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Reflection paper on resistance in ectoparasites

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1. Introduction

A wide variety of ectoparasite species of importance to animal health is found in Europe. Ectoparasite infestation is seen in both food-producing and companion animals. It may be associated with a significant decline in animal health and welfare and may result in production losses within farming systems. Infested animals may act as a source of infestation to both animals and, in the case of ectoparasites of zoonotic importance, humans. Furthermore, some ectoparasite species act as important vectors of bacterial, viral, helminth or protozoan pathogens, some of which pose a serious threat to animal and public health.

Regulation (EU) 2019/6 promotes responsible use of antiparasitic products in order to minimize the occurrence of resistance, and considers the risk of antiparasitic resistance development as part of the benefit-risk balance assessed when making decisions for product approval.

The scope of this reflection paper is to provide an overview of the currently known resistance situation in ectoparasites to active substances used in veterinary medicinal products (and also in biocides, where relevant to animal health) with a special focus on Europe, and to provide a review of the current knowledge on resistance mechanisms, detection methods and possible control strategies. This information might be useful for the elaboration of guidance related to ectoparasiticide development or use, or to prepare future applications.

2. Definition of resistance

Resistance to ectoparasiticides is the selection of a specific heritable trait (or traits) in an ectoparasite population as a result of exposure of that population to an active substance, resulting in a significant increase in the percentage of the population that will survive to a standard dose of that chemical when used as recommended (Coles and Dryden, 2014; WHO, 2010).

An ectoparasite may be resistant to several active substances, through cross-resistance or multiple resistance. Cross-resistance is most often defined as the sharing of resistance among different acaricides with a similar mode of action, likely resulting from the alteration of their common target site. Multiple resistance refers to a decreased susceptibility to several active substances with different modes of action. In that case, different resistance mechanisms can coexist in the same parasite, or a common mechanism may be involved, this not being target site resistance (see section 4 for an overview of the known resistance mechanisms) (Abbas *et al.*, 2014; National Science Foundation Center for Integrated Pest Management; Balabanidou *et al.*, 2018). Some sources refer to cross-resistance when any common resistance mechanism is involved and thus resistance to one substance confers resistance to another one even where the parasite has not been exposed to the latter (IRAC, 2022). The term "side-resistance" is used by some authors instead of cross-resistance, when it concerns several substances within the same chemical class.

For ectoparasites, the level of resistance is usually quantified as the ratio of the ectoparasiticide concentrations required to obtain the same mortality rate respectively on a test field population and on a susceptible reference population of a same parasite species (e.g. LC50 resistant / LC50 susceptible), generally obtained by exposing these parasites to a range of concentrations under controlled conditions. This ratio is named resistance factor (RF) or resistance ratio (RR) (modified from Research and Reflection Ring on Pesticide Resistance). The concentration considered as fully effective against a reference sensitive population (e.g. the LD95) can be termed discriminating dose.

An ectoparasite population with reduced susceptibility may be qualified as "tolerant" or "not yet resistant" when the RF is below a given threshold e.g. 5 or 10; such threshold is however not strictly

correlated to treatment outcome in the field (Mota-Sanchez *et al.*, 2008; Eiden *et al.*, 2015). Tolerance is also used to describe natural differences in susceptibility to a substance, between species, strains or life stages (Coles and Dryden, 2014).

While lack of efficacy might be due to resistance, it may however also be caused by other factors (see section 8.2).

3. Current state of ectoparasite resistance

Reports of resistance or susceptibility status in a number of ectoparasite species of veterinary importance have been published worldwide. However, literature on ectoparasite resistance in Europe is currently relatively scarce.

3.1. Ticks

Most reports on resistance in ticks refer to *Rhipicephalus* (formerly *Boophilus*) *microplus*, also known as the southern cattle tick, which is a one-host tick preferring cattle and buffalo. All stages remain on a single individual host, which makes them more sensitive to resistance selection after treatment compared to multi-host ticks. At present, the tick is endemic in subtropical and tropical regions worldwide, but not in continental Europe. It was meanwhile eradicated in the USA.

Resistance to all major ectoparasiticides, including macrocyclic lactones and fipronil, is described in *R. microplus*, in several regions of the world such as Latin America, India, Australia and sub-Saharan Africa. This possibly includes multiple resistance (Reck *et al.*, 2014). An overview on the global resistance situation of this tick is given by Agwunobi *et al.* 2021; Rodriguez-Vivas *et al.*, 2018; Dzemo *et al.*, 2022.

Resistance has also been reported, to a lesser extent, in several other tick species, mainly of the *Rhipicephalus* genus. In 2016, multiresistant *R. appendiculatus* and *R. decoloratus* (regarding pyrethroids, organophosphates and amitraz) were evidenced for the first time in Uganda (Vudriko *et al.*, 2016).

Notably, there are documented cases of resistance to several veterinary medicinal products for the brown dog tick *Rhipicephalus sanguineus*, of which the entire life cycle can occur in one animal and its direct environment (Coles and Dryden, 2014; Becker *et al.*, 2019; Rodriguez-Vivas *et al.*, 2017; Eiden *et al.*, 2015; Tucker *et al.*, 2021; Sunkara *et al.*, 2022). Among these publications, one is from the EU (Estrada-Peña, 2005). This *in vitro* study in Spain reported a high resistance rate in *R. sanguineus* ticks to deltamethrin, and variable sensitivity to propoxur. However, all tested *R. sanguineus* strains still appeared to be sensitive to amitraz.

No other reports were found on tick resistance in Europe.

Fipronil resistance emergence was evidenced in India in *Hyalomma anatolicum* (Gupta *et al.*, 2020).

Currently, there is no documented evidence of resistance in *Ixodes ricinus*, *Ixodes hexagonus*, or *Dermacentor reticulatus* to ectoparasiticides.

3.2. Mites

Dermanyssus gallinae

In Europe, the first evidence of tolerance of the red poultry mite *Dermanyssus gallinae* to synthetic pyrethroids and carbamates was reported from Italy in the 80's of the last century (Genchi *et al.*,

1984). A survey in the former Czechoslovakia indicated resistance of *D. gallinae* to the synthetic pyrethroids permethrin and tetramethrin as well as to the organophosphate trichlorfon at few farms; resistance to the banned dichlorodiphenyltrichloroethane (DDT) was, however, widespread (Zeman, 1987). Resistance to synthetic pyrethroids in *D. gallinae* has also been reported in France (Beugnet *et al.*, 1997), Sweden (Nordenfors *et al.*, 2001) and Italy (Marangi *et al.*, 2009).

In the UK, comparisons with laboratory-reared susceptible mites suggested the presence of resistance to malathion, bendiocarb, cypermethrin and permethrin in field isolated mites (Fiddes *et al.*, 2005). A first comprehensive testing of *D. gallinae* from 10 big laying hen companies in Germany in 1999 to 2000 ascertained partial or nearly complete resistance to organophosphates, synthetic pyrethroids and carbamates.

Overall, resistance to a range of active substances among pyrethroids, organophosphates and carbamates is widely reported in *D. gallinae*. This holds true for Europe and for other parts of the world (Thomas *et al.*, 2018; Decru *et al.*, 2020; Sparagano and Ho, 2020; Koç *et al.*, 2022).

Varroa destructor

In Europe, resistance of *Varroa destructor* mites to synthetic pyrethroids, i.e. flumethrin and tau-fluvalinate, has been reported since the early 1990s in the Lombardy region in Italy, spreading quickly to Switzerland, Slovenia, France, Belgium, and Austria (Faucon *et al.*, 1995, Lodesani *et al.*, 1995; Trouiller, 1998). From there, it continued its spread throughout Europe following the established colony trade routes in France, reaching Germany in 1997 and Finland in 1998 via possible bee movement from Italy (Martin, 2004). In 2001, the resistance surveillance programme of the UK's National Bee Unit confirmed the first cases of pyrethroid resistance in the UK (Thompson *et al.*, 2002 and 2003; Martin, 2004; Lea, 2015). Pyrethroid resistance is now considered widespread in the UK (Lea, 2015). More recently, resistance to the synthetic pyrethroids acrinathrin and tau-fluvalinate as well as to the formamidine amitraz was detected in *V. destructor* mites from hives in the Czech Republic, using *in vitro* test methods (Kamler *et al.*, 2016). In 2001, the first laboratory and field detection of *V. destructor* resistance to the organophosphate coumaphos was reported in Italy (Spreafico *et al.*, 2001).

