Table 3). The mean numbers of live free and attached live ticks from topically treated and control animals should be used to calculate efficacy values by using the formula given in Section 4.2.1. For animals treated with systemically acting acaricides, the use of only live attached tick counts may be the most appropriate measure of efficacy for some species. This should be supported by pharmacokinetic/pharmacodynamic data and/or by assessment of the relative numbers of live free ticks on nontreated and treated animals (these should be equivalent; a greater number of free ticks on treated animals could indicate that the treatment may have an expellent effect). The assessment of ticks as alive by confirmation of movement should be similarly performed as for topically applied acaricides. The methodology used for the assessment of efficacy should be defined and justified in the study protocol. For specific label claims such as 'aids in' or 'prevents the transmission of tick-borne pathogens', studies specifically designed to demonstrate such a claim should be performed. Reliable models have been developed (McCall et al., 2011; Jongejan et al., 2011) to assess the efficacy of compounds in preventing the transmission of tick-borne pathogens.

As for fleas, the short-term persistent efficacy is measured by re-infesting the animals in each study group at ~7-day intervals and counting the ticks ~48 h later. If a longer period of attachment can be justified, e.g., a systemic mode of action and/or taking into account potential transmission of pathogens, counting ticks ~72 h or longer post-infestation may be acceptable. However, the period of attachment and physiological effects on ticks during this period should also be considered and described.

In long-term evaluation studies, the interval between each re-infestation should be no less than 4 weeks as more frequent application of ticks may induce a sufficiently strong immune response to reduce parasite establishment and provoke skin reactions. For greater precision, a final infestation after ~2 weeks may be indicated.

Tick species. Species of ticks common to dogs and cats within geographical regions (Table 2) should be tested to demonstrate sufficient biological control activity to support label claims. For a restricted (single species) claim, data to support a single tick species is adequate in the USA and certain geographical regions. Generally the tick species controlled should be determined by the laboratory data and specified in the claims, however, some authorities like the USA-EPA may allow a general "tick control" claim. In this case, laboratory data using animals should be provided for the Brown Dog tick (*R. sanguineus*) and at least one other tick species common on that host, for example, the American Dog tick (*D. variabilis*) in the USA, the European dog tick (*D. reticulatus*) in Europe, the Asian Bush tick (*Haemaphysalis longicornis*) for Asia and Japan, or the Yellow Dog tick (*H. elliptica*) in Southern Africa.

4.2.4. Repellency and anti-feeding effects

Two types of repellency are defined: "*sensu stricto*" for repellency characterized by an irritant effect, causing the tick to move away from the treated animal or leading it to fall off soon after contact with the treated haircoat within ~6–8 h and "*sensu lato*" for all other tick repellency (or expellency) up to ~24 h. The first, repellency *sensu stricto*, may be attributed to the vapor phase of a compound or irritant effect through direct contact (for example, oil of Citronella but also some synthetic pyrethroids), while the second repellency (or expellency) causes inhibition of attachment or detachment of already attached ticks, (for example, some synthetic pyrethroids or amitraz). Some insecticides/acaricides may act both ways. This distinction should be reflected in the design of *in vitro* techniques to test whether newly emerged unfed adult fleas or ticks become randomly scattered within an experimental apparatus, or if their spatial distribution can be significantly influenced by the presence of the investigational compound. Flea repellent *in vitro* test systems include Y- or T-tube olfactometers as well as the four-arm airflow olfactometer (Vet et al., 1983). Useful reviews on tick repellents (Bissinger and Roe, 2010; Halos et al., 2012) and *in vitro* test systems for tick repellents (Carroll et al., 2003; Dautel, 2004) should be consulted for testing compounds and formulations for tick repellency. The Moving-object bioassay (Dautel et al., 1999; Dautel, 2004) allows ticks to display host seeking behavior more closely related to the natural situation and has been used successfully to evaluate products (Schwantes et al., 2008: Dautel and Cranna, 2006). Further regulatory testing guidelines for the evaluation of repellency against fleas and ticks can be found in the USA-EPA OPPTS810.3700 Insect Repellents for Human Skin and Outdoor Premises, 712-C-99-369, December 1999, and EMEA/CVMP/005/00-Rev 2.

Various approaches have been employed in the past to gain an *in vivo* estimate of repellency (Endris et al., 2002). Recently, tick control terminology with respect to repellency has been proposed (Halos et al., 2012) along with an *in vivo* study design to measure the effects (early expellency and repellency *sensu stricto*, disruption of attachment, and acaridical 'killing' effect) based on the status of the tick over a time course of 48 h post-treatment on dogs. With reference to the effect/efficacy of an acaricide on pre-existing tick infestations, it can either cause detachment, expellency which results in detachment and disengagement (leaving the host), and lastly killing, in which case attached ticks may die *in situ*. A very specific approach will be required to determine repellency *sensu stricto*. A proposed method would be to place sedated treated and control dogs in infestation crates and to release ticks in the crates (not on the dogs) and conduct assessments ~30 min–1 h later (only counts of ticks remaining in crates) (Fourie, personal communication). A modification of this methodology was used to assess prevention of tick attachment of a fipronil, amitraz and (S)-methoprene topical combination by placing ticks on the bottom of a crate, placing the treated dog in the crate for 2 h, removing the dog to a second crate for 2 h, and then thumb counting the attached ticks on the dog (Prullage et al., 2011b). Ticks will move toward a potential host over short distances attracted by host odors, radiant heat and other stimuli (*Ixodes*, *Dermacentor* and *Amblyomma* spp.), but this approach may differ from the natural encounter where in some tick species appetence takes the form of preparing an ambush. To determine repellency/expellency *stricto lato*, ticks can be placed directly on the sedated animals in the infestation crates and assessments can be made for periods up to ~24 h.

With specific reference to an anti-feeding effect of a compound, 97% of cat fleas will normally have fed within 1 h of arriving on their host (Cadiergues et al., 2000). The anti-feeding effect of an investigational product can be evaluated from as early as ~15 min post-infestation for topical contact products by collecting all on-host live/moribund/dead fleas from treated and untreated animals. For systemically active compounds that require the flea to ingest the compound, as early as ~15 min post-treatment or post-infestation can be evaluated, but effects on feeding in the case of nitenpyram was not observed until 1 h post-dosing (McCoy et al., 2008). To quantify flea feeding, a Real-Time PCR assay can be used to determine the amount of host DNA in the fleas collected from treated and untreated animals allowing for the appropriate comparative statistical analysis (Wang et al., 2012). Other quantitative methods include Drabkin's reagent and radionuclides (^{51}Cr-ethyrocyte and ^{125}I-albumin) which have been used to measure blood consumption (Dryden and Gaafar, 1991). Simple hemoglobin test strips or viewing crushed fleas under a microscope can provide qualitative information on flea blood feeding.

The anti-feeding effect of a compound against ticks can be determined in a similar manner with the exception of microscopic confirmation as the blood cells become hemolyzed and difficult to identify.

