Title

Acaricidal activity of fluralaner against O*rnithodoros moubata* and O*rnithodoros erraticus* argasid ticks evaluated through *in vitro* feeding.

Authors

Pérez-Sánchez, R.; Oleaga, A.

Author's affiliations:

Parasitología Animal, Instituto de Recursos Naturales y Agrobiología de Salamanca (IRNASA, CSIC), Cordel de Merinas, 40-52, 37008 Salamanca, Spain.

Author's e-mail address:

ricardo.perez@irnasa.csic.es; ana.oleaga@irnasa.csic.es

* **Corresponding author**:

Ricardo Pérez-Sánchez

Parasitología Animal, IRNASA, CSIC,

Cordel de Merinas, 40-52, 37008 Salamanca, Spain.

Tel.: +34 923219606;

fax: +34 923219609.

e-mail address: ricardo.perez@irnasa.csic.es

Abstract

Ornithodoros erraticus and *Ornithodoros moubata* are argasid tick vectors that transmit severe diseases such as African swine fever and human relapsing fever. Elimination of the synanthropic populations of these vectors would facilitate the control of these diseases. Fluralaner is a novel isoxazoline that selectively blocks the GABA- and glutamate-gated channels, providing potent insecticidal and acaricidal activity. The aim of the current study was to provide quantitative data on the susceptibility of males, females and third nymphal instar of *O. erraticus* and *O. moubata* to fluralaner through *in vitro* feeding exposure. Fluralaner activity against these developmental stages and species was assessed by feeding the ticks on ovine blood medicated with decreasing fluralaner concentrations between 1 and 10^{-8} µg/mL. Tick mortality was measured at 4, 24 and 48 h and 1, 2 and 3 weeks post-feeding. Tests included solvent-treated and untreated blood controls. Fluralaner was extremely active against *O. erraticus,* with mean lethal concentrations 50 (LC_{50}) and 95 (LC_{95}) of 2.0 x 10^{-8} and 5.4 x 10^{-8} µg/mL, respectively. Fluralaner was also highly active against *O. moubata*, showing a mean LC_{50} of 1.5 x 10^{-6} µg/mL and a mean LC_{95} of 1.8 x 10^{-3} µg/mL. In the latter species, the most susceptible life stages were the females (LC_{95} 1.4 x 10^{-4} µg/mL). Fluralaner demonstrated potent acaricidal activity against all developmental stages of *O. erraticus* and *O. moubata* tested, in the first 48 h after *in vitro* feeding. Therefore, fluralaner has the potential to provide very high acaricidal efficacy to multiple argasid tick species *via* feeding exposure and could be included as an acaricidal agent in integrated programmes for the control of argasid tick vectors and argasid tick-borne diseases.

Keywords

Ornithodoros erraticus, *Ornithodoros moubata*, argasids, Fluralaner, acaricidal activity.

1. Introduction

Ticks are blood-feeding ectoparasites that transmit a wealth of pathogenic microorganisms that cause severe diseases in livestock, companion animals and people (de la Fuente et al., 2008; Jones et al., 2008).

Ticks belong to two main families, the Argasidae (soft ticks) and the Ixodidae (hard ticks), which share a haematophagous lifestyle but display important morphological and biological differences (Sonenshine and Roe, 2014). Most ixodid ticks are non-nidicolous exophiles, which live in open environments such as forests, brush lands, savannahs, meadows and even semi-deserts (Randolf, 2014; Sonenshine and Roe, 2014). Ixodid ticks typically feed over a period of several days (slow feeders) and ingest amounts of blood up to 100 times their unfed body weight. Ixodid females feed only once and die after laying several thousands of eggs (Apanaskevich and Olivier, 2014). In contrast, most argasid ticks are nidicolous endophiles, which live in nests, burrows, caves or other shelters used by their hosts or live close by (Gray et al., 2014; Sonenshine and Roe, 2014). Argasid ticks typically complete their blood meal within minutes or hours (fast feeders), ingesting from 5 to 10 times their body weight in blood. In addition, adult argasids can feed and reproduce up to 10 times, laying several hundreds of eggs *per* trophogonic cycle (Oleaga-Pérez et al., 1990; Vial, 2009).

Among the argasids, *Ornithodoros erraticus* and *Ornithodoros moubata* have great medical and veterinary importance as vectors of the African swine fever (ASF) virus and of several *Borrelia* spirochetes that cause human relapsing fever (HRF) (Cutler, 2010; Sánchez-Vizcaíno et al., 2015).