In Spain, resistance to coumaphos, tau-fluvalinate and amitraz was recently reported in mite populations from different locations (Hernández-Rodríguez *et al.*, 2021; Higes *et al.*, 2020). In a French study conducted in 2018-2019, mites collected from different apiaries presented resistance to amitraz and tau-fluvalinate (Almecija *et al.*, 2020). Recent reviews confirm that in *V. destructor*, resistance is described for all major synthetic compounds used for its control, in various geographical areas in and outside of Europe; in contrast, no significant resistance is reported to natural compounds considered as "soft acaricides", such as thymol, oxalic acid or formic acid (Jack and Ellis, 2021; Vilarem *et al.*, 2021).

It is of note that some degree of reversal of fluvalinate resistance may occur after a period with no exposure (Elzen and Westervelt, 2004).

Psoroptes ovis

Resistance in sheep scab to the organophosphate propetamphos and the synthetic pyrethroid flumethrin has been reported in the UK (Synge *et al.*, 1995; Clark *et al.*, 1996; Coles, 1998; Bates, 1998). Populations of sheep *Psoroptes* spp. that were resistant to flumethrin already showed side-resistance to high cis cypermethrin (HCC) levels (Bates, 1998). Moreover, and despite that resistance in this parasite might be easily confounded with inappropriate treatment application or poor management, there is evidence of macrocyclic lactone resistance in sheep *Psoroptes* mites in the UK

(Doherty *et al.*, 2018; Sturgess-Osborne *et al.*, 2019), and in Belgian Blue beef farms in Belgium and the Netherlands (van Mol *et al.*, 2020).

Macrocyclic lactone resistance in *P. ovis* has also developed outside of Europe, in both sheep and cattle (Soler *et al.*, 2022)

Sarcoptes scabiei

Very few reports are published indicating potential acquired resistance in *S. scabiei*. In Japan, a case report on two dogs treated with 300 µg/kg bw ivermectin suggested that *S. scabiei* in these dogs was clinically refractory to the treatment (Terada *et al.*, 2010).

3.3. Lice

Resistance to synthetic pyrethroids of lice species in livestock has been reported in a number of regions.

Based on data from a survey of OIE member countries and FAO questionnaires, in Europe, lice insecticide resistance has been mapped for UK and France (FAO, 2004).

Most scientific reports concern biting (chewing) lice and in particular the sheep louse *Bovicola (Damalinia) ovis* in Australia and New Zealand.

Reduced efficacy was first reported after only a few years following the introduction of synthetic pyrethroid formulations in 1981 in Australia (Boray *et al.*, 1988).

In 1995, results of bioassays indicated resistance in field populations of *B. ovis* whose response fell within the modal range with maximum RF of 26 (cypermethrin), 12 (deltamethrin), 20 (cyhalothrin) and 30 (alphacypermethrin). Two highly resistant strains displayed high RF (408 and 746) to cypermethrin with side-resistance conferred to the other synthetic pyrethroids. This level of resistance was sufficient to cause pour-on products to fail (Levot *et al.*, 1995). At nearly the same time in New Zealand, low to moderate cypermethrin-RF ranging up to 12.4 in the *B. ovis* field populations were identified (Wilson *et al.*, 1997). In a retrospective study, the resistance level of an Australian strain of *B. ovis* that had been highly resistant to pyrethroids dropped after having been left untreated for 10 years (RF₅₀ of 5). However, the RF increased again up to 321 within two years after resumption of cypermethrin backline treatment (pour-on). A return to using pyrethroid pour-on after 15 years of the insect growth regulators (IGRs) use was hence not considered sustainable in the long term (Levot, 2012). Resistance of *B. ovis* to pyrethroids is now widespread in Australia and New-Zealand (Heath and Levot, 2015).

Resistance against IGRs (triflumuron and diflubenzuron) was confirmed in Australian lice populations using both a molting inhibition test (James *et al.*, 2008) and a louse egg hatch test (Levot and Sales, 2008). While the resistance to IGRs is now widespread in Australia, resistance to the neonicotinoid imidacloprid and to spinosad is not described (James *et al.*, 2008; Levot, 2000; Colvin *et al.*, 2022).

Organophosphate resistance was apparently rare in Australia when the Australian Pesticides and Veterinary Medicines Authority decided in 2007 to phase out diazinon use as a dipping and jetting treatment for the control of lice.

In northern England, resistance developed to γ-benzene hexachloride (γ-BHC), aldrin and dieldrin, used in plunge dips, in populations of sheep lice in the mid 1960s (Barr and Hamilton, 1965; Page *et al.*, 1965). In Scotland, a sheep flock was suspected of being infested with a synthetic pyrethroid resistant population of *B. ovis*. A bioassay demonstrated a deltamethrin RF of 14.1, which is greater

than a resistant reference strain (Devon isolate) that showed a RF of 10.4. Laboratory and field data thus indicated possible resistance to deltamethrin (Bates, 2001).

In 2012, a population of *Bovicola (Werneckiella) ocellatus*, collected from donkeys in the UK, displayed a high level of pyrethroid (permethrin and cypermethrin) tolerance, which is likely to reflect development of resistance (Ellse *et al.*, 2012).

In the UK and Ireland, deltamethrin tolerance in the cattle louse *Bovicola bovis* was recently recorded (Sands *et al.*, 2015; McKiernan *et al.*, 2021).

Low levels of resistance to deltamethrin *in vitro* in the buffalo louse (*Haematopinus tuberculatus*) were also described in India, while cypermethrin and flumethrin were effective (Shakya *et al.*, 2022).

Information on insecticide resistance in the sucking louse *Haematopinus suis* is overall rare. In Europe, a population resistant to the organophosphate insecticide dichlorvos was described by Müller and Bülow in Germany in 1988.

3.4. Fleas

Against the banned synthetic organochlorine methoxychlor, only a single case of cat flea (*Ctenocephalides felis*) resistance was reported in Europe (Denmark) in 1986. Other cases were reported outside Europe with a total of 28 and 12 documented cases for *C. felis* and *C. canis*, respectively (Mota-Sanchez and Wise, 2017).

In their review, Coles and Dryden (2014) report *C. felis* resistance to carbamates, organochlorines, organophosphates, pyrethrins and pyrethroids. Resistance ratios (RR₅₀) were typically less than 20, and the reports essentially concern single strains. Exposure to carbaryl has been suspected to induce resistance to carbamates (carbaryl, propoxur, bendiocarb). Although it can be expected that most of these cases are from the USA, their geographical origin is not always easy to identify.

One paper reports pyrethroid resistance in response to intensive use of cypermethrin, in *Ctenocephalides felis* collected from goats in Turkey (Erkunt Alak *et al.*, 2020).

The susceptibility of 12 field isolates from cats and dogs and four laboratory reference strains of the cat flea *C. felis* collected throughout Australia, the United States and Europe was determined following the topical application of insecticides to adult fleas. In the field isolates, the LD₅₀ values in fleas following fipronil and imidacloprid administration were consistent with published baseline figures, suggesting the tested populations remain susceptible to these active substances. Results for the synthetic pyrethroids permethrin and deltamethrin, however, suggested a level of resistance in all isolates, whilst for tetrachlorvinphos only one field-collected isolate from Australia showed a 21-fold resistance at LD₅₀ compared to the reference strains (Rust *et al.*, 2015).

Large-scale monitoring of the imidacloprid resistance status in *C. felis* has been carried out in several countries including Australia, Germany, France, the UK and the USA. No evidence of a decreased susceptibility to imidacloprid over the period 2002-2017 was reported (Rust *et al.*, 2011; Kopp *et al.*, 2013; Rust *et al.*, 2018).

According to the reviews of Rust (2016, 2020), resistance to pyrethroids, carbamates and organophosphates in fleas may be widespread. Since the introduction of lufenuron, fipronil and imidacloprid in the mid-1990s, there has been little direct evidence of resistance developing to them, and failures may in most cases be due to operational factors. It is hypothesised that this could be due to rapid adult killing before feeding, or to the combination of insecticides and IGRs. However, a field strain collected from a complaint case in the USA, with a RR₅₀ of 26 for fipronil, was confirmed to be

poorly susceptible to treatment with fipronil *in vivo*, while treatment with nitenpyram was effective (Schenker *et al.*, 2001).

3.5. Flies

Insecticide resistance in the house fly *Musca domestica* to numerous active substances is widespread, with reports from a huge number of countries around the world (see for example Abbas and Hafez, 2021). The following overview for *M. domestica* is limited to Europe.