4.3. Clinical phase in vitro tests

4.3.1. Flea eggs – enumeration and ovicidal effects

Flea egg studies might be indicated, for example, to demonstrate that eggs produced by fleas after exposure to an IGR or IDI are infertile, or that a residual pulicidal treatment prevents flea-egg output (*i.e.* by killing newly-acquired fleas before they start to lay eggs). In both cases, residual activity is likely to decline with time and consequently data should include observations made at the end of the claimed period of persistency. Extraneous factors can influence egg-production and fertility and so statistical comparisons should be made with untreated controls. Eggs from non-treated hosts may also be collected as a source of material for conducting larvicidal studies (Section 4.3.2). Should eggs be required for this purpose from insecticide-treated animals, large infestations (~50–200 fleas per animal, dependent on the sensitivity of the fleas to the adulticide), may be necessary to generate adequate numbers. Fleas from untreated controls should be removed at the earliest opportunity on welfare grounds.

The eggs which are collected for up to ~24–96 h post-infestation provide for a determination of the ovicidal, larvicidal and prevention of new emergence activities. Infested animals are placed in individual cages with a mesh floor that allows eggs and other debris to pass through and accumulate on a paper-lined tray. Precautions are needed to prevent food, water and urine from soiling the tray. Sufficient eggs are collected up to ~24 h and counted under magnification using a needle or fine-tipped camel's hair brush to manipulate the eggs. Hatchability can be estimated by placing known numbers of eggs onto flea-larval rearing media in glass Petri dishes, incubating as described below and counting the number of resulting larvae. If there are sufficient eggs, two or more replicate dishes should be prepared as these biological processes are very sensitive to ambient effects. As flea larvae will sometimes eat unhatched eggs, enhanced accuracy can be obtained by gluing the eggs to the Petri dish lid so that they are out of reach of the hatched larvae that drop down into the medium below. This enables hatched and unhatched eggs to be counted by direct observation (Rust et al., 2002).

A model of assessing the efficacy of an IGR in an adulticide plus IGR combination has been published (Franc et al., 2007) wherein actively reproducing fleas (50 per cat, approximately 50:50 sex ratio) from untreated cats are transferred to treated cats. The eggs produced provides for a determination of the ovicidal, larvicidal and prevention of new flea emergence activities.

4.3.2. Flea larvicidal effects

Flea larvicidal activity may result from (1) interference with egg-development within the adult flea, (2) ingestion by larvae of active compound adhering to or contained within debris falling from a treated animal (such as skin scales or flea feces), and (3) direct transfer of active material from the animal's coat to in-contact surfaces (such as the pet's bedding). The basic experimental design described below can be modified to test any of the above circumstances and to accord with local established practices. The resulting *in vitro* 'efficacies' do not necessarily provide a quantitative indication of performance in the field as this may be affected by extraneous factors. Coat debris, for example, may be scattered over variable areas affecting the amount of active material falling on flea habitats.

Equal numbers of freshly collected eggs (usually ~20–100, if available) are counted into replicate glass Petri dishes containing flea-larval rearing medium (Moser et al., 1991; Richman et al., 1999). Control and test dishes will have weighed quantities of debris from untreated or treated animals, respectively, mixed into the medium. Stringent precautions are necessary to prevent transfer of active material to control dishes, work surfaces, instruments or equipment as trace amounts can often have measurable effects on larval development. The containers are incubated at ~21–29 °C and ~70–90% relative humidity. After ~72 h, the numbers of hatched larvae are recorded and the dishes replaced in the incubator. After a further 9 days, the contents of each dish are poured through a 40-mesh (425 μm) sieve to separate the pupae from the rearing medium. These are counted and placed in new containers with sealed lids to prevent the escape of emerging adult fleas. Approximately ~28–35 days after egg collection, the containers are removed from the incubators, shaken, and adult fleas immobilized with CO_2, by freezing or by pouring into hot water, before being counted. Remaining pupae are dissected and any normally developed adults added to the total flea count.

Comparisons of the mean numbers of hatched eggs, larvae, pupae or newly-emerged adult fleas in replicated control and treatment dishes may be made with the formula given in Section 4.2.1. Another approach is to calculate an 'efficacy' value based on the relative proportions of eggs that eventually give rise to adult fleas by application of the formula of Abbott (1925):

$$\text{Efficacy}\ \ (\%) = \frac{(C - T)}{C} \times 100$$

where C and T are the percentages of adult emergence for the control and treated dishes, respectively.

4.3.3. Tick in vitro tests

Several disposable pipette, vial or Petri dish methods can be used to evaluate the susceptibility of ticks to acaricides and establish lethal concentration values (Barnard et al., 1981; Prullage et al., 2011a, 2011c). The inner surface of the containers is treated with acaricide/acetone solutions and air-dried for ~24 h. Mature or immature ticks are placed into treated and control pipettes and incubated in a Bell jar for ~24 h or ~7 days (residual activity) at ~21 °C and ~90% relative humidity. The viability of the ticks based on movement, normal posture, and leg coordination are evaluated after the incubation period. Egg hatch reduction bioassays can be conducted on replete female ticks to evaluate the effect of acaricides and IGRs (Donahue et al., 1997).

4.4. Simulated home environment studies

In the case of pets that are mainly confined to the environs of the home, the longer-term clinical benefits of a pulicidal, IGR or IDI treatment are to a large extent attributable to the impact of that treatment on the off-host parasite population ('on-animal environmental control'). This comes about because stopping flea-egg output or inhibiting flea fertility will eventually lead to a general reduction in the number of host-seeking fleas in the domestic environment. This effect will be accelerated if there is any additional direct or indirect lethal effect on off-host life-cycle stages. Although not an essential step in product evaluation, laboratory models have been developed to demonstrate this accumulative impact on the total flea population using dogs (Blagburn et al., 1995) or cats (Fisher et al., 1996; Jacobs et al., 2001). In these studies, the pen (rather than the animal) is the experimental unit as animal flea-counts are used as an (imperfect) indicator of the magnitude of the total flea population (*i.e.* all on- and off-animal life-cycle stages). Animals are assigned to treated and untreated control groups, as indicated in Section 4.2.1, and placed in partly carpeted pens. Purpose built facilities may be constructed to allow animal's access to both indoor and outdoor components to further simulate the household environment. Fleas are placed on the animals and flea-larval rearing medium may be dusted on the carpet. The flea life cycle is thereby established in each pen. Animals can be treated and assessed for fleas at appropriate intervals, so that a comparison can be made of the build-up of the flea populations in the two sets of pens.

The simulated home environment model can be used to replicate two clinical scenarios: (1) prevention, and (2) management of existing flea problems. In the former case, animals in the treated group will receive their first treatment prior to initiation of the flea life cycle. In the second instance, the flea population is allowed to establish before the first treatment is given. Since this model evaluates the success of a flea population allowed to complete its life-cycle, the method of assessment of flea burdens should be designed to have least impact on the fleas. Options include thumb counting (using a rigorous and well-defined method); or remove-and-replace flea comb counting.

After a period of acclimation and buildup of flea infestations, flea counts are performed to (1) verify that the infestations have built up to sufficient levels, and (2) to provide data used to allocate animals/pens to groups on the basis of pre-treatment flea burdens. Since the experimental unit is the pen and animal, this design requires that animals are not relocated from their pens when the allocation procedures are performed.