O. moubata is distributed throughout southern and eastern Africa, where it can be found in wild and domestic habitats (Vial, 2009). It feeds on warthogs, domestic swine and humans, and transmits both the ASF virus and the spirochete *Borrelia duttoni*, which is the agent of the East African HRF. In the countries where these diseases are endemic, *O. moubata* populations inhabiting anthropic habitats contribute to the persistence of both diseases and, potentially, to their spread to other countries (Cutler, 2010; Sánchez-Vizcaíno et al., 2015; Quembo et al., 2016). *O. erraticus* is the type species of the *O. erraticus*

78 complex, which includes several species distributed throughout the Mediterranean basin,
79 the Middle East, the Caucasus and the Russian Federation (Rebaudet and Parola, 2006; Vial,
80 2009). In southern Europe, *O. erraticus* are found in free-range pig farms, hidden in cracks
81 and fissures inside and around pig-pens, and primarily feeds on swine when it acts as vector
82 for the ASF virus (Oleaga-Pérez et al., 1990; Boinas et al., 2014; Díaz-Martín et al., 2015a;
83 Sánchez-Vizcaíno et al., 2015). Furthermore, species in the *O. erraticus* complex can also
84 feed on humans and transmit several HRF spirochetes such as *B. hispanica* and *B. crocidurae*
85 to them (Diatta et al., 2012; Trape et al., 2013). The presence of *O. erraticus* populations in
86 anthropic environments has contributed to the persistence of ASF and HRF in endemic areas
87 in Eurasia (Pérez-Sánchez et al., 1994; Rebaudet and Parola, 2006; Boinas et al., 2011, 2014).
88 Thus, to be effective, any programme aimed at the prevention and control of ASF and
89 HRF requires the elimination of, at least, the synanthropic populations of these argasid ticks
90 (Díaz-Martín et al., 2015a). However, control of ticks and tick-borne diseases is a difficult
91 task, and none of the methods that have been used hitherto has been found to be fully
92 effective against all ticks and the diseases they transmit.
93 Anti-tick vaccines are a promising, cost-effective and environmentally friendly
94 method for control of tick infestations (de la Fuente et al., 2016), but an effective vaccine
95 against *Ornithodoros* ticks is still lacking (Díaz-Martín et al., 2015a). In addition, the
96 application of acaricides in the habitats colonised by *Ornithodoros* ticks has been ineffective
97 because their nidicolous lifestyle offers shelter from exposure to acaricides (Astigarraga et
98 al., 1995).
99 Consequently, the recent availability of an ectoparasiticide such as fluralaner, which
100 is systemically distributed in the host, instead being applied to the environment, could offer
101 an efficient, alternative method for the control of *Ornithodoros* tick infestations.
102 Fluralaner is a novel, recently developed molecule belonging to the isoxazoline class.
103 It provides potent ectoparasiticidal activity by selectively blocking both the GABA- and
104 glutamate-gated channels of arthropod, but not mammalian, neurons (Gassel et al., 2014).
105 The efficacy of fluralaner against fleas and numerous ixodid tick species has already been
106 demonstrated and comprehensively reviewed (Pfister and Armstrong, 2016). More recently,

the acaricidal efficacy of fluralaner against several species of mites, including *Sarcoptes scabiei* in dogs (Taenzler et al., 2016), *Lynxacarus radovskyi* in cats (Han et al., 2016), *Psoroptes cuniculi* in rabbits (Sheinberg et al., 2017) and *Otodectes cynotis* in dogs and cats (Taenzler et al., 2017), has been also reported.

In contrast, information regarding the susceptibility of argasid ticks to fluralaner is scant and only refers to the third nymphal instar (nymphs-3) of *O. moubata* (Gassel et al., 2014; Williams et al., 2015) and *O. turicata* (McTier et al., 2016). Since more information regarding the susceptibility of other developmental stages and species of argasid tick vectors to fluralaner is needed, the current study was designed to provide quantitative data on the susceptibility of male, female and nymphs-3 ticks of *O. erraticus* and *O. moubata* to fluralaner through *in vitro* feeding exposure.

2. Material and Methods

2.1. *Ornithodoros erraticus and Ornithodoros moubata ticks*

Tick specimens were obtained from laboratory colonies maintained at the Instituto de Recursos Naturales y Agrobiología de Salamanca (IRNASA) (CSIC), Salamanca, Spain. The colony of *O. erraticus* was established from specimens collected in Salamanca, western Spain, and the colony of *O. moubata* was established from specimens submitted from the Institute for Animal Health, Pirbright, Surrey, UK. The ticks were kept in controlled conditions (28 °C, 85% relative humidity (RH) and 12-h photoperiod) and allowed regularly feeding on rabbits. For these experiments, newly moulted unfed males, females and nymphs-3 of *O. erraticus* and *O. moubata* were obtained.

All animal manipulations were performed according to the rules from the ethical and animal welfare committee of the Institution where the experiments were conducted (IRNASA, CSIC), following the corresponding EU rules and regulations.