M. domestica strains resistant to organophosphates and synthetic pyrethroids have been identified on German farms (Pospischil *et al.*, 1996). In a more recent study, 58 out of 60 *M. domestica* field populations from dairy farms in Germany showed varying degrees of resistance towards the pyrethroid deltamethrin using the on-farm FlyBox test method (Jandowsky *et al.*, 2010). The deltamethrin-resistance of 15 isolates selected from these field populations could be confirmed in the laboratory by topical application of lambda-cyhalothrin.

In Denmark, pyrethroid resistance in *M. domestica* has also been observed. Four out of 21 field populations showed more than 100-fold resistance (RR_{95}) to bioresmethrin synergised by piperonyl butoxide. These farms had a history of heavy pyrethroid use. In addition, resistance to the organophosphate azamethiphos was found to be widespread (Kristensen *et al.*, 2001).

In Turkey, field strains of *M. domestica* collected between 2004 and 2006 from cow farms revealed year to year variable resistance levels against synthetic pyrethroids. Very high resistance levels against cypermethrin were reported for the Antalya strain (Akiner and Çağlar, 2012). Furthermore, neonicotinoid-resistant houseflies are present at a detectable level in Danish field populations from livestock farms. The field populations were 6 to 76-fold resistant to the neonicotinoid thiamethoxam. The cross-resistance seen between the neonicotinoids thiamethoxam and imidacloprid let the authors to conclude that their use as a replacement for each other should be avoided (Kristensen and Jespersen, 2008).

In the UK, low-level resistance of fly eggs (RF 2.9) and L1 larvae (RF 2.4) to the IGR cyromazine was reported in a field strain of *M. domestica* from a pig farm (Bell *et al.*, 2010). In Denmark, resistance towards the benzoylphenyl urea IGR diflubenzuron was observed. Two out of 21 populations had larvae surviving 6.1 times the LC_{95} of diflubenzuron. The authors also found field populations with some resistance to the IGR cyromazine. Eight out of the 21 field populations had larvae surviving 2.2 times the LC_{95} of a susceptible strain, and one population had larvae surviving 4.4 times the LC_{95} (Kristensen and Jespersen, 2003). According to the review of Junquera *et al.*, 2019, field resistance of houseflies to diflubenzuron has been reported in numerous countries, including Denmark, Turkey and Hungary in Europe, although mostly at low to moderate levels (i.e. $RR_{50} < 10$).

In another study, the susceptibility of 31 Danish field populations of *M. domestica* from livestock farms to spinosad, a compound of the spinosyn class, varied from RF_{50} 2.2 to 7.5-fold compared to the susceptible WHO reference strain. Based on the steep dose-response curve determined and the limited variation of spinosad activity against the field populations, it was considered that, overall, these field populations are still susceptible to spinosad (Kristensen and Jespersen, 2004).

Resistance to pyrethroids in stable flies (*Stomoxys calcitrans*) is reported in different areas over the world, including France, and in association to different resistance mechanisms (Olafson *et al.*, 2019). In France, a *S. calcitrans* strain, collected from cattle commonly treated with synthetic pyrethroids, showed an LD_{90} for blood-engorged flies of 7.1 and 22.6 times over the recommended dose of both deltamethrin and fenvalerate, respectively (Salem *et al.*, 2012). In another French study, none of the five populations tested using impregnated filter paper showed full susceptibility to pyrethroids;

however they were susceptible to phoxim (Tainchum *et al.*, 2018). In Germany, 100% of the *S. calcitrans* populations tested on 40 dairy farms were suspected to be resistant against deltamethrin after using the FlyBox test method. The on-farm observations were confirmed in the laboratory, demonstrating that, 24 h after topical application of a discriminating dose of deltamethrin, the mortality rate was below 80%. Using a discriminating dose of azamethiphos all stable fly colonies also turned out to be resistant. In contrast, exposure to the IGRs cyromazine and pyriproxyfen at their recommended concentrations demonstrated 100% efficacy (Reissert-Oppermann *et al.*, 2019).

In the horn fly *Haematobia irritans*, pyrethroid and organophosphate resistance is reported in tropical and subtropical areas of the globe; high resistance levels to pyrethroids were evidenced in Brazil based on bioassays and in association to genetic analysis (Brito *et al.*, 2019).

Resistance to the benzoylphenyl urea (BPU) diflubenzuron and to organophosphates, with cross-resistance evidenced between the two classes, was reported in sheep blowflies in Australia (Junquera *et al.*, 2019).

3.6. Mosquitoes and sand flies (*Nematocera*)

Studies regarding the examination of resistance or susceptibility of products with insecticidal efficacy focus on vector control programs put in place in the context of human health. There are numerous reports from different areas of the world that describe the occurrence of resistance in mosquitoes and sand flies against commonly used chemical classes (Alexander and Maroli, 2003; Dhiman and Yadav, 2016; Fawaz *et al.*, 2016; Salim-Abadi *et al.*, 2016; Moyes *et al.*, 2017; Balaska *et al.*, 2021). It can be assumed that such data also has relevance for the efficacy of veterinary medicinal products if these contain insecticidal substances of the same class as used for vector control programs or in agriculture. However, there is only limited information on the resistance situation in Europe (including for the Mediterranean region). The information that could be identified is summarised below.

Pyrethroid resistance appears increasingly reported in *Culex pipiens* throughout Mediterranean EU countries. Following a bioassay examination of the resistance status of 13 *Cx. pipiens* populations from 5 regions in Greece (according to the standard methodology of WHO) over a three-year period, susceptibility to deltamethrin could be demonstrated in 12 populations; one population in the Attika region was found to be resistant (Kioulos *et al.*, 2014). In another study conducted in Greece using the CDC bottle bioassay (Centers for Disease Control and Prevention) according to the guideline for evaluating insecticide resistance in vectors (CDC, 2012), resistance of *Cx. pipiens* to deltamethrin was shown for the Evros and the Thessaloniki region (Fotakis *et al.*, 2017). In 2022, Fotakis *et al.* further reported concerning frequencies of mutations associated to pyrethroid and diflubenzuron resistance in *Cx. pipiens* in Crete. In Italy, phenotypic resistance to pyrethroids was shown by Pichler *et al.* (2022a). In a survey conducted in Northern Spain (Paaijmans *et al.*, 2019), all tested *Cx. pipiens* populations revealed resistance to all four classes of insecticides available, i.e. pyrethroids, carbamates, organophosphates and organochlorines, while *Aedes albopictus* populations were susceptible to those classes, except for one of the tests performed with pirimiphos-methyl (an organophosphate).

In *Aedes albopictus*, the level of resistance was assumed to be relatively low as reviewed by Vontas *et al.* (2012). Concerning pyrethroids, the data indicated that deltamethrin and permethrin seemed to be effective against *Ae. albopictus* adults as all populations that had been tested from a wide geographical area over a range of years remained susceptible. The data collection included bioassay results from *Ae. albopictus* populations from Greece and Italy of the year 2009, which showed clear susceptibility to deltamethrin.

Knockdown resistance mutations (*kdr*; see section 4) associated with mild pyrethroid resistance phenotypes were however detected in Crete by Fotakis *et al.*, 2022 and in several area of the world (Auteri *et al.*, 2018). Pyrethroid resistance was recently reported in *Ae. albopictus* populations from Italy and Spain and associated with the V1016G point mutation in the voltage-sensitive sodium channel gene conferring knockdown resistance (*kdr*) (Pichler *et al.*, 2022b).

In *Phlebotomus* spp., resistance against deltamethrin and permethrin was detected in the west of Turkey where both insecticides have been applied for a long time. No resistance was found in a neighbouring province without insecticide use. Susceptibility tests and determination of the resistance status were performed according to current WHO standards (Karakus *et al.*, 2017).

Two Italian sand fly populations (of *Phlebotomus (P.) perniciosus* and *P. papatasi*) were found to be susceptible to 3 different insecticides (DDT, lambda-cyhalothrin and permethrin) compared to a known susceptible laboratory reference strain, based on bioassay tests according to the WHO standard protocols (Maroli *et al.*, 2002).

3.7. Sea lice (Copepods)

In Europe, reduced sensitivity of *Lepeophtheirus salmonis* (the salmon louse) to organophosphates, pyrethroids and emamectin benzoate has been documented (Ljungfeld *et al.*, 2014; Sevatdal *et al.*, 2005; Espedal *et al.*, 2013; Grontvedt *et al.*, 2014). Resistance to hydrogen peroxide is described (Agusti-Ridaura *et al.*, 2020). No resistance seems evidenced in any part of the world against the benzoylphenyl urea compounds, potentially because of a relatively modest use in salmon (Junquera *et al.*, 2019).