Investigators should be aware that reinfestation pressure in these studies can become extremely high and they must be vigilant to protect the welfare of the untreated controls. If necessary, parasite challenge can be lowered by reducing the carpet size and/or combing off adult fleas from the animals, or raised, by introducing flea pupae or re-infesting the animals. Any such interventions should be documented, as they will of course influence the statistical interpretation of these data. Another approach is to allow for "salvage" treatments with a short-acting non-residual pulicide to be used when flea numbers reach a threshold (high) level on individual animals. The number of salvage treatments required can then be used as an inverse measure of the success of flea control, additional to flea count results. Flea counts in these studies can be high and it is acceptable to terminate flea counts at the threshold level (attributing a count of e.g. ">200"), perhaps in combination with salvage treatment.

4.5. Efficacy thresholds against flea and tick species

The acceptable level of efficacy for a label claim is an arbitrary figure that will depend on the intended use of the product (*i.e.* the control objective) and local regulatory requirements. Generally, a reduction in flea or tick numbers of 90% or more may be expected to provide immediate relief from irritation and blood-loss, to ameliorate clinical signs in hypersensitive cases and, in closed environments, to bring further benefits by reducing future parasite challenge. A higher level of control may, however, be indicated in particular circumstances such as the management of flea allergic dermatitis in highly sensitive individuals; to protect against dangerous paralysis-inducing ticks such as *I. holocyclus*; and to reduce transmission of pathogenic organisms.

Label claims should specify the time interval (min, h, days, weeks or months) over which the stated degree of efficacy is maintained.

4.6. Statistical analysis

Appropriate statistical methods should be used to analyze data. These should be documented in the protocol giving details of methodology, references and calculations. Any questions regarding the selected procedures should have been discussed with statisticians and if relevant, to the appropriate authority, in the early stages of protocol development.

5. Types of study

5.1. Dose determination studies

The purpose of this type of study is to determine the minimum effective point dose to be recommended. Ideally, the final formulation of inert ingredients (carriers, emulsifiers, etc.) with the exception of the varying concentrations of the active ingredient should be used. In exceptional cases, where justified, data generated with an equivalent formulation may be accepted. The effectiveness of the investigational product should be evaluated using appropriate studies such as the controlled test with the major parasite species (Sections 4.2.1–4.2.3). Ideally, four groups, each consisting of a sufficient number of animals to allow statistical analysis, may be administered at 0, 0.5, 1 and 2 times the anticipated recommended dose. Each group should harbor or be uniformly infested with adequate numbers of each species of ectoparasite. Single or mixed tick/flea infestations may be used. Groups should be held under the same study conditions and husbandry practices should be described in the protocol. The route and

technique of administration should be the same as that proposed for marketing. The time intervals for parasite counts should be justified, especially with regard to the biology of the ectoparasite(s). Data obtained for each parasite at the recommended dose in the dose determination study(s) should be acceptable as one of the dose confirmation studies provided that: the formulation used was equivalent to the formulation intended for marketing, the investigational product was applied according to the labeling, adequate infestation of ectoparasite species was established, and the number of study animals was adequate.

5.2. Dose confirmation studies

At least two controlled studies, wherever possible with different isolates of the target parasite, are recommended to demonstrate the efficacy of an investigational product against each ectoparasite species and stage of development as indicated on the investigational product labeling. An adequate number of treated and control animals are necessary for each study and group sizes should be justified. Studies should be conducted using the formulation and administration technique intended for marketing and using the recommended unit doses for the weight ranges of animals to be treated. Studies should be conducted with different laboratory isolates genetically enriched with parasites from field isolates (~every 3 years). The history of the isolate of flea and tick should be well documented. If resistance problems are widespread in an ectoparasite species for which efficacy is claimed, additional controlled dose confirmation studies may be necessary using defined resistant strains of that parasite, particularly if the new active ingredient has a similar mode of action or is a close chemical analog to that of existing ectoparasiticides.

Studies should also be undertaken to evaluate the effect, if any, of shampooing or wetting of animals on the effectiveness of the topically applied investigational product against the target flea and tick species. If studies permit, access to open runs allows the photostability of the investigational product on the animal to be evaluated.

5.3. Field studies

The off-host life-cycle stages of both fleas and ticks are affected by climatic fluctuations. Studies should be conducted with contemporary field populations of flea or tick species, and performed in different geographic and climatic regions. Clinical studies should be conducted at a time including the usual peak parasite season for the region. Some parasites such as *R. sanguineus* and *C. felis felis*, however, are affected to a lesser extent as they can thrive within heated housing. Field studies may be used as dose confirmation or confirmatory field efficacy and safety evaluations.

5.3.1. Field studies – fleas

Field studies involve privately owned pet animals living in households. The investigators must take into account the increased risks and responsibilities of conducting research studies in this environment.

Field studies should be conducted in the final stages of the development program after the investigational product has been used in pen studies confirming the efficacy of the product at the intended dose and formulation, its safety in the target animal has been confirmed, and the label treatment method has been decided.

By their nature, field studies involve a high degree of variability between cases and often inconsistencies in compliance and study management by owners. To maximize the quality of data it is important to recruit animals and households which, as much as possible, have a consistent history with respect to factors affecting flea populations; and which are likely to remain consistent with factors affecting the flea population apart from the imposition of the study treatment. Well-considered selection criteria should be employed to ensure the enrolment of cases which are acceptable with respect to key criteria such as flea burden, flea treatment history, duration of prior residency at premises (depending on the claim to be investigated), likelihood of exposure to flea burden (e.g., visiting other flea-infested sites), washing/hydrobathing, clipping and grooming and so on.

Clinical field studies are required primarily for follow-up evaluation of the performance of the investigational product as employed by the user in the field, and to gain experience on the efficacy and safety of the investigational product when applied under various clinical conditions. Depending on the target claim and product, studies should normally be performed for at least 1 month, but 2–3 months (~8–12 weeks) during the "flea season" in varying geographical regions, which should include, as scientifically indicated, regions with different husbandry practices, environmental conditions and ectoparasite resistance profile, are often indicated. Enrolled animals should have a confirmed naturally acquired infestation (minimum of 5 fleas) and include a variety of breeds kept under different conditions.

Carefully managed multi-centric, randomized and blinded (masked) studies may allow data to be pooled for this purpose. The recruited animals should be treated according to proposed/actual label directions. In multi-pet households, all dogs and cats should, wherever possible, be treated with the same product since the longer-term benefits of treatment are associated with a reduction in environmental infestation pressure (Section 4.4). However, only one animal per host species (1 dog and/or 1 cat, if different statistical analysis population) may serve as the representative of the household as the statistical unit. It should be noted, however, that not all dog products can be used safely on cats. In studies where the test product cannot be used on cats, justifiable options include: (1) selecting only households which have no cats; (2) selecting households with cats and treating them with another product, on the condition that the cats and dogs do not contact each other or share resting places; or (3) selecting households with cats and not treating them, on the condition that the cats and dogs do not live in the same environments (for example, dog lives only outside and the cat lives only inside).