2.2. Blood preparation

The acaricidal effect of fluralaner on males, females and nymphs-3 of *O. erraticus* and *O. moubata* was assessed through *in vitro* feeding on fresh defibrinated ovine blood. In accordance with previous data obtained by Gassel et al. (2014) and Williams et al. (2015) for *O. moubata* nymphs-3, blood was medicated with the following serially decreasing concentrations of fluralaner: 1, 10^{-2}, 10^{-4}, 10^{-6} and 10^{-8} µg/mL (ppm) (Table 1).

Fluralaner was dissolved in dimethyl sulfoxide (DMSO) to produce a stock solution of 50 mg/mL, and then diluted in blood to produce a 1,000 µg/mL (1,000 ppm) preparation. This was further diluted in series with ovine blood to obtain the desired test concentrations.

Untreated blood and blood treated with 0.002% DMSO were included as controls. This DMSO concentration was the highest concentration to which the ticks were exposed, which was equivalent to the DMSO concentration in the 1 ppm fluralaner preparation.

2.3. *In vitro* feeding procedure

For the *in vitro* feeding procedure, a feeding device similar to that described by Kröber and Guerin (2007) was set up (Fig. 1), but substituting the silicone membrane by a Parafilm membrane, in accordance with recommendations of Schwan et al. (1991).

The ticks to be fed were counted, weighed and placed into the feeding units, which consisted of plastic vials (26 mm diameter, Deltalab S.L., Barcelona, Spain) with their bottom removed and then sealed with a stretched Parafilm membrane, fixed by means of an elastic band.

Ten males, 10 females and 20 nymphs-3 of each species were included per treatment group in each feeding unit, and three repeats per treatment were done (Table 1). Feeding units were set up in six-well plates with 10 ml of blood per well and the plates were incubated at 37 °C with gentle agitation (200 rpm) on a heater-shaker platform. The ticks were allowed to feed to engorgement for a maximum of 2 h (Fig. 1).

When the fully engorged ticks detached themselves, they were immediately recovered and transferred to dry filter paper in Petri dishes (one dish per treatment group). Ticks were then weighed and incubated at 28 °C, 85 % RH and a 12-h photoperiod for 4 weeks.

At 4, 24 and 48 h and 1, 2 and 3 weeks post-feeding, the dishes were examined and numbers of live and dead ticks recorded. Finally, at 4 weeks post-feeding, the moulting rates of the surviving nymphs and the fecundity and fertility rates of the surviving females were also recorded as described by Díaz-Martín et al. (2015b). The whole experiment was performed in triplicate.

2.4. Data analysis

The percentage mortality (M) for each tick stage and species in the control and fluralaner-treated groups at every time-point after feeding were calculated using the following formula:

M (%) = number of dead ticks per treatment x 100 / number of fully engorged ticks per treatment

Corrected mortality percentages were then calculated at each time-point, for each fluralaner concentration, and for each tick stage and species using the Schneider-Orelli's formula (Williams et al., 2015):

Corrected mortality (%) = $100 \times (M_F - M_C)/ (100 - M_C)$,

where "M_F" was the mortality for each tick stage and species and fluralaner concentration, and "M_C" was the arithmetic mean mortality obtained from all replicates in the corresponding untreated and DMSO-treated blood controls.

Corrected mortality percentages were then summarised for each tick stage and for each species, as the arithmetic mean of three replicas at every fluralaner concentration and each time-point post-feeding.

Corrected mortality data were then used to calculate the lethal concentrations LC_{50} and LC_{95}, defined as the fluralaner concentrations causing 50% and 95% killing effect at each tick stage and in each species. For this, a Probit regression analysis was performed using the MedCalc® Version 17.2 software.

Moulting rates of the nymphs-3 and fecundity and fertility rates of the females were summarised as the mean ± standard deviation of three replicas per fluralaner concentration.

3. Results

3.1. Susceptibility of *Ornithodoros erraticus* to fluralaner

All *O. erraticus* males, females and nymphs-3 fed until engorgement in the control and fluralaner-exposed groups, and ingested normal amounts of blood (2.5 mg/male, 7.7 mg/female and 1.5 mg/nymph), with no difference between control and fluralaner groups.

The mean mortalities obtained from both controls (untreated blood and DMSO-treated blood) were 0% for all the developmental stages between 4 h and 3 weeks post-feeding.

The corrected mortality percentages obtained for the fluralaner-treated groups, summarised in table 2. Fluralaner concentrations as low as 10^{-6} ppm caused >95% mortality in the first 24 h post-feeding (hpf) and 100% mortality in 48 hpf.