4. Mechanisms of resistance

Two major types of resistance mechanisms have been identified:

1. Enzyme-based detoxification resistance (metabolic resistance) occurs when enhanced activity levels of e.g. esterases, oxidases, or glutathione S-transferases (GST) prevent the ectoparasiticide from reaching its target site. This can be caused by a change in a single amino acid altering the catalytic centre activity of the enzyme, by constitutive gene overexpression or by amplification of gene copies in resistant ectoparasites.
2. Alterations preventing the ectoparasiticide from acting at the target site; these are usually associated to point mutations.

Jonsson and Hope (2007) concluded that the development of resistance will occur faster if resistance is dependent on only a single gene mutation, especially if this single gene mutation forms a dominant allele. If multiple genes play a role in causing resistance, the spread of resistance will be slower within the population.

However, other mechanisms are described, notably structural alterations of the arthropod cuticle conferring penetration resistance, and often combined to mechanisms described above (Balabanidou *et al.*, 2018). Among others, penetration resistance may involve the overexpression of ATP binding cassette (ABC) transporters playing a potential role in the transport of both cuticular components and drug substances. Behavioural resistance is described as well, although it may not always be a heritable character (Zalucki and Furlong, 2017; Carrasco *et al.*, 2019).

4.1. Pyrethroids

Resistance mechanisms to pyrethroids in many ectoparasites are extensively described in the literature and are mainly based on point mutations altering the target site, i.e. the voltage-gated sodium channel in the axonal membrane. These mutations and the associated resistance mechanism are often referred to as *kdr* (knockdown resistance), following the initial observation of “knockdown” after which the arthropod recovers; they are known to confer cross-resistance to organochlorines and in particular DDT. As an example, a specific sodium channel gene mutation has been shown to be associated with resistance to permethrin in *Rhipicephalus microplus* (Foil *et al.*, 2004; FAO, 2004). Now a number of similar mutations have been described or are under investigation in that species (Kumar *et al.*, 2020a). Also, point mutations in a sodium channel gene confer tau-fluvalinate (pyrethroid) resistance in *Varroa destructor* (Hubert *et al.*, 2014, González-Cabrera *et al.*, 2013). A molecular study identified sodium channel gene mutations that could lead to *kdr* phenotypes to pyrethroids in several insect species, including the housefly (Martinez-Torres *et al.*, 1997). Resistance to both pyrethroids and DDT has been observed in *Aedes aegypti*, and was suggested to be caused by the *kdr*-type resistance mechanism (Bregues *et al.*, 2003). An overview of the position of resistance-associated point mutations in the sodium channel genes is given by Rinkevich *et al.* (2013), with a focus on arthropod pests of importance to agriculture or human health. A *kdr* associated mutation was evidenced in *Aedes albopictus* in different areas of the world including China, USA, Brazil, India and Mediterranean countries (Auteri *et al.*, 2018).

Enzyme-based detoxification resistance to pyrethroids is also well known. A specific metabolic esterase with permethrin-hydrolysing activity, CzEst9, has been purified and its gene coding region cloned. This esterase has been associated with high resistance to permethrin in *R. microplus* (Foil *et al.*, 2004). Since then, various mechanisms of metabolic resistance were described in that species (Kumar *et al.*, 2020b). An overview of metabolic resistance mechanisms in vector mosquitoes can be found in the publication of Black *et al.*, 2021.

In *L. salmonis*, resistance to deltamethrin has been associated to mutations of mitochondrial DNA, which could reveal a particular mode of action of deltamethrin in that ectoparasite species (Tschesche *et al.*, 2022).

4.2. Organophosphates

Pruett (2002) showed that an insensitive acetylcholinesterase (AChE), i.e. the target site for these substances, was involved in organophosphate resistance in two strains of *R. microplus*. It is suggested that point mutations within the AChE gene may be the molecular basis for target site insensitivity as shown by studies with *Drosophila melanogaster* (Mutero *et al.*, 1994).

In salmon lice across the North Atlantic, a Phe362Tyr mutation of the AChE gene was found to be strongly linked to lice survival following chemical treatment with azamethiphos, demonstrating that this mutation represents the primary mechanism for organophosphate resistance. It was observed that the Phe362Tyr mutation is not a *de novo* mutation but probably existed in salmon lice before the introduction of organophosphates in commercial aquaculture (Kaur *et al.*, 2017).

Similar findings have been reported in *Culex quinquefasciatus*, where greater oxidase and esterase activities were observed when Ace-1^R was absent (Corbel *et al.*, 2007). The likely implication of metabolic mechanisms in bendiocarb resistance in *Anopheles gambiae* populations from Cameroon was also stressed by Antonio-Nkondjio *et al.* (2016). Sanil and Shetty (2010) studied the genetic basis of propoxur resistance in *An. stephensi* and showed that the resistance gene *pr* is autosomal,

monofactorial, and incompletely dominant; however, no clear link with (a) resistance mechanism(s) seems to be made.

4.3. Neonicotinoids

Kavi *et al.* (2014) investigated the mechanisms underlying imidacloprid resistance in house flies. Their results suggested that resistance is not due to detoxification changes by cytochrome P450 (Cyt P450), in contrast to earlier findings (Markussen and Kristensen, 2010) but results from a different resistance mechanism that could be linked to autosomes 3 and 4 of the house fly. This mechanism could be related to the target nicotinic acetylcholine receptor (nAChR) but was not completely specified. It confers high RFs but unstable resistance.

Investigations in crop plant insects reveal the occurrence of both types of resistance mechanisms, i.e. linked to the upregulation of metabolic pathways (notably in relation to Cyt P450 enzymes), or to modifications of the nAChR (Ihara and Matsuda, 2018). Recently, behavioural resistance to imidacloprid has been described in the house fly (Hubbard and Gerry, 2021).

4.4. Macroyclic lactones

There is evidence for the participation of ATP-binding cassette (ABC) transporters in ivermectin resistance in the cattle tick *R. microplus*. ABC transporters are known as efflux transporters, and found in all organisms reducing cellular concentrations of toxic compounds (Pohl *et al.*, 2011). However, presently, the exact mechanism of resistance is still unknown (Abbas *et al.*, 2014).

4.5. Insect Growth Regulators

As opposed to other, neurotoxic insecticides or acaricides, IGRs interact with highly insect-specific hormonal or biochemical pathways systems that are key to the reproduction of arthropods, which confers them a particularly low toxicity level to the host. They belong to two main groups corresponding to different mechanisms of action. Resistance mechanisms were described against both groups of IGR:

Juvenile hormone analogues (JHA):

Increased levels of microsomal Cyt P450 monooxygenases and related enzymes were found to play an important role in the pyriproxyfen resistance of houseflies (*M. domestica*) in Japan (Zhang *et al.* 1998).

An *Aedes albopictus* population from Florida showed significant resistance against two juvenile hormone analogues, methoprene and pyriproxyfen. The population presented over-expressed Cyt P450, esterases (ESTs), and glutathione-S transferase (GSTs), suggesting that the global overexpression of the detoxification enzyme families may cause the reduced susceptibility towards these IGRs (Marcombe *et al.*, 2014).

Chitin Synthesis inhibitors (CSI):

A study from Douris *et al.* (2016) provided evidence that benzoylphenyl urea insecticides (BPUs) etoxazole and buprofezin share the same molecular mode of action by direct interaction with chitin synthase 1 (CHS1). They detected a mutation (I1042M) in the CHS1 gene of a BPU-resistant *Plutella xylostella* (diamondback moth) at the same position as the I1017F mutation reported in spider mites that confers etoxazole resistance. Using a genome-editing CRISPR/Cas9 approach, homozygous lines of *Drosophila melanogaster* bearing either of these mutations were highly resistant to etoxazole and all tested BPUs (diflubenzuron, lufenuron, triflumuron). These findings have immediate effects on

resistance management strategies of major agricultural pests but also on mosquito vectors of serious human diseases (e.g. Dengue, Zika), as diflubenzuron, the standard BPU, is one of the few effective larvicides in use.

Several mutations in the chitin synthase putative binding site of diflubenzuron have been previously reported in *Cx. pipiens* from Italy and France and associated with high levels of resistance against this larvicide (Fotakis *et al.*, 2020).

According to the review of Junquera *et al.*, 2019, cross-resistance to diflubenzuron and organophosphates occurs in sheep blowflies, through a detoxification mechanism. The authors point out that in laboratory resistance selection studies with houseflies, multiple mechanisms have been selected, including e.g. reduced cuticular penetration and accelerated excretion. An elevation in microsomal oxygenases and esterase activity in *R. microplus* ticks confers resistance to fluzuron.