Flea treatment history criteria require careful consideration. Owners will have used products with vastly differing efficacies; some of which have negligible efficacy or persistence, and others which have more than one month of high efficacy. This may influence both efficacy and safety. The flea treatment history of each enrolled animal needs careful consideration and it should justify the inclusion based on the efficacy characteristics of the particular products currently and previously used, and the consistency of use of these products.

Protocols also need to stipulate conditions during the study which promote management of a stable environment with respect to flea population influences (e.g. control over animal incursions and excursions and chemical use). The timing of flea counts will be determined by the nature of the label claim and the results of the earlier laboratory studies. Flea burdens should be quantified in a methodical standardized manner using a recognized method such as comb counting (Appendix A.2). Numerical counts are recommended for analytical purposes.

Other approaches such as area and thumb counting methods are quicker and easier but are markedly less accurate (Dryden et al., 1994; Heckenberg et al., 1994). Consequently, they should only be used for recruiting flea-infested animals.

The percentage of animals in a group on which no evidence of flea infestation is found is known as the "zero flea" or "flea free" value. While this is a useful descriptive measure of product performance, it should not be used as the only means of evaluating field efficacy.

Collected fleas can be identified to species level if required.

5.3.2. Field studies – ticks

Clinical field studies should be conducted during periods of peak seasonal activity of the tick species under investigation and should be performed in more than one geographical region. All enrolled animals should be tick infested and the initial intensity of infestation should be documented. Well-controlled field studies with a total number of individual animal cases to satisfy regional regulatory requirements should be available for efficacy confirmation and to evaluate administration in conjunction with other frequently used veterinary products, such as vaccines, anthelmintics, antibiotics, steroids, flea and tick control products, anesthetics, NSAIDs, antihistamines, alternative/herbal remedies, shampoos, and prescription diets. The animals should belong to a variety of breeds with different husbandry. Furthermore, animals exposed to a high risk of infestation (e.g. hounds, hunting dogs, etc.) should be included if possible. The final unit dose formulation intended for marketing should be used at the recommended dose and route of administration.

The number of ticks per host animal should be determined by a reliable standardized method (e.g. by use of a comb, thumb counting and/or forceps). All or a subsample of ticks may be collected to identify to species level and then numerically counted by species.

5.4. Selection, preparation and welfare of animals

The choice of study animals for laboratory studies should be justified based on breed characteristics, sex, age, size, behavior, husbandry, hair coat length and source. Study animals should be properly vaccinated and treated for internal parasites including heartworm prevention medication in heartworm endemic areas as long as the product does not have any flea or tick efficacy that may adversely affect the establishment of flea or tick infestations and confound interpretation of the efficacy results. For studies utilizing paralysis-inducing ticks, hyperimmune dogs from commercial suppliers and lower tick infestation rates are recommended for animal welfare reasons. Animals treated with experimental or commercial topical or systemic endo- and/or ectoparasiticides and/or endectocides should not be considered for studies until an adequate time has passed since treatment was given. The number of animals should be large enough to permit good statistical evaluation. The validity of the results of the testing program is directly related to the degree of variability within the study. Therefore, increasing the number of study animals increases the reliability of the study results. In general, a minimum of 6 animals per group is recommended for laboratory based dose determination and confirmation studies. Six animals is typically the minimum number to have adequate statistical power to determine significant differences against the control at a 90% level of efficacy. Higher numbers of animals can be used and should be used in products with high animal to animal variability in flea and tick counts.

Candidate animals for laboratory studies may be infested with ~50–100 cat fleas and/or target species ticks ($\leq$50) per animal to determine their suitability as a host for these parasites and their ability to tolerate the effects of infestation. Such pre-study parasite counts may serve to select animals for a study and to allocate them to homogenous groups based on the criterion "capable to harbor flea and/or tick burdens" (Section 4.2.1). Any animal displaying more than minimal signs of flea-bite allergy or reaction to tick-bites should be excluded from the study. Animals with hair coat characteristics that do not allow thorough and complete comb counting should not be considered for use. Animals may be washed with water and a mild non-insecticidal shampoo ~7–14 days prior to treatment. Bathing may facilitate combing during flea counts, but may also remove important skin oils that could influence the duration of parasiticide activity. All animals selected for study should be weighed prior to treatment (between ~Day −3 and 0) in order to calculate the treatment dosage and to serve as another criterion for allocation purposes, if necessary.

The welfare of animals used in efficacy studies must be a prime consideration. Proper supervision by qualified personnel must ensure parasite infestations do not reach levels causing undue stress, and such studies should not extend for unnecessarily prolonged periods. Individual animals unduly affected should be withdrawn from the study and treated. Where treatments under evaluation are obviously not achieving a satisfactory control level, studies should be terminated.

5.5. Allocation of animals

Flea and tick numbers on dogs and cats are influenced by grooming behavior, which varies considerably between species and individuals. In controlled laboratory tests, a pre-allocation infestation with fleas and/or ticks is therefore necessary to determine the parasite-carrying capacity of each animal (Section 5.4). Animals can then be ranked by pre-allocation flea or tick counts and, if necessary, by sex or body weight to form replicates (blocks). Within each replicate, animals are then randomly allocated to treated or control groups. If dogs are co-infested with both fleas and ticks in the same study then either parasite counts can be used for allocation purposes. In field studies, infested animals can be allocated completely at random to treatment or control groups, or randomly based on order of recruitment within each study site.

5.6. Housing of animals

In laboratory studies, all animals should be housed or confined under similar environmental and husbandry conditions. If cross-contamination with active compound is a potential problem, for example in some IGR or IDI studies, treated and control animals may be housed in separate areas or rooms, providing that ambient conditions are identical. Otherwise, pen allocation should be random or determined by study and/or statistical design. A diagram of the building and placement of the animals should be provided. Water and food sources should be free of any potential contaminants that might interfere with the study and provided to maintain proper body weight and nutritional requirements. For animals housed over long periods of time, exercise and socializing routines should be included to meet animal welfare requirements. Care needs to be taken in the selection of plastic toys, if supplied for environmental enrichment, as many contain phthalic acid ester plasticizing agents with documented arthropod repellency properties (Hartley and West, 1969). Caging and runs for the housing of animals should be of adequate size to comply with regulatory and local animal welfare guidelines. Health observations should be conducted for each animal starting on the first day of acclimatization or in accordance with the protocol requirements and continuing until the end of the in-life animal phase of the study.

Conflict of interest

Authors Alan A. Marchiondo and Douglas Rugg are current employees of Zoetis inc. (formerly Pfizer Animal Health), USA, Peter A. Holdsworth is a current employee of Animal Health Alliance (Australia) Ltd., Australia, Leon F. Fourie is a current employee of ClinVet International (Pty) Ltd., Republic of South Africa, and Klaus Hellmann is the current general manager of Klifovet AG, Germany. Daniel E. Snyder is a current employee of Elanco Animal Health, Greenfield, IN, USA, and Michael W. Dryden is a University Distinguished Professor at Kansas State University, Manhattan, KS, USA. The authors were directly involved in the preparation and review of the manuscript, and there were no conflicting interests that may have biased the information reported herein.