Finally, 10^{-8} ppm of fluralaner caused noticeably lower mortality. With this concentration, cumulative mortalities peaked at 48 hpf and did not further increase between 48 hpf and 3 weeks post-feeding.

At 4 weeks post-feeding, the surviving nymphs-3 in the 10^{-8} ppm group had moulted normally and showed mean moulting rates of 84.3% ± 3.8, which were similar to moulting rates of the nymphs-3 in the control groups.

The surviving females in this group reproduced normally, laying on average 65 ± 13 fertile eggs/female, which was within the range of females in the control groups.

3.2. Susceptibility of *Ornithodoros moubata* to fluralaner

All the *O. moubata* males, females and nymphs-3 fed until repletion in the control and fluralaner-exposed groups, and ingested normal amounts of blood (30 mg/male, 116 mg/female and 21 mg/nymph), without showing differences between control and fluralaner groups.

Mortality rates in the untreated blood controls were 0% at 48 hpf, but these slightly increased to 3% in females and to 4.6% in nymphs-3 between 1 and 3 weeks post-feeding

(males remained unaffected). In contrast, mortality rates in the DMSO-treated blood controls reached 4.2% in males, 45.5% in females and 33.9% in nymphs-3 in the first 48 hpf, and between 48 hpf and 3 weeks post-feeding no more dead ticks were recorded. Accordingly, mean mortality rates for both control groups were 2.1% for males, 22.8% for females and 15.9% for nymphs-3 at 48 hpf, showing increases between 48 h and 3 weeks post-feeding that were not statistically significant.

In the fluralaner-treated groups, 1 ppm of fluralaner caused 100% mortality in the first 4 hpf, while 10^{-2}, 10^{-4} and 10^{-6} ppm caused progressively lower mortality rates, which peaked at 48 hpf (Table 3). These mortality rates did not increase, or increased insignificantly, between 48 h and 3 weeks post-feeding.

At 4 weeks post-feeding, surviving ticks in the fluralaner-treated groups moulted and reproduced normally. Moulting rates of the nymphs-3 in the fluralaner groups were on average 90.2% ± 1.2, similar to those of the nymphs-3 in the control groups. Female fertility rates in the fluralaner groups were also similar to those in the control groups (on average, 125 ± 22 fertile eggs/female).

3.3. Lethal concentrations (LC) for *O. erraticus* and *O. moubata*

Table 4 shows LC_{50} and LC_{95} values for fluralaner in males, females and nymphs-3 of both species throughout feeding exposure.

For *O. erraticus,* the average LC_{50} was 2.0 x 10^{-8} µg/mL and the average LC_{95} was 5.4 x 10^{-8} µg/mL. It is notable that both LC_{95} and LC_{50} values were of the same order of magnitude, and that values were similar for the different developmental stages tested, although somewhat lower for nymphs-3, indicating that this was the most susceptible stage.

For *O. moubata*, the average LC_{50} value was 1.5 x 10^{-6} and LC_{95} value 1.8 x 10^{-3} µg/mL, the latter value being up to 3 orders of magnitude higher than the former. In this species, both LCs varied substantially (by one or two orders of magnitude) for all of the developmental stages tested, the lowest values occurring in females.

4. Discussion

In vitro feeding of soft ticks through artificial membranes, such as silicone and Parafilm, has already been used successfully for the maintenance of *O. coriaceus* and *O. moubata* (Osborne and Mellor, 1985; Hokama et al., 1987; Schwan et al., 1991). This method has also been used for studying the effects of ASF virus infection on *O. moubata* and for testing the acaricidal efficacy of fluralaner to *O. moubata* and *O. turicata* nymphs-3 (Rennie et al., 2000; Gassel et al., 2014; Williams et al., 2015; McTier et al., 2016). The method has also been adapted for hard ticks, allowing its application in a variety of research areas, including the testing of novel acaricides, vaccines and antibodies that target tick-protective antigens and the study of tick-pathogen relationships (Kroeber and Guerin, 2007; de la Fuente et al., 2016).

In the current study, we have set up an *in vitro* device to feed both *O. moubata* and *O. erraticus* on ovine blood, which is similar to the apparatus formerly used by Kröber and Guerin (2007) to feed hard ticks. Good feeding performance and normal moulting and fertility rates that were recorded for specimens fed on non-medicated blood confirmed the reliability of the method for maintenance of both species, extending its applicability to investigations of *O. erraticus*. As a consequence, the *in vitro* feeding method was applied to an investigation of acaricidal efficacy of fluralaner against adults and nymphs-3 of these two species, in order to confirm and expand on previously reported data for *O. moubata* and to assess the susceptibility of another important argasid vector, *O. erraticus*.