4.6. Carbamates

Carbamate insecticide resistance in *An. gambiae* s.l. was mainly considered due to target-site insensitivity arising from a single point mutation in the AChE gene (*Ace-1^R*), since the mean *Ace-1^R* mutation frequency had increased significantly after a two years campaign of indoor residual spraying using the carbamate insecticide bendiocarb in Benin (Aikpon *et al.*, 2014 a, b). However, a low *Ace-1^R* mutation frequency in *An. gambiae* populations, associated with the resistance to carbamate and organophosphate detected in a further study (Aikpon *et al.*, 2014c), strongly supported the involvement of metabolic resistance based on the high activities of non-specific esterases, Glutathione-S-transferases and mixed function oxidases. Similar findings have been reported in *Culex quinquefasciatus* and *An. gambiae*, where greater oxidase and esterase activities were observed when *Ace-1^R* was absent (Corbel *et al.*, 2007). The likely implication of metabolic mechanisms in bendiocarb resistance in *An. gambiae* populations from Cameroon was also stressed by Antonio-Nkondjio *et al.* (2016). Sanil and Shetty (2010) studied the genetic basis of propoxur resistance in *An. stephensi* and showed that the resistance gene *pr* is autosomal, monofactorial, and incompletely dominant; however no clear link with (a) resistance mechanism(s) seems to be made.

4.7. Formamidines - Amitraz

The target of amitraz activity has been proposed to be one of the biogenic amine receptors, most likely the adrenergic or octopaminergic receptors. In resistant tick strains two nucleotide substitutions in the octopamine receptor sequence have been detected resulting in amino acids that differ from all the susceptible strains (Chen *et al.*, 2007; Corley *et al.*, 2013). These mutations provided the first evidence for an altered target site as a mechanism of amitraz resistance in ticks. However, since the target site of amitraz has not been definitively identified, the exact mechanism of resistance to amitraz is still not completely understood (Leeuwen *et al.*, 2010; Guerrero *et al.*, 2012a; Pohl *et al.*, 2012).

Resistance to amitraz in *Varroa destructor* is associated with mutations in the β -adrenergic-like octopamine receptor (Hernandez-Rodriguez *et al.*, 2022).

The mechanisms of resistance to amitraz in *R. microplus* and other tick species is further addressed in the review of Jonsson *et al.*, 2018. Besides target site resistances, these authors show that elevated expression of ABC transporters as xenobiotic pumps also constitutes a resistance mechanism, and is potentially associated with cross-resistance to organophosphate and organochlorine acaricides.

4.8. Phenylpyrazoles - Fipronil

In the cattle tick *R. microplus*, resistance to the phenylpyrazole fipronil was associated to target site mutations (Castro Janer *et al.*, 2019), including mutations homolog to the *rdl* one (“resistance to dieldrin”), which is conserved across a number of arthropods and confers cross-resistance to organochlorines. In the dog brown tick *R. sanguineus*, no relation was found between the level of metabolic activity and tolerance to fipronil (Eiden *et al.*, 2017).

5. Methods of detecting resistance

In vivo efficacy trials are carried out directly on animals by means of administering the product according to the recommended dose rate and application mode, and the number of arthropods pre- and post-treatment (or their status, e.g. fed or unfed) is subsequently compared. There are no widely recognized standard methodologies or interpretation criteria for these tests, although they may refer to VICH (International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products) or WAAVP (World Association for the Advancement of Veterinary Parasitology) efficacy criteria. These tests will not be further discussed below.

In vitro susceptibility tests (bioassays) are numerous and vary according to the specific chemical and arthropod being investigated. Some validated test methods are given by FAO (2004), CDC (2012), WHO (2005) and IRAC (consulted 2022). Most but not all of the tests require laboratory conditions. Tests which can be performed under field conditions are e.g. the CDC bottle test (CDC, 2012) or the “FlyBox” mobile test kit (Jandowsky *et al.*, 2010). Threshold values (e.g. discriminating doses) vary among different arthropod species and different ectoparasiticides. The validity of any of these methods is evaluated by using defined reference strains of arthropods (either susceptible or resistant).

Obaid *et al.* (2022) have reviewed the available *in vitro* methods with focus on resistance surveillance in the tick *R. microplus*.

Biochemical and molecular tools have been developed to confirm the occurrence of some specific resistance traits.

5.1. Exposing adults or larvae to treated surfaces

This approach usually requires the direct contact between a surface treated with the chemical under investigation and the adult arthropods. It involves exposing adult arthropods to surfaces treated with different dilutions of the chemical under investigation for a predetermined period of time. At defined time points, the mortality of the arthropods is evaluated. Materials used for these surfaces may vary, e.g. paper, fabric or glass, but the principle remains the same (Thompson *et al.*, 2002; Jandowsky *et al.*, 2010; Rust *et al.*, 2014; Sternberg *et al.*, 2014).

A method for testing the susceptibility of tick larvae on treated surfaces is the larval packet test (LPT) promoted by the FAO (2004). It has been suggested that this assay when combined with the discriminating concentration concept may be used as an inexpensive and rapid resistance diagnostic technique (Eiden *et al.*, 2016).

These types of tests are not suitable for testing resistance to IGRs which act by disrupting the moulting process and/or inhibiting the hatching of eggs.

5.2. Topical application to adults or larvae

A method often used is the topical application on the body surface of the adult arthropod. Using different dilutions, small droplets of the chemical under investigation are applied by micro-syringe to the arthropods that are immobilised, for example by carbon dioxide or cooling. At the end of the test, the mortality of the arthropods is evaluated (Pessoa *et al.*, 2015).

Another type of topical application test is the adult immersion test (AIT). During this test the arthropods are submerged in different dilutions of the chemical under investigation (Castro-Janer *et al.*, 2009).

For larvae an analogous test is the Larval Immersion Test (LIT) (Shaw, 1966). This test is not widely used and has not been promoted by the FAO.

5.3. Feeding tests with treated rearing media

The basic principle is that the tested chemical, at different concentrations, is added to the culture rearing media for the larval stages of the ectoparasite. The larvicidal efficacy can be tested with such bioassays (Kelly *et al.*, 1987; Rust *et al.*, 2014).

A bioassay to determine resistance in sea lice has been described by Sevatdal *et al.* (2005). Pre-adult II sea lice are put in boxes and placed in seawater. The sea lice are then exposed to different doses of ectoparasiticides for 30 to 60 minutes. Twenty-four hours after exposure, survival rates of sea lice can be evaluated.

For testing IGR resistance in temporary pests like flies, the fly eggs are usually incubated in rearing media with increasing concentrations of the IGR (Jandowsky *et al.*, 2010). For ectoparasites that remain on the host permanently, like fleas, eggs can be collected from treated animals or from adults exposed *in vitro*, and monitored for development in specific test conditions (Young *et al.*, 2004). The use of wool or skin scrapings of the host are considered essential for egg hatching in lice (Levot and Sales, 2008; James *et al.*, 2008).

5.4. Biochemical and molecular assays

These tests have the potential to investigate biochemical or genetic resistance markers in an individual ectoparasite and thus confirm and further characterize resistance following a susceptibility assay. However, these tests are currently only used for research purposes. Several biochemical and immunological assays are described by the WHO (1998) to test the elevation or alteration of ectoparasite enzymes involved in higher tolerance to ectoparasiticides. For example, the biochemical microtitre plate tests allow for the same ectoparasite to be used for all assays to test enzyme activity, e.g. for detecting altered acetylcholinesterase, or elevated esterase or glutathione-S-transferase. It should be stressed that biochemical assays do not exist for all known resistance mechanisms.

Kumar (2019), has reviewed the available molecular markers for the detection of resistance against different types of acaricides in the tick *R. microplus*, including the detection of mutations on target genes of sodium channel, acetylcholinesterase, carboxylesterase, and β -adrenergic octopamine receptor.

6. Resistance monitoring programmes

There are currently no systematic monitoring programmes for resistance in ectoparasites in Europe, except monitoring programmes for resistance occurrence in salmon lice in Norway (Helgesen *et al.*,

2022). As stated above, an international program to monitor cat flea populations for susceptibility to imidacloprid has been run from 2002 to 2017 (Rust *et al.*, 2018).