Acknowledgements

The authors wish to acknowledge Drs. Robert G. Arther (Bayer Health Care, USA), Fred Beugnet (Merial, France), Byron L. Blagburn (Auburn University, USA), Jacques Guillot (Alfort, France), Dennis E. Jacobs (Emeritus Professor of Veterinary Parasitology, Royal Veterinary College, UK), Marcelo E. Labruna (Universidade de São Paulo, Brazil), Dorothee Stanneck (Bayer AG, Germany), Jozef Vercruysse (University of Gent, Belgium), and Andrew von Berky (Von Berky Veterinary Services, Australia) for commenting and reviewing this manuscript.

Appendix A.

A.1. Methods of flea and tick infestation

Flea infestations should be established using newly emerged unfed adults from an established colony maintained on cats or dogs. Fleas obtained from an artificial membrane system should not be used for laboratory studies, as they will have become adapted to that feeding system. Adult ticks are recommended for infestation studies and should be unfed and relatively uniform in age. As a general rule, animals should be infested with ~50–100 fleas and/or ~25–50 ticks. The sex ratio of the fleas should be ~1:1 or weighted in favor of females ($\geq$2:1), especially for the evaluation of IGRs or IDIs. The sex ratio for ticks should be approximately balanced, but appropriate for the biology of the species, *i.e.*, *Ixodes*. Flea and tick infestation is done by spreading the parasites over the body of the animals but avoiding the application site of any topical product. Tick infestations may be facilitated by holding the vials of ticks in the test area of the control animals for ~12–18 h prior to infestation and by lining the grated flooring of the test animal cage with carpeting during the infestation period (~12–18 h) (Marchiondo, personal communication). Anesthesia and/or Elizabethan collars may be used to facilitate infestation of the animal and for testing the repellency activity of a compound. Anesthesia and collars may also increase the tick infestation rate. Elizabethan collars may be useful in facilitating and maintaining infestations on cats. For specific design, ticks may be deposited on the bottom of a crate and not directly on the animal. Attachment rates of *I. ricinus* can be increased by using a higher number of ticks (60) and a lower number of males compared to females (~25:75) as male *I. ricinus* attach only briefly or not at all (Kiszewski et al., 2001; Kužner et al., 2012) but the presence of male *I. ricinus* is sufficient to stimulate female tick attachments and to achieve greater attachment rates. The source and propagation history of fleas and ticks used should be documented and their known susceptibility or resistance to major classes of ectoparasiticide/endectocide documented. Recipient animals should be free of fleas or ticks prior to infestation.

A.2. Flea counting methods

The accurate assessment of the number of fleas infesting an animal is paramount to generating accurate efficacy data, but is difficult to achieve as fleas can move through the pelage very quickly. Methods for determining the number of adult fleas on an animal include: (1) comb counts in which an animal is combed with a fine-toothed comb (~11–13 teeth per centimeter) over the entire body for a set time (usually 5–20 min depending on the accuracy required) or until no further fleas are recovered after 50 strokes (Dryden et al., 1994; Marchiondo, personal communication), (2) total flea removal in which the animal is sprayed thoroughly with an alcohol-based pyrethrin flea spray, or dosed orally with nitenpyram, and then brushed or combed until all fleas are removed, using a non-resistant flea population to one of the actives (Baker and Farver, 1983; Dryden, 1992), and (3) thumb- or area counting in which fleas are counted/estimated by visual inspection over a set time period while the hair is parted manually at predetermined locations on the animal (Fox et al., 1969). Comb-counting has been shown to be more sensitive than the thumb-counting method (Gregory et al., 1995). Personnel involved in combing and counting the ectoparasite must be trained, experienced, and masked to treatment. Changing protective clothing between treated and control groups is highly recommended as well as the use of separate combs for each group.

A.3. Tick counting methods

Tick counting methods include locating attached ticks, preferably with gloved fingers, and counting while they are still in place (thumb counting) or removing attached ticks, preferably with gloved fingers, forceps or a flea comb. In the latter case, the animal's entire body is combed with a fine-toothed (~11–13 teeth per centimeter) comb for a set time-period such as 5–20 min or until no further ticks are recovered after 50 strokes. The ears, pinna, pinnal fold, and interdigital spaces of dogs should be carefully checked for ticks. A flea comb is particularly effective in recovering Brown Dog (*R. sanguineus*) and American Dog (*D. variabilis*) ticks that are not visible or apparent by palpation (Marchiondo, personal communication).

Appendix B.

A number of regulatory authorities have issued direction regarding their specific requirements. The following section summarizes sources available at the time of publication.

B.1. Efficacy

Guidance documents for evaluating the efficacy of parasiticides for treatment and control of fleas and ticks on dogs and cats have been adopted by the EMA (EMEA/CVMP/005/2000-Rev 2-as modified 12 November 2007 and Questions and Answers on the CVMP Guideline on "Testing and evaluation of the efficacy of antiparasitic substances for the treatment and prevention of tick and flea infestations in dogs and cats (EMEA/CVMP/055/00-Rev 2, March 2009, November 2011). Another revision of EMEA/CVMP/005/2000-Rev 2 is currently on going.

The USA-EPA has published evaluation guidelines that address externally applied parasiticides not internally absorbed by the treated pet, have been revised and harmonized into a single set of guidelines by the Office of Prevention, Pesticides and Toxic Substances (OPPTS 810.3300, Product Performance Test Guidelines, Treatments to Control Pests of Humans and Pets, EPA 712-C-98-411, March 1998, to minimize variations among the testing procedures that must be performed to meet the data requirements of the EPA under the Toxic Substances Control Act (TSCA, 15 U.S.C. 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA, 7 U.S.C. 136, *et seq.*). The Australian Pesticides and Veterinary Medicines Authority has published two guidelines: No. 16 Guidelines for acceptable claims based on currently approved constituents for parasiticides used on cats, dogs and ornamental caged birds, May 1996, and No. 17 Guidelines for small animal ectoparasiticide efficacy submission, July 1996. The CVM has not published formal guidelines for systemically active flea and tick parasiticides. However, such products are regulated as New Animal Drug Applications (NADA) under the Code of Federal Regulations FDA 21 CFR Part 514, April 1, 2004 (http://www.fda.gov/cvm/). Basically, the FDA requirements parallel the guidelines of EPA with respect to testing procedures and efficacy evaluation criteria with the additions of a clinical field efficacy study and target animal safety/tolerance studies. However, the FDA often requires a concurred protocol that specifies the use of only the lower half of the unit dose range in pivotal dose confirmation studies. In Japan, the Industrial Chemicals Control Law (corresponding to TSCA in the USA) and the Agricultural Chemicals Regulation Law (corresponding to FIFRA in the USA) primarily address flea and tick parasiticides.

B.2. Animal welfare

All studies must comply with existing country guidelines governing animal studies of the countries where such studies are conducted such as the United States Department of Agriculture's USDA Animal Welfare Act (9 CFR), Public Health Service Policy on the Humane Care and Use of Laboratory Animals, 1966, Institutional Animal Care and Use Committee (IACUC), Directive 2010/63/EU of the European Parliament and of the Council (22 September 2010) on the Protection of Animals Used for Scientific Purposes, Good Clinical Practices, (VICH GL9, 2000), and Good Target Animal Study Practices: Clinical Investigators and Monitors, FDA, CVM, May 1997.