The results showed that *O. erraticus* was very susceptible to fluralaner; feeding exposure to doses as low as 10^{-6} µg/mL caused >95% mortality in the first 24 hpf and 100% mortality by 48 hpf. These results prompted us to test an even lower dose of fluralaner (10^{-8} µg/mL), which allowed us to establish an efficacy limit for this species with an average LC_{95} value of 5.4 x 10^{-8} µg/mL, nymphs-3 representing the most susceptible life stage (LC_{95} 4.7 x 10^{-8} µg/mL). This LC_{95} value was extremely low, approximately 10^{6}-fold lower than the lowest LC_{95} previously reported for any tick species in similar feeding experiments (Gassel et al., 2014; Williams et al., 2015; McTier et al., 2016; Pfister and Amstrong, 2016), indicating that fluralaner can be a very effective agent for control of *O. erraticus*.

Regarding *O. moubata*, results of these experiments indicated that this species was also very susceptible to fluralaner, although not as susceptible as *O. erraticus*, since higher fluralaner doses (10^{-2} µg/mL) were required to effectively kill 100% of the *O. moubata* specimens in the same time period (48 h). The mean LC_{95} for *O. moubata* was 1.8 x 10^{-3} µg/mL, females being the most affected life stage (LC_{95} 1.4 x 10^{-4} µg/mL).

The LC_{95} values obtained here were lower than those previously reported by Williams et al. (2015) for *O. moubata* nymphs-3 after feeding exposure to fluralaner in a similar experiment (LC_{95} 9.104 x 10^{-2} µg/mL). It is possible that use of different *O. moubata* strains could account for the observed difference in susceptibility to fluralaner. It was also noteworthy that in our experiments *O. moubata* was more affected by use of DMSO as a solvent (15.9% mean mortality in controls) than the *O. moubata* nymphs-3 tested by the former authors (2.5% mean mortality in their equivalent controls). This observation and the fact that *O. erraticus* was unaffected by the DMSO (0% mortality among the specimens fed on DMSO-treated blood) would suggest a particular sensitivity of this *O. moubata* strain to both fluralaner and DMSO. Irrespective of the basis of these differences in *O. moubata* sensitivity to fluralaner, our current results confirm the previous findings of Gassel et al. (2014) and Williams et al. (2015) concerning the high acaricidal effect of fluralaner on the *O. moubata* nymphs-3 and provide novel data showing an even greater acaricidal effect against *O. moubata* females. Therefore, these data indicate that fluralaner can be a very efficient acaricidal agent for the control of *O. moubata*.

Results of the current study, combined with those obtained by the previously mentioned authors, indicate that fluralaner has the potential to provide very high acaricidal efficacy against multiple argasid tick species via feeding exposure.

Finally, it is notable that pharmacokinetic studies in dogs have shown that following single oral administration of 12.5 mg/kg of fluralaner, maximum plasma levels of fluralaner (above 1,000 ng/ml) are on average reached within 24 h. These levels slowly decrease, remaining higher than 10 ng/ml for at least the following 12 weeks (Kilp et al., 2014). This means that, in dogs, plasmatic concentrations of fluralaner would range between 1 ppm and 10^{-2} ppm during the first 12 weeks after treatment. Accordingly, the current results suggest

that dogs treated in this way would be protected against any hypothetical infestation by *O. erraticus* and *O. moubata* during this time period. Currently, fluralaner is not labeled for use in pigs, which are the main hosts to be protected against these two *Ornithodoros* species, and no pharmacokinetic data are as yet available for pigs. As metabolism can be significantly different in pigs compared to dogs, specific data should be obtained for pigs in order to define the acaricidal efficacy of fluralaner in this species. In addition, as pig meat is consumed on a regular basis by many human populations across the world, specific pharmacokinetic data are considered necessary in order to quantify the possible impact on consumer safety of exposure to fluralaner residues.

Conclusions

In vitro feeding of argasid ticks on blood through Parafilm membranes is a long-stablished method than can be used for the maintenance of laboratory colonies of soft ticks and for testing novel acaricidal compounds. Fluralaner has demonstrated potent acaricidal efficacy in the first 48 h after *in vitro* feeding exposure against all the tested developmental stages of *O. moubata*, females being most affected. Fluralaner has also demonstrated an even higher acaricidal efficacy against all the developmental stages of *O. erraticus* that were tested, nymphs-3 being the developmental stage most affected. Fluralaner has the potential to provide very high acaricidal efficacy to multiple argasid tick species *via* feeding exposure and it could be included as acaricidal agent in integrated programmes for the control of argasid tick vectors and argasid tick-borne diseases.

Authors´ contributions

Both authors contributed equally in the study design and protocol, and in monitoring the study, which was carried out in the facilities of IRNASA (CSIC) in Salamanca (Spain). Both authors contributed equally to interpreting the results and in the writing and revision of the manuscript.