Various projects monitor the environment and the health status of honey bee colonies including distribution of *Varroa* mite infestation at a national level in EU member states (e.g. Italy with BeeNet, i.e. an Italian beekeeping monitoring network, the German bee monitoring project, Spain, etc.). However, they do not specifically study levels of resistance and there is no EU-wide monitoring project that homogeneously collects data on *Varroa* resistance according to a standardized study protocol. In France, the field efficacy of products authorised against *V. destructor* in bees is monitored annually on a voluntary basis supervised by FNOSAD (Federation Nationale des Organisations Sanitaires Apicoles Departementales) in order to detect any lack of expected efficacy. This is carried out using *in vivo* efficacy tests. The international honey bee research association COLOSS (prevention of honey bee COLony LOSSes) has a *Varroa* control task force; however, it does not primarily focus on resistance monitoring (<https://coloss.org/activities/taskforces/varroa/>).

Pharmacovigilance system

Lack of expected efficacy should be reported within the EU pharmacovigilance system. These reports could be supportive in providing evidence of potential development of resistance to a specific active substance.

However, the system has its limitations as resistance is difficult to recognise in the field, and lack of expected efficacy is generally underreported. Thus, the true incidence of lack of efficacy is likely to be underestimated. Consequently, the current pharmacovigilance system is of limited value to detect and monitor resistance.

7. Management strategies to delay the development of resistance

According to the WHO (2014) the occurrence of resistance is of focal nature and requires local decisions. From a general perspective, however, the following measures for reducing the development of resistance, based on the knowledge of the factors that drive resistance development, are addressed in the related literature.

7.1. Monitoring

Regular resistance monitoring over a given geographical area to support the choice of an appropriate ectoparasiticide for application has been recommended in the public literature (FAO, 2004; Abbas *et al.*, 2014; Karakus *et al.*, 2017; Kumar *et al.*, 2020b; Esteve-Gasent *et al.*, 2020). Monitoring requires a recognised laboratory responsible for resistance testing, a defined standard methodology including a susceptible reference strain and, if necessary, also a strain that is known to be resistant (FAO, 2004).

7.2. Prudent use of ectoparasiticides

7.2.1. Reduction of number of treatments

There is a general consensus that the reduction of the selection pressure for resistance in the field may delay the emergence of resistance (FAO, 2004; Thullner *et al.*, 2007), and it has been recommended to reduce the use of ectoparasiticides (e.g. timing the treatments according to epidemiology) (FAO, 2004; Heath and Levot, 2015).

This was supported by a case control study performed in Australian dairy farms (Queensland), where regional differences were noted in the prevalence of acaricide resistance to the cattle tick *R. microplus*. Certain regions and the frequency of acaricide application were consistently associated with resistance; for instance, it was observed that the risk of resistance to synthetic pyrethroids and to amitraz increased when more than 5 applications of acaricide were made in the previous year (Jonsson *et al.*, 2000). In their recent review on resistance status in ixodid ticks, again with a focus on *R. microplus*, Agwunobi *et al.* (2021) and Rodriguez-Vivas *et al.* (2018) also pointed out the significant relationship between a frequent application of acaricides and the development of resistance, which has been demonstrated in various studies globally.

Other experiences in Australia, in the sheep louse *Bovicola ovis*, show that frequent use of ectoparasiticides of the same class over an extended period of time is considered a risk factor for the development of ectoparasiticide resistance (Levot *et al.*, 1995; Wilson *et al.*, 1997).

Furthermore, it may be a problem when the same products are used to control different species of parasites and where the epidemiology of the different species also differs, e.g. the use of macrocyclic lactones (endectocides) as anthelmintics may also select for resistance in ectoparasites (FAO, 2004). It is of note that conversely, the use of endectocides against ticks has been associated to resistance selection in helminths (Molento and Brandao, 2022). Therefore, the knowledge of preceding treatment practices may help to find the reason for an observed lack of efficacy of an ectoparasiticide.

7.2.2. Dosing and method of administration of a veterinary medicinal product

The method of administration has also been taken into account by several authors. For tick eradication programmes, topical application via plunge dips or spray races were considered superior with regard to efficacy compared to administration with a hand-held spray apparatus, since the latter method might provide insufficient distribution and/or wetting of the animals with the possibility of ticks being exposed to sublethal concentrations; nevertheless, the hand-spray method could be favourable in terms of delaying the development of resistance due to completely unexposed parasites (Jonsson *et al.*, 2000; WHO, 2014; Agwunobi *et al.*, 2021).

It appears that the negative impact on ectoparasite resistance development of underdosing, or even overdosing (e.g. due to persistence of residues), is unclear and poorly discussed in the literature (Rodriguez-Vivas *et al.*, 2018; Vilarem *et al.*, 2021).

7.2.3. Targeted selective treatment

According to the concept of targeted selective treatment, a certain proportion of the infested animals (as individual animals or subgroups) is left untreated, based on physiological, pathological or parasitological criteria.

This is based on the notion of *refugia*, i.e. parasites in environmental or animal reservoirs with no exposure to ectoparasiticides, which do not undergo the selection pressure for genes that confer resistance, and will dilute the corresponding alleles if involved in the next parasite generation. The existence of natural *refugia* for an insect or acari species hampers resistance development (Coles and Dryden, 2014). The maintenance of *refugia* for susceptible ectoparasites through strategic treatment and husbandry management has been shown to be beneficial in delaying resistance development against anthelmintics, and has been considered as a means to delay resistance also in ectoparasites, e.g. in ticks, lice, mites, by several authors (Kunz and Kemp, 1994; Goss *et al.*, 1996; FAO, 2004; Clloyd, 2010; Abbas *et al.*, 2014; McNair, 2015; WHO report, 2014; Heath and Levot, 2015), although

this might be difficult to apply in practice and further investigation in clinical trials is deemed necessary.

7.2.4. Rotation of different classes of ectoparasiticides

The alternate use of ectoparasiticide substances, provided they have different modes of action and supposedly present no cross-resistance, is recommended by many authors in order to delay, overcome or even reverse resistance (Obaid *et al.*, 2022; Agwunobi *et al.*, 2021; Abbas *et al.*, 2014; WHO report, 2014; Soler *et al.*, 2022; Hafez, 2022).

With regard to acaricides there is evidence from a study performed under laboratory conditions with defined *R. microplus* tick strains that rotation of pyrethroids (deltamethrin) with organophosphate acaricides (coumaphos) could delay the development of pyrethroid resistance. However, further field trials are considered necessary to confirm such strategy (Thullner *et al.*, 2007). The field study of Jonsson *et al.*, 2010, demonstrated the possibility to overcome pre-existing amitraz resistance in ticks, using a rotation of amitraz and spinosad.

It thus appears that there is experimental evidence of the benefits of such strategy, although it remains relatively scarce and the benefit may vary depending on the substances considered, the target parasite and other conditions. Notably, this approach assumes that within an ectoparasite population the frequency of resistant individuals to each chemical used before will decline during the application of the alternate substances. This could imply that concurrent maintenance of refugia is required (Kunz and Kemp, 1994), or that the involvement of substances to which resistance is less stable due to fitness cost could be important for success (Kavi *et al.*, 2014; Rodriguez-Vivas *et al.*, 2018; Alam *et al.*, 2020). Also, it remains unclear how the benefit differs with the occurrence or absence of pre-existing resistance.

7.2.5. “Multi-active products”

A further strategy to delay the development of resistance that is still under discussion is the use of products containing two or more ectoparasitidal substances with different modes of action against the same parasite (multi-active products). This approach is based on the assumption that an individual parasite is unlikely to carry resistant alleles for two or more acaricides or insecticides with different modes of action (Kunz and Kemp, 1994; Abbas *et al.*, 2014; WHO report, 2014; Agwunobi *et al.*, 2021). This strategy requires that the active substances in a multi-active product are compatible, of equal persistence (to prevent that sublethal concentrations of one component would select for resistant heterozygotes) and that they are used at recommended concentrations. According to Taillebois and Thany (2022), the data available in the literature confirm that neonicotinoids and pyrethroids could be effective in multi-active combinations, and in particular towards pyrethroid-resistant arthropods, partly due to a synergism between these classes. Similarly, the results of El Sherif *et al.* (2022) led the authors to conclude that an abamectin/chlorfenapyr mixture could be used as a promising resistance management strategy based on a potentiation effect.

However, the potential risk of the development of multiple resistance cannot be fully excluded. Moreover, the actual benefits in delaying resistance when used preemptively in fully susceptible arthropod populations, rather than in overcoming existing resistance, were apparently not evaluated. Further thorough clarification on this strategy appears necessary before firm conclusions on its usefulness can be drawn.

Products combining a neurotoxic insecticide/acaricide to an IGR are sometimes likened to multi-active products, although they target different parasitic stages. They might possibly confer some specific

benefits in regard of resistance, as suggested by Rust, 2016, in relation to fleas; however, no experimental data seem currently available to substantiate this.