References

Abbott, W.S., 1925. A method of comparing the effectiveness of an insecticide. J. Econ. Entomol. 18, 265–267.

Anon., 1971. Recommended methods for the detection and measurement of resistance of agricultural pests to pesticides. Tentative method for larvae for the cattle tick, *Boophilus* spp. FAO Plant Protect. B., 15, FAO Method No. 19.

Apanaskevich, D.A., Horak, I.G., Camicas, J.L., 2007. Redescription of *Haemaphysalis (Rhipistoma) elliptica* (Koch, 1844), an old taxon of the *Haemaphysalis (Rhipistoma)* leachi group from east and

southern Africa, and of *Haemaphysalis (Rhipistoma) leachi* (Audouin, 1826) (Ixodida, Ixodidae). Onderstepoort J. Vet. Res. 74, 181–208.
Baker, N.F., Farver, T.B., 1983. Failure of brewer's yeast as a repellent to fleas on dogs. J. Am. Vet. Med. Assoc. 183, 212–214.
Barnard, D.R., Jones, B.G., Rodgers, G.D., Mount, G.A., 1981. Acaricide susceptibility in the lone star tick: assay techniques and baseline data. J. Econ. Entomol. 74, 466–469.
Bissinger, B.W., Roe, R.M., 2010. Tick repellents: past. present and future. Pestic. Biochem. Physiol. 96, 63–79.
Blagburn, B.L., Hendrix, C.M., Vaughn, J.L., Lindsay, D.S., Barnett, S.H., 1995. Efficacy of lufenuron against developing stages of fleas (*Ctenocephalides felis felis*) in dogs housed in simulated home environments. Am. J. Vet. Res. 56, 464–467.
Cadiergues, M.C., Hourcq, P., Cantaloube, B., Franc, M., 2000. First bloodmeal of *Ctenocephalides felis felis* (Siphonaptera: Pulicidae) on cats: time to initiation and duration of feeding. J. Med. Entomol. 37 (4), 634–636.
Carlotti, D.N., Jacobs, D.E., 2000. Therapy, control and prevention of flea allergy dermatitis in dogs and cats. Vet. Dermatol. 11 (2), 83.
Carroll, J.F., Solberg, V.B., Klun, J.A., Kramer, M., Debboun, M., 2003. Comparative activity of Deet and AI3-37220 repellents against the tick *Ixodes scapularis* and *Amblyomma americanum* (Acari: Ixodidae) in laboratory bioassays. J. Med. Entomol. 41, 249–254.
Coles, G.C., Bauer, C., Borgsteede, F.H.M., Klei, T.R., Taylor, M.A., Waller, P.J., 1992. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) methods for the detection of anthelmintic resistance in nematodes of veterinary importance. Vet. Parasitol. 44, 35–44.
Dautel, H., 2004. Test systems for tick repellents. Int. J. Med. Microbiol. 293 (Suppl. 37), 182–188.
Dautel, H., Kahl, O., Siems, K., Oppenrieder, M., Muller-Kuhrt, L., Hilker, M., 1999. A novel test system for detection of tick repellents. Entomol. Exp. Appl. 91, 431–441.
Dautel, H., Cranna, R., 2006. Assessment of repellency and mortality of imidacloprid plus permethrin spot-on solution against *Ixodes holocyclus* using a moving object bioassay. Aust. Vet. Pract. 36, 138–147.
Dobson, R.J., Sangster, N.C., Besier, R.B., Woodgate, R.G., 2009. Geometric means provide a biased efficacy result when conducting a faecal egg count reduction test (FECRT). Vet. Parasitol. 161, 162–167.
Donahue, W.A., Teel, P.D., Strey, O.F., Meola, R.W., 1997. Pyriproxyfen effects on newly engorged larvae and nymphs of the lone star tick (Acari: Ixodidae). J. Med. Entomol. 34 (2), 206–211.
Drummond, R.O., Crust, S.F., Trevino, J., Gladney, I., Graham, W.J.O.H., 1973. *Boophilus annulatus* and *B. microplus*: laboratory studies of insecticides. J. Econ. Entomol. 66, 130–133.
Dryden, M.W., 1992. Differential activity of cythioate against female and male *Ctenocephalides felis* on cats. Am. J. Vet. Res. 53, 801–802.
Dryden, M., Gaafar, S., 1991. Blood Consumption by the Cat Flea, *Ctenocephalides felis felis* (Siphonaptera: Pulicidae). J. Med. Entomol. 28 (3), 394–400.
Dryden, M.W., Rust, M.K., 1994. The cat flea: biology, ecology and control. Vet. Parasitol. 52, 1–19.
Dryden, M.W., Boyer, J.E., Smith, V., 1994. Techniques for estimating on-animal populations of *Ctenocephalides felis* (Siphonaptera: Pulicidae). J. Med. Entomol. 21, 631–634.
Dryden, M.W., Payne, P.A., 2004. Biology and control of ticks infesting dogs and cats in North America. Vet. Ther. 5 (2), 139–154.
Duncan, J.L., Abbott, E.M., Arundel, J.H., Eysker, M., Klei, T.R., Krecek, R.C., Lyons, E.T., Reinemeyer, C., Slocombe, J.O.D., 2002. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.): second edition of guidelines for evaluating the efficacy of equine anthelmintics. Vet. Parasitol. 103, 1–8.
Düwel, D., Barth, D.W., Battle, E.G., Berger, H., Stewart, T.B., Theodorides, V.J., 1986. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) guidelines for evaluating the efficacy of anthelmintics in swine. Vet. Parasitol. 21, 69–82.
Endris, R.G., Cooke, D., Amodie, D., 2002. Repellency and efficacy of 65% permethrin and selamectin against *Ixodes ricinus*. Vet. Ther. 3 (1), 64–71.
Estrada-Pena, A., Ascher, F., 1999. Comparison of an amitraz-impregnated collar with topical administration of fipronil for prevention of experimental and natural infestations by the brown dog tick (*Rhipicephalus sanguineus*). J. Am. Vet. Med. Assoc. 214, 1799–1803.
Estrada-Peña, A., Bouattour, Camicas, J.L., Walker, A.R., 2004. Ticks of Domestic Animals in the Mediterranean Region: A Guide to Identification of Species. University of Zaragosa, Spain.
Finney, D.J., 1971. Probit Analysis. Cambridge University Press, Cambridge.
Fisher, M.A., Jacobs, D.E., Hutchinson, M.J., Dick, I.G., 1996. Evaluation of flea control programmes for cats using fenthion and lufenuron. Vet. Rec. 138 (4), 79–81.
Fox, I., Rivera, G.A., Bayona, I.G., 1969. Controlling cat fleas with dichlorvos-impregnated collars. J. Econ. Entomol. 62, 1246–1249.
Franc, M., Beugnet, F., Vermot, S., 2007. Efficacy of Fipronil–(S)-Methoprene on fleas, flea egg collection, and flea egg development following transplantation of gravid fleas onto treated cats. Vet. Ther. 8, 285–295.
Gregory, L.M., Zakson, M., Endris, R.G., Shoop, W.L., 1995. A further comparison of the thumb-counting and comb-counting techniques used to determine *Ctenocephalides felis* infestation levels on dogs. Vet. Parasitol. 56, 349–352.
Halos, L., Baneth, G., Beugney, F., Bowman, A.S., Chomel, B., Farkas, R., Franc, M., Guillot, J., Inokuma, H., Kaufman, R., Jongejan, F., Joachim, A., Otranto, D., Pfister, K., Pollmeier, M., Sainz, A., Wall, R., 2012. Defining the concept of 'tick repellency' in veterinary medicine. Parasitology 139, 419–423.
Hartley, G.S., West, T.F., 1969. Repellents, Attractants and other Behaviour Controlling Compounds. In Chemicals of Pest Control. Pergamon Press, London.
Heckenberg, K., Costa, S.D., Gregory, L.M., Michael, B.F., Endris, R.G., Shoop, W.L., 1994. Comparison of thumb-counting and comb-counting methods to determine *Ctenocephalides felis* infestation levels on dogs. Vet. Parasitol. 53, 153–157.
Holdsworth, P.A., Conway, D.P., McKenzie, M.E., Dayton, A.D., Chapman, H.D., Mathis, G.F., Skinner, J.T., Mundt, H.C., Williams, R.B., 2004. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) guidelines for evaluating the efficacy of anticoccidial drugs in chickens and turkeys. Vet. Parasitol. 121, 189–212.
Holdsworth, P.A., Vercruysse, J., Rehbein, S., Peter, R., De Bruin, C., Letonja, T., Green, P., 2006a. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) guidelines for evaluating the efficacy of ectoparasiticides against biting and nuisance flies on ruminants. Vet. Parasitol. 136, 3–13.
Holdsworth, P.A., Vercruysse, J., Rehbein, S., Peter, R., De Bruin, C., Letonja, T., Green, P., 2006b. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) guidelines for evaluating the efficacy of ectoparasiticides against myiasis causing parasites on ruminants. Vet. Parasitol. 136, 15–28.
Holdsworth, P.A., Kemp, D., Green, P., Peter, R.J., De Bruin, C., Jonsson, N.N., Letonja, T., Rehbein, S., Vercruysse, J., 2006c. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) guidelines for evaluating the efficacy of acaricides against ticks on ruminants. Vet. Parasitol. 136, 29–43.
Holdsworth, P.A., Vercruysse, J., Rehbein, S., Peter, R., Letonja, T., Green, P., 2006d. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) guidelines for evaluating the efficacy of ectoparasiticides against biting lice, sucking lice and sheep ked on ruminants. Vet. Parasitol. 136, 45–54.
Jacobs, D.E., Arakawa, A., Coutrney, C.H., Gemmell, M.A., McCall, J.W., Myers, G.H., Vanparijs, O., 1994. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) guidelines for evaluating the efficacy of anthelmintics for dogs and cats. Vet. Parasitol. 52, 179–202.
Jacobs, D.E., Hutchinson, M.J., Stanneck, D., Mencke, N., 2001. Accumulation and persistence of flea larvicidal activity in the immediate environment of cats treated with imidacloprid. Med. Vet. Entomol. 15 (3), 342–345.
Jongejan, F., Fourie, J.J., Chester, S.T., Manavella, C., Mallouk, Y., Pollmeier, M.G., Baggott, D., 2011. The prevention of transmission of *Babesia canis canis* by *Dermacentor reticulatus* ticks to dogs using a novel combination of fipronil, amitraz and (S)-methoprene. Vet. Parasitol. 179, 343–350.
Kiszewski, E., Matuschka, F.R., Spielmann, A., 2001. Mating strategies and spermiogenesis in ixodid ticks. Annu. Rev. Entomol. 46, 167–182.
Kužner, J., Turk, S., Fourie, J.J., Grace, S., Marchiondo, A.A., Rugg, D., 2012. Efficacy of a novel fipronil spot-on for the treatment and control of induced infestations of adult cat fleas (*Ctenocephalides felis*) and castor bean ticks (*Ixodes ricinus*) on cats. Parasitol. Res. 111, 1–8, Online First.
Marchiondo, A.A., 1993. Safe and effective flea control for cats. Vet. Tech. 14 (4), 235–245.
Marchiondo, A.A., Riner, J.L., Sonenshine, D.E., Rowe, K.F., Slusser, J.H., 1990. Ovicidal and larvicidal modes of action of fenoxycarb against the cat flea (Siphonaptera: Pulicidae). J. Med. Entomol. 27, 913–921.
Marchiondo, A.A., Holdsworth, P.A., Green, P., Blagburn, B.L., Jacobs, D.E., 2007. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) guidelines for evaluating the efficacy of parasiticides for the treatment, prevention and control of flea and tick infestations on dogs and cats. Vet. Parasitol. 143, 332–344.