Funding

MSD Animal Health Innovation GmbH provided the Fluralaner and the costs for this study.

The authors declare that there are not conflicts of interest.

References

Apanaskevich, D.A. and Olivier, J.H.Jr., 2014. Life Cycles and Natural History of Ticks. In: Sonenshine, D.E. y Roe, R.M. (Ed.) Biology of Ticks, Vol I. Oxford University Press. pp. 59-73.

Astigarraga, A., Oleaga-Pérez, A., Pérez-Sánchez, R., Encinas-Grandes, A., 1995. A study of the vaccinal value of various extracts of concealed antigens and salivary gland extracts against *Ornithodoros erraticus* and *Ornithodoros moubata*. Vet. Parasitol. 60, 133-147.

Boinas, F.S., Wilson, A.J., Hutchings, G.H., Martins, C., Dixon L.J., 2011. The persistence of African swine fever virus in field-infected *Ornithodoros erraticus* during the ASF endemic period in Portugal. PLoS ONE. 6: e20383. doi:10.1371/journal.pone.0020383.

Boinas, F., Ribeiro, R., Madeira, S., Palma, M., Lopes de Carvalho, I., Núncio, S., Wilson, A.J., 2014. The medical and veterinary role of *Ornithodoros erraticus* complex ticks (Acari: Ixodida) on the Iberian Peninsula. J. Vector Ecol. 39, 238-248.

Cutler, S.J., 2010. Relapsing fever--a forgotten disease revealed. J. Appl. Microbiol. 108, 1115-1122.

de la Fuente, J., Estrada-Pena, A., Venzal, J.M., Kocan, K.M., Sonenshine, D.E., 2008. Overview: Ticks as vectors of pathogens that cause disease in humans and animals. Front. Biosc. 13, 6938-6946.

de la Fuente, J., Kopacek, P., Lew-Tabor, A., Maritz-Olivier, C., 2016. Strategies for new and improved vaccines against ticks and tick-borne diseases. Parasite Immunol. 38, 754-769.

Diatta, G., Souidi, Y., Granjon, L., Arnathau, C., Durand, P., Chauvancy, G., Mane, Y., Sarih, M., Belghyti, D., Renaud, F., Trape, J.F., 2012. Epidemiology of tick-borne borreliosis in Morocco. PLoS Neg. Trop. Dis. 6, e1810. doi:10.1371/journal.pntd.0001810.

Díaz-Martín, V., Manzano-Román, R., Obolo-Mvoulouga, P., Oleaga, A., Pérez-Sánchez, R., 2015a. Development of vaccines against *Ornithodoros* soft ticks: an update. Ticks Tick Borne Dis. 6, 211–220.

Díaz-Martín, V., Manzano-Román, R., Oleaga, A., Pérez-Sánchez, R., 2015b. New salivary anti-haemostatics containing protective epitopes from *Ornithodoros moubata* ticks: Assessment of their individual and combined vaccine efficacy. Vet. Parasitol. 212, 336-

349.

Gassel, M., Wolf, C., Noack, S., Williams, H., Ilg, T., 2014. The novel isoxazoline ectoparasiticide fluralaner: Selective inhibition of arthropod ᵞ-aminobutyric acid- and L-glutamate-gated chloride channels and insecticidal/acaricidal activity. Insect Biochem. Mol Biol. 45, 111-124.

Gray, J.S., Estrada-Peña, A., Vial, L., 2014. Ecology of Nidicolous ticks. In: Sonenshine, D.E. y Roe, R.M. (Ed.) Biology of Ticks, Vol II. Oxford University Press. pp. 39.

Han, H.S., Noli, C., Cena, T., 2016. Efficacy and duration of action of oral fluralaner and spot-on moxidectin/imidacloprid in cats infested with *Lynxacarus rad*ovskyi. Vet. Dermatol. 27, 474–e127. DOI: 10.1111/vde.12390.

Hokama, Y., Lane, R.S., Howarth, J.A., 1987. Maintenance of adult and nymphal *Ornithodoros coriaceus* (Acari: Argasidae) by artificial feeding through a Parafilm membrane. J. Med. Entomol. 24, 319–323.

Jones, K.E., Patel, N.G., Levy, M.A., Storeygard, A., Balk, D., Gittleman, J.L., Daszak, P., 2008. Global trends in emerging infectious diseases. Nature 451, 990-993.

Kilp, S., Ramirez, D., Mark, J.A., Roepke, R.K.A., Nuernberger, M.C., 2014. Pharmacokinetics of fluralaner in dogs following a single oral or intravenuous administration. Parasit. Vectors 7, 85. doi:10.1186/1756-3305-7-85.