7.3. Synergists

Synergists are active substances with typically little or no direct activity against parasites, but when combined with an antiparasitic, they provide a degree of control greater than what would be expected from the simple additive effects of each compound. Most synergists act by inhibiting the enzymatic detoxification of antiparasitic substances by the parasite. A common example of a synergist is piperonyl butoxide (PBO). PBO is used as a synergist to certain ectoparasiticides (e.g. pyrethroids, carbamates) for the control of arthropods. It has no direct killing properties against arthropods and is practically non-toxic to birds and mammals (NPIC, 2017). PBO inhibits numerous enzymes in the arthropods that can break down the active substance before they can operate. Specifically, PBO inhibits the detoxification of ectoparasiticides by binding to the Cyt P450 dependent mixed function oxidases (MFOs), which are responsible for the degradation of active substances (Weber, 2005).

It seems however that PBO is the only substance of that type approved in the EU in VMPs, and there is no recent or widely distributed EU VMP containing PBO; the reasons for this are unclear.

By adding PBO to a product, existing resistance might be overcome to some extent (Taillebois and Thany, 2022; Gleave *et al.*, 2021), thereby preserving the efficacy of carbamates and synthetic pyrethroids. It is however currently unclear to which extent such strategy would be sustainable in the face of increased metabolic resistance.

7.4. Environmental control measures

To delay the development of resistance, additional measures which may reduce the infestation pressure and thereby the frequency of ectoparasiticide application have been addressed in relevant literature: pasture management (e.g. pasture alternation and/or rotation, in combination with ectoparasiticides) and/or housing management (e.g. good ventilation, thorough manure removal, optimum animal density, low stress) (Jonsson *et al.*, 2000; Abbas *et al.*, 2014). Treatment of the surroundings to reduce or eliminate reinfestations is also a common strategy to reduce the infestation pressure, e.g. as practiced in the case of fleas. Management measures may include mosquito traps, horsefly traps and fly traps (lights, sticky strips) (Heath and Levot, 2015).

Moreover, quarantine of bought-in livestock may be considered as a strategy to prevent possible infestation and the need for treatment of the whole flock at a later time. This has been recommended for ticks, lice and mites (non-flying obligatory ectoparasites) (FAO, 2004).

A series of practicable environmental measures, in the particular context of a veterinary faculty in Northern Europe, are presented by Humblet *et al.*, 2020a.

7.5. Alternative management strategies

Alternative (non-chemical) methods for controlling ectoparasiticide infestations are, for example, the use of natural enemies (e.g. predatory mites) and vaccination.

7.5.1. Natural enemies

The black dump fly *Hydrotaea aenescens* (formerly *Ophyra*) has been used successfully for controlling house fly populations on swine and poultry farms in Europe and the United States (Betke *et al.*, 1989, Ruzsler, 1989; Turner and Carter, 1990; Jespersen, 1994; Hogsette and Jacobs, 1999).

Leclercq *et al.* (2014) studied the efficacy of cleaner fish (wrasse, *Labridae*), which feed on the skin of other fish, as a biological control against sea lice. The authors concluded that farmed Ballan wrasse (*Labrus bergylta*) are highly effective controls against sea lice.

In poultry production, the release of predator mites such as *Androlaelaps casalis* that consume the poultry red mite *D. gallinae* is used. However, although commercially available, the use of predator mites under field conditions needs further research (Sparagano *et al.*, 2014).

Entomopathogenic fungi (such as *Beauveria bassiana* and *Metarhizium anisopliae*) and bacteria (notably *Bacillus thuringiensis*) are promising tools able to provide a high level of host and environmental safety, and likely to bear a low risk of inducing resistance. Numerous publications show their biological activity in parasites of veterinary importance, i.e. various tick species, *D. gallinae*, *Psoroptes* spp. and *V. destructor* (see for review, Ebani and Mancianti, 2021). Although none is currently part of an authorised VMP in the EU, these can be commercially produced and are currently marketed for other applications, e.g. the fight against mosquito larvae.

Treatment strategies relating to arthropod endosymbionts like *Wolbachia* spp. are also currently subject to research efforts (Madhav *et al.*, 2020; Ebani and Mancianti, 2021).

7.5.2. Vaccination

For few arthropod species the development of vaccines is considered a possible alternative approach for the control of ectoparasites. For many years research efforts focused on the development of a vaccine against the southern cattle tick *R. microplus*, which has considerable negative impact on livestock production (De la Fuente *et al.*, 2007; Vargas *et al.*, 2010; Guerrero *et al.*, 2012b; McNair, 2015; Schettters *et al.*, 2016). Presently, a few vaccines, containing the gut antigen Bm86 of *R. microplus* or polyprotein, are commercially available. However, efficacy of these vaccines appears to be variable (Pereira *et al.*, 2022). Cattle tick vaccine research is ongoing in order to develop improved vaccines.

Similar approaches for other veterinary infestations are also considered useful (e.g. dog ticks, sheep scab, sea lice) (McNair, 2015; Ribeiro *et al.*, 2021). However, the selection of suitable antigens as vaccine candidates is generally a major constraint (Smith *et al.*, 2001; Smith and Pettit, 2004), and so far no vaccine against ectoparasites is available in the EU.

7.5.3. Plant-derived products

There is a considerable body of literature on the potential use of plant-based products, in particular essential oils, for the treatment of ectoparasite infestations in animals. This has been investigated in various arthropod species, in particular *R. microplus* and *D. gallinae*. However, at present there are not many products authorised as veterinary medicinal products containing such active substances, for several reasons, including short efficacy periods and standardisation issues (George *et al.*, 2014; Salman *et al.*, 2020; Selles *et al.*, 2021; Pugliese *et al.*, 2021). Some products indicated against *Varroa* infestation in honeybees are authorised within the EU and contain pure phytotherapeutic compounds (e.g. thymol), possibly in combination with essential oils (e.g. eucalyptus and peppermint).

8. Discussion

Although the available published data may not give a full view of the resistance status in ectoparasites in the EU, due to a possibly variable level of investigation, worldwide expanding resistance against ectoparasiticide substances contained in veterinary medicinal products and biocides is a major concern for animal welfare, for livestock production and potentially for human safety. The development of resistance to antiparasitics is known to be influenced by the host, the parasite, the frequency of use of antiparasitic products and the environment/husbandry system.

8.1. Resistance mechanisms

Presently, in ectoparasites several types of resistance mechanisms have been identified, mainly including target site alterations and enzyme-based detoxification mechanisms, but also mechanisms involving efflux transporters or decreased penetration. For several ectoparasite species resistance mechanisms against some of the relevant substance classes have been determined. However, clinically relevant resistance has also been observed for which the underlying resistance mechanism is presently not exactly known, e.g. for amitraz or macrocyclic lactone compounds in ticks. The possibility that resistance against a substance or substance class is complex i.e. based on more than one mechanism needs to be taken into account. To summarise, more information in this area including the inheritance of resistance genes is needed not least for the benefit of establishing resistance management programs. Therefore, continuation of research in the highly complex process of resistance development is needed.

8.2. Detection of resistance

Usually, resistance will be suspected through lack of efficacy during clinical use. However, lack of efficacy could also occur due to other factors including inadequate application of a product, e.g. underdosing, inappropriate dosing frequency or timing of treatment, or poor application techniques. Such inappropriate practices could also lead to further selection of resistant ectoparasite species. In 1994 Dryden and Rust suspected that the reason for lack of efficacy of ectoparasiticides in fleas is most likely not linked to resistance development but rather to treatment deficiencies related to the absence of environmental control, i.e. poor penetration of insecticides into carpets with subsequent re-emergence of adult fleas.

A complicating factor is that it is generally difficult to confirm that lack of efficacy observed in the field is due to resistance against the veterinary medicinal product. Currently, most of the methods available to verify suspected resistance require time-consuming laboratory conditions. In addition, special expertise is necessary to propagate an ectoparasite population in the laboratory before resistance testing. As resistance detection is the basis for efficient monitoring (at the farm and regional level) and subsequent treatment choice, there is a need for the development of resistance detecting methods that can be routinely performed under field conditions, and which can provide results in a timely manner with regard to the resistance/susceptibility status of an ectoparasite population.

8.3. Monitoring of resistance

In Europe, published information on resistance in ectoparasites is rather sporadic, focusing predominantly on mites, sea lice, lice and flies and to a lesser extent on ticks, fleas and mosquitoes.

Only in very few EU Member States structured resistance surveillance programs are available, and only for specific parasites. Apart from this, there is a huge absence of information on the resistance

situation and possible trends over time in most ectoparasite species in European countries in relation to currently used ectoparasiticides. Experience from countries outside Europe (e.g. Australia, New Zealand) show that such information is a prerequisite to manage ectoparasite resistance development (Jonsson *et al.*, 2000; FAO, 2004; Abbas *et al.*, 2014; Karakus *et al.*, 2017). Thus, there is a need for systematic monitoring throughout the European region.

8.4. Strategies to delay resistance development

To reduce the risk of resistance development and to achieve the expected treatment benefits, a sufficiently confirmed diagnosis as well as a correct application of the veterinary medicinal product, including an appropriate choice of pharmaceutical form and route of administration, are inherent measures.

In order to ensure that a suitable ectoparasiticide is selected, regular regional monitoring for the development of resistance against different chemical classes would be useful. The present difficulties, however, connected to the establishing of a monitoring program or to on-farm testing for resistance, have already been addressed above.

Targeted selective treatment based on the refugia concept has been shown to be beneficial in delaying resistance development against anthelmintics, and is described by several authors in the field of ectoparasites. Currently available data, however, is insufficient to draw final conclusions on the usefulness of this concept for ectoparasites. Nevertheless, targeted treatment in a broad sense could be applied to ectoparasites, which would consist in avoiding unnecessary or systematic use and base treatment decisions on thorough clinical, epidemiological and parasitological assessment.

Apart from targeted selective treatment there are further strategies proposed in the literature, which could delay resistance development, e.g. rotation in the use of ectoparasiticide classes or use of multi-active product combinations with different mode of action targeted against the same parasite species. These strategies are theoretically most successful if implemented before resistance develops to any of the active substances included. However, it is described that in practice such strategies are often implemented when resistance has already developed against one or more active substances, which is likely to make them less effective. Moreover, there is always the potential risk of selection of multiresistant parasite species. It is of note also that fitness cost and the subsequent persistence of resistance likely plays a role in the effectiveness of these methods (Rodriguez-Vivas *et al.*, 2018; Jonsson *et al.*, 2010).

This emphasises the need for further scientific evaluation of the optimal conditions for use of rotation strategies or multi-active products.

Furthermore, there are treatment-independent options to reduce the need for treatment and, thus, selection for resistance. These are environmental control measures like appropriate pasture and/or housing management, the use of specific insect traps or the use of natural enemies of particular ectoparasites. The latter strategy, however, is often negatively influenced by concomitant use of ectoparasiticides. Nevertheless, it is generally agreed that non-chemical strategies could contribute to a reduced use of ectoparasiticides and, thus, to the delay of development of resistance. Therefore, it would be beneficial to put further emphasis on the research and the integration of such sustainable strategies into management programs and on the education of responsible persons.

Overall, many authors recommend Integrated Pest Management (IPM) of arthropod infestations, i.e. strategies that would combine several methods of delaying resistance; for example, appropriate resistance monitoring could be used to facilitate the choice of treatment or treatment rotation, while environmental control measures are used to decrease treatment frequency. Discussions on Integrated

Pest Management can be found in the recent literature, again with focus on the cattle tick *R. microplus* (Kumar *et al.*, 2020b; Rodriguez-Vivas *et al.*, 2018; Githaka *et al.*, 2022), but also in regard of other parasitosis and for Europe, e.g. stable flies (Rochon *et al.*, 2021), poultry mite infestation (Decru *et al.*, 2020) or *Varroa* infestations (Vilarem *et al.*, 2021). A practical example is available relating to vector arthropods and to the specific environment of a European veterinary faculty including an educational farm (Humblet *et al.*, 2020b).

8.5. Assessment of product applications for ectoparasiticides

In marketing authorisation applications, information on the potential emergence of resistant arthropod species of clinical relevance is required. Applicants are also requested to provide data on the resistance mechanism(s) as far as known. Furthermore, it would be useful if scientifically supported risk mitigation measures aimed at reducing the risk for resistance development could be presented. It is acknowledged, however, that the possibility to provide information on resistance is restricted due to the current lack of both surveillance programs and useful methods for detecting resistance. Even though there is some information on resistance mechanisms available in the literature, the available data is currently limited, particularly with regard to information on the mode of heredity. Nevertheless, applicants are encouraged to provide all available data in the field of resistance development in the target parasites against the active substance in the product to be authorised.

Until recently, precautionary and informative statements relating to resistance were recommended only for anthelmintic products intended to treat ruminants and horses, in the *Guideline on the summary of product characteristics for anthelmintics*. The scope of the document was revised by the CVMP, and is now extended to ectoparasiticides and all target species (*Guideline on the summary of product characteristics for antiparasitic veterinary medicinal products*, EMA/CVMP/EWP/170208/2005-Rev.1). However, considering that information on the resistance situation in the EU is relatively scarce, there may be limited information to include in the product literature for some substances and parasites.

In addition, as addressed in the revised guideline, appropriate pack sizes should be made available on the market to allow treatment of different numbers of animals without causing leftovers that could be used inappropriately and favour resistance development.

9. Conclusion

There is limited knowledge on ectoparasiticide resistance in Europe, as documentation on this subject is scattered and incidental. For most ectoparasite species in European countries there is no comprehensive database that provides an overview regarding the resistance situation against commonly used ectoparasiticides and possible trends over time. However, such information is considered essential for managing resistance development. In order to establish a sound basis for action regarding resistance management it would not only be important to initiate systematic monitoring programs, but also to continue the research regarding resistance mechanisms.

In relation to this lack of systematic data, resistance to ectoparasiticides is difficult to determine in the field. It can be assumed by lack of efficacy of an insecticidal/acaricidal product; however, other causes may also result in insufficient or lacking efficacy, e.g. inadequate application of a product. In addition, reinfestations from the surroundings or from untreated animals could simulate lack of efficacy. Therefore, suspected resistance should require verification by laboratory tests where available. Since most of the available tests are arduous and time-consuming, further research regarding the

development of reliable and easy to use tests for resistance diagnostic purposes, that can be routinely used, should be encouraged.

Even though there is an insufficient knowledge on the current situation of ectoparasiticide resistance in Europe, the CVMP deemed it appropriate to include prudent use warnings and information on the resistance status in the SPC of ectoparasitocidal products, according to the available data; this is why the scope of the *Guideline on the summary of product characteristics for antiparasitic veterinary medicinal products* was extended to ectoparasiticides and all target species. It should, however, be stressed that current knowledge on the prudent use of ectoparasiticides is by no means complete.

10. Recommendations

There are several issues related to the use of ectoparasiticides with the purpose of reducing the risk for resistance development that do not fall within the mandate of the CVMP. For many issues, action requiring interdisciplinary professional expertise and input from other parties is needed to improve understanding, monitoring, management practices, and the prudent use of ectoparasiticides so as to reduce inappropriate use and consequently delay resistance development.

10.1. Recommendations within the responsibility of the CVMP

- Use of an ectoparasiticide VMP should be based on the confirmation of ectoparasitic infestation, using appropriate diagnostic methods, or on thorough assessment of the risk of infestation. This is particularly important considering the use of fixed combination products extending the antiparasitic spectrum. Such products should be restricted to situations where all active substances are necessary at the time of administration, through appropriate statements in the product literature. The CVMP should ensure that an adequate SPC wording and prudent use warnings as appropriate, based on the latest scientific knowledge and on the *Guideline on the summary of product characteristics for antiparasitic veterinary medicinal products*, are implemented.
- According to this guideline, appropriate pack sizes should be made available for the market to allow treatment of different numbers of animals without causing leftovers that could be used inappropriately and favour resistance development.
- With the purpose of collecting relevant information on the circumstances of cases of lack of efficacy and in order to be able to identify its possible reasons, further guidance with regard to the information expected when cases of lack of efficacy of ectoparasiticides are reported to the pharmacovigilance system should be considered.
- The CVMP should consider whether there is a need to revise the *Guideline on demonstration of efficacy of ectoparasiticides (7AE17a)* in light of current scientific knowledge and the experience gained to date in product approval procedures.
- Annex II of Regulation (EU) 2019/6 (Commission Delegated Regulation (EU) 2021/805) sets out general requirements for documenting the antiparasitic resistance status of a product submitted for marketing authorisation. The CVMP should develop, as appropriate, more specific guidance on the resistance data that should be included in marketing authorisation applications for ectoparasiticides (e.g. published literature addressing the concerned regions in Europe) based on these requirements. This could possibly be done through the revision of the *Guideline