McCall, J.W., Baker, C.F., Mather, T.N., Chester, S.T., McCall, S.D., Irwin, J.P., Young, S.L., Cramer, L.G., Pollmeier, M.G., 2011. The ability of a topical novel combination of fipronil, amitraz and (S)-methoprene to protect dogs from *Borrelia burgdorferi* and *Anaplasma phagocytophilum* infections transmitted by *Ixodes scapularis*. Vet. Parasitol. 179, 335–342.

McCoy, C., Broce, A.B., Dryden, M.W., 2008. Flea blood feeding patterns in cats treated with oral nitenpyram and the topical insecticides imidacloprid, fipronil and selamectin. Vet. Parasitol. 156 (3–4), 293–301.

Moser, B.A., Koehler, P.G., Patterson, R.S., 1991. Effect of larval diet on cat flea (Siphonaptera: Pulicidae) developmental times and adult emergence. J. Econ. Entomol. 84, 1257–1261.

Powers, K.G., Wood, I.B., Eckert, J., Gibson, T., Smith, H.J., 1982. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) guidelines for evaluating the efficacy of anthelmintics in ruminants (bovine and ovine). Vet. Parasitol. 10, 265–284.

Prullage, J.B., Cawthrone, E.G., Le Hir de Fallois, L.P., Timmons, P.R., 2011a. Synergism between fipronil and amitraz in a *Rhipicephalus sanguineus* tick residual contact test. Exp. Appl. Acarol. 54 (2), 173–176.

Prullage, J.B., Hair, J.A., Everett, W.R., Yoon, S.S., Cramer, L.G., Franke, S., Cornelison, K., Hunter, J.S., 2011b. The prevention of attachment and the detachment effects of a novel combination of fipronil, amitraz and (S)-methoprene for *Rhipicephalus sanguineus* and *Dermacentor variabilis* on dogs. Vet. Parasitol. 179 (4), 311–317.

Prullage, J.B., Tran, H.V., Timmons, P., Harriman, J., Chester, S.T., Powell, K., 2011c. The combined mode of action of fipronil and amitraz on the motility of *Rhipicephalus sanguineus*. Vet. Parasitol. 179, 302–310.

Richman, D.L., Koehler, P.G., Brenner, R.J., 1999. Spray-dried bovine blood: an effective laboratory diet for *Ctenocephalides felis felis* (Siphonaptera: Pulicidae). J. Med. Entomol. 36, 219–221.

Rust, M.K., Reierson, D.A., 1989. Activity of insecticides against the preemerged adult cat flea in the cocoon (Siphonaptera: Pulicidae). J. Med. Entomol. 21, 727–731.

Rust, M.K., 2005. Advances in the control of *Ctenocephalides felis* (cat flea) on cats and dogs. Trends Parasitol. 21 (5), 232–236.

Rust, M.K., Waggoner, M., Hinkle, N.C., Mencke, N., Hanson, O., Vaughn, M., Dryden, M.W., Payne, P., Blagburn, B.L., Jacobs, D.E., Bach, T., Bledsoe, D., Hopkins, T., Mehlhorn, H., Denholm, I., 2002. Development of a larval bioassay for susceptibility of cat fleas (Siphonaptera: Pulicidae) to imidacloprid. J. Med. Entomol. 39 (4), 671–674.

Schapiro, S.J., Everitt, J.I., 2006. Preparation of animals for use in the laboratory: issues and challenges for the Institutional Animal Care and Use Committee (IACUC). ILAR J. 47, 370–375.

Schwantes, U., Dautel, H., Jung, G., 2008. Prevention of infectious tick-borne diseases in humans: comparative studies of the repellency of different dodecanoic acid-formulations against *Ixodes ricinus* ticks (Acari: Ixodidae). Parasit. Vectors 1 (1), 8–19.

Stone, B.F., Haydock, K.P.A, 1962. Method for measuring the acaricide susceptibility of the cattle tick *Boophilus microplus* (Can.). Bull. Entomol. Res. 53, 563–578.

Tatchell, R.J., 1974. The use of the packet test for detecting acaricide resistance in African ticks. In: Proceedings of the 3rd International Congress of Parasitology, Volume 2, pp. 993–994.

Vercruysse, J., Rehbein, S., Holdsworth, P.A., Letonja, T., Peter, R., 2006. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) guidelines for evaluating the efficacy of ectoparasiticides against (mange and itch) mites on ruminants. Vet. Parasitol. 136, 55–66.

Vet, L.E.M., Van Lanteren, J.C.V., Heymans, M., Meelis, E., 1983. An airflow olfactometer for measuring olfactory responses. Phys. Entomol. 8, 97–106.

Walker, J.B., Keirans, J.E., Horak, I.G., 2000. The Genus *Rhipicephalus* (*Acari Ixodidae*): A Guide to the Brown Ticks of the World. Cambridge University Press, Cambridge.

Wang, C., Mount, J., Butler, J., Gao, D., Jung, E., Blagburn, B.L., Kaltenboeck, B., 2012. Real-time PCR of the mammalian hydroxymethylbilane synthase (HMBS) gene for analysis of flea (*Ctenocephalides felis*) feeding patterns on dogs. Parasit. Vectors 5, 1–8.

Wood, I.B., Amaral, N.K., Bairden, K., Duncan, J.L., Kassai, T., Malone Jr., J.B., Pankavich, J.A., Reinecke, R.K., Slocombe, O., Taylor, S.M., Vercruysse, J., 1995. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) guidelines for evaluating the efficacy of anthelmintics in ruminants (bovine, ovine, caprine). Vet. Parasitol. 58, 181–213.

Yazwinski, T.A., Chapman, H.D., Davis, R.B., Letonja, T., Pote, L., Maes, L., Jacobs, D.E., 2003. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) guidelines for evaluating the effectiveness of anthelmintics in chickens and turkeys. Vet. Parasitol. 116, 159–173.

Glossary of terms

Acaricide: an agent that kills members of the order Acarina, specifically ticks and mites.

Anti-feeding effect: interference with the natural process of feeding, avoiding any blood meal.

Blinding or masking: a procedure to reduce potential study bias in which designated personnel are kept uninformed ("blinded" or "masked") of the treatment assignments in a study.

Control: the overall beneficial effect of a clinical intervention. For example, in the case of a pulicidal compound, it might be the combined effect of the ability of the product to kill fleas on the animal and the progressive reduction in environmental infestation pressure thereby achieved.

Dose determination study: a controlled study, utilizing animals in pen facilities, to determine the optimum effective dose against the target parasite(s). This type of study is also used to determine the least susceptible parasite species that is likely to be present in the field.

Dose confirmation study: a controlled study, utilizing animals usually in pen facilities, to confirm the effective dose against the target parasite(s) as determined in the dose determination study. This type of study is also used to define the persistent efficacy against the target parasite(s) following re-infestation.

Efficacy (=therapeutic efficacy): the actual therapeutic response produced by a product against an ectoparasite as determined in a number of controlled studies using infested animals.

Engorged tick: an adult female ixodid ('hard tick') that has taken a single large blood meal to produce a conspicuous filling of the alloscutum. (Prior to feeding they are known as 'unengorged ticks').

Immediate or curative efficacy: the therapeutic effect of a product on a resident (pre-existing) ectoparasite population within a defined period from treatment (for example, ~4 up to 72 h).

Knockdown: the immediate initial action of some chemicals on the nervous system of arthropods, characterized by inability to move in a coordinated fashion (immobilization). Knockdown may be followed by recovery or by death of the parasite.

On-animal environmental control: the application of therapeutic substances (for example, insecticides, IGRs or IDIs) to an animal for the purpose of progressively reducing or eliminating the numbers of host-seeking off-host life-cycle stages in that animal's close environment.

Persistent (residual or sustained) efficacy: extended therapeutic activity of a product measured in days or weeks after the treatment day. Sometimes referred to as prophylactic or protective effect.

Prevention: the action of stopping ectoparasitic infestations from establishing, usually for a defined post-treatment time interval.

Protective period: the time period, expressed in days or weeks after the treatment, that a product will prevent re-infestation of the animal by the ectoparasite. Sometimes referred to as the prophylactic period or the persistent efficacy period.

Pulicide: an agent that kills members of the family Pulicidae, specifically fleas, used for flea control.

Repellency (sensu stricto): Characterized by an irritant effect, causing the tick to move away from the treated animal leading it to fall off soon after contact with its hair coat (Halos et al., 2012).

Speed of kill (action): the time after therapeutic treatment for a product to kill a stated percentage of the parasite population. It is determined by the rate at which the active material covers the body surface, the rate at which the active compound is taken up by the parasite, the mode of action of the toxicant and the susceptibility of the target organism.

Tick expellency (=repellency "sensu lato"): causing ticks to fall off the host animal by disrupting the mechanisms of attachment, *i.e.* either by causing detachment of already attached ticks or by preventing attachment of new infesting ticks (Halos et al., 2012).

Treatment (Therapeutic treatment): an intervention designed to kill (speed of kill or action) and/or eliminate parasites already on the animal at the time of treatment, *i.e.* an existing infestation.

'Zero flea stage' or "flea free stage": Determination by a reliable evaluation method that no evidence of flea infestation (adults, eggs or flea feces) is present on an animal at an evaluation time-point. The "zero flea value" for a treatment group is a useful descriptive measure of product performance in clinical field studies but should not be used as the only means of evaluating field efficacy.