Krobër, T., Guerin, P.M., 2007. *In vitro* feeding assays for hard ticks. Trends Parasitol. 23, 445-449

McTier, T.L. , Chubb, N., Curtis, M.P., Hedges, L., Inskeep, G.A., Knauer, C.S., Menon, S., Mills, B., Pullins, A., Zinser, E., Woods, D.J., Meeus, P., 2016. Discovery of sarolaner: A novel, orally administered, broad-spectrum, Isoxazoline ectoparasiticide for dogs. Vet. Parasitol. 222, 3-11.

Oleaga-Pérez, A., Pérez-Sánchez, R., Encinas-Grandes, A., 1990. Distribution and biology of *Ornithodoros erraticus* in parts of Spain affected by African swine fever. Vet. Rec. 126, 32-37.

397 Osborne, R.W., Mellor, P.S. 1985. Use of a silicone membrane feeding technique in the
398 laboratory maintenance of a colony of *Ornithodoros moubata*. Trop. Anim. Health. Prod.
399 17, 37-38.
400 Pérez-Sánchez, R., Astigarraga, A., Oleaga-Pérez, A., Encinas-Grandes, A., 1994. Relationship
401 between the persistence of African swine fever and the distribution of *Ornithodoros*
402 *erraticus* in the province of Salamanca, Spain. Vet. Rec. 135, 207-209.
403 Pfister, K., Armstrong, R., 2016. Systemically and cutaneously distributed ectoparasiticides: a
404 review of the efficacy against ticks and fleas on dogs. Parasites & Vectors 9, 436-451.
405 Quembo, C.J., Jori, F., Pérez-Sánchez, R., Heath, L., Vosloo, W., 2015. Investigation into the
406 epidemiology of African swine fever virus at the wildlife–domestic interface of the
407 Gorongosa National Park, Central Mozambique. Transbound. Emerg. Dis. 63, 443-451.
408 Randolf, S.E., 2014. Ecology of Non-nidicolous ticks. In: Sonenshine, D.E. y Roe, R.M. (Ed.)
409 Biology of Ticks, Vol II. Oxford University Press. pp. 3-38.
410 Rebaudet, S. and Parola, P., 2006. Epidemiology of relapsing fever borreliosis in Europe.
411 FEMS Immunol. Med. Microbiol. 48, 11–15.
412 Rennie, L., Wilkinson, P.J., Mellor, P.S., 2000. Effects of infection of the tick *Ornithodoros*
413 *moubata* with African swine fever virus. Med. Vet. Entomol. 14, 355–360.
414 Sánchez-Vizcaíno, J.M., Mur, L., Gómez-Villamandos, J.C., Carrasco, L., 2015. An Update on
415 the Epidemiology and Pathology of African swine fever. J. Comp. Pathol. 152, 9-21.
416 Schwan, E.V., Hutton, D., Shields, K.J.B., Townson, S., 1991. Artificial feeding and successful
417 reproduction in *Ornithodoros moubata moubata* (Murray, 1877) (Acarina: Argasidae).
418 Exp. Appl. Acarol. 13, 107-115.
419 Sheinberg, G., Romero, C., Heredia, R., Capulin, M., Yarto, E., Carpio, J., 2017. Use of oral
420 fluralaner for the treatment of *Psoroptes cuniculi* in 15 naturally infested rabbits. Vet
421 Dermatol. doi: 10.1111/vde.12429.
422 Sonenshine, D.E. and Roe, R.M., 2014. Overview: Ticks, People and Animals. In: Sonenshine,
423 D.E. y Roe, R.M. (Ed.) Biology of Ticks, Vol I. Oxford University Press. pp. 3-16.
424 Taenzler, J., Liebenberg, J., Roepke, R.K.A., Frénais, R,, Heckeroth, A.R., 2016. Efficacy of
425 fluralaner administered either orally or topically for the treatment of naturally acquired

426 *Sarcoptes scabiei var. canis* infestation in dogs. Parasit. Vectors 9, 392. DOI
427 10.1186/s13071-016-1670-7.
428 Taenzler, J., de Vos, C., Roepke, R.K.A., Frénais, R., Heckeroth, A.R., 2017. Efficacy of
429 fluralaner against *Otodectes cynotis* infestations in dogs and cats. Parasit. Vectors 10,
430 30. DOI 10.1186/s13071-016-1954-y.
431 Trape, J.F., Diatta, G., Arnathau, C., Bitam, I., Sarih, M., Belghyti, D., Bouattour, A., Elguero,
432 E., Vial, L., Mane, Y., Balde, C., Prugnolle, F., Chauvancy, G., Mahe, G., Granjon, L.,
433 Duplantier, J.M., Durand, P., Renaud, F., 2013. The epidemiology and geographic
434 distribution of relapsing fever borreliosis in West and North Africa, with a review of the
435 *Ornithodoros erraticus* complex (Acari: Ixodida). PLoS One 8, e78473.
436 Vial, L., 2009. Biological and ecological characteristics of soft ticks (Ixodida: Argasidae) and
437 their impact for predicting tick and associated disease distribution. Parasite 16, 191-202.
438 Williams, H., Zoller, H., Roepke, R.K.A., Zschiesche, E., Heckeroth, A.R., 2015. Fluralaner
439 activity against life stages of ticks using *Rhipicephalus sanguineus* and *Ornithodoros*
440 *moubata* in *in vitro* contract and feeding assays. Parasit. Vectors, 8, 90. DOI
441 10.1186/s13071-015-0704-x.
442

Figure captions.

Figure 1. Feeding apparatus (A), and engorging *Ornithodoros moubata* females (B) and nymps-3 (C).

Table 1

Table 1. Summary of assays to test the acaricidal efficacy of fluralaner against *Ornithodoros* spp.

Species	Developmental stage	Ticks per treatment group	Number of treatments: untreated blood, blood + DMSO, blood + fluralaner ($1, 10^{-2}, 10^{-4}, 10^{-6}, *10^{-8}$ ppm)	Replicates	Total tick number
Ornithodoros moubata	female	10	6	3	180
	male	10	6	3	180
	nymph-3	20	6	3	360
Ornithodoros erraticus	female	10	7*	3	210
	male	10	7*	3	210
	nymph-3	20	7*	3	420

* the 10^{-8} ppm dilution was tested only with *O. erraticus*.

Table 2

Table 2. *Ornithodoros erraticus* cumulative corrected mortality in the first 48 h after feeding exposure to fluralaner. Numbers represent the arithmetic mean of 3 replicates per developmental stage (M, males; F, females; N3, nymphs-3) or the arithmetic mean mortality from all the developmental stages tested.

Fluralaner (ppm)	**Time post-feeding**											
	4 hours				**24 hours**				***48 hours**			
	M	**F**	**N3**	**All**	**M**	**F**	**N3**	**All**	**M**	**F**	**N3**	**All**
1	100	100	100	**100**								
10^{-2}	62.9	0	0	21	100	100	100	**100**				
10^{-4}	0	0	11.5	3.8	92.6	93.6	100	95.4	100	100	100	**100**
10^{-6}	0	0	0	0	91.5	98.0	100	96.5	100	100	100	**100**
10^{-8}	0	0	0	0	3.3	2.8	2.7	3.0	9.4	8.2	22.5	**13.4**

* After 48 hours post-feeding there were no additional mortality and the surviving ticks moulted and reproduced normally.

Table 3

Table 3. *Ornithodoros moubata* corrected cumulative mortality in the first 48 h after feeding exposure to fluralaner. Numbers represent the arithmetic mean of 3 replicates per developmental stage (M, males; F, females; N3, nymphs-3) or the arithmetic mean mortality from all the developmental stages tested.

Fluralaner (ppm)	**Time post-feeding**											
	4 hours				**24 hours**				***48 hours**			
	M	**F**	**N3**	**All**	**M**	**F**	**N3**	**All**	**M**	**F**	**N3**	**All**
1	100	100	100	**100**								
10^{-2}	0	0	2.4	0.8	49.5	79.3	67.1	65.3	86.9	100	93.9	**93.6**
10^{-4}	0	0	0	0	55.1	67.4	82.5	68.3	74.6	84.3	82.5	**80.5**
10^{-6}	0	0	0	0	49.0	79.6	62.4	63.7	71.8	80.1	62.4	**71.4**

*After 48 hours post-feeding, the surviving ticks moulted and reproduced normally. Between 48 hours and 4 weeks post-feeding, the mortality rates did not increase or increased insignificantly.

Table 4

Table 4. Lethal concentrations (LC) of fluralaner in ppm for males, females and nymphs-3 of *Ornithodoros erraticus* and *Ornithodoros moubata* in the first 48 hours after feeding exposure.

	O. erraticus				***O. moubata***			
	Males	**Females**	**Nymphs-3**	**All stages**	**Males**	**Females**	**Nymphs-3**	**All stages**
LC_{50}	2.2×10^{-8}	2.3×10^{-8}	1.6×10^{-8}	2.0×10^{-8}	2.5×10^{-6}	6.5×10^{-7}	2.7×10^{-6}	1.5×10^{-6}
LC_{95}	5.9×10^{-8}	6.1×10^{-8}	4.7×10^{-8}	5.4×10^{-8}	1.8×10^{-2}	1.4×10^{-4}	2.6×10^{-3}	1.8×10^{-3}

Figure 1

Figure 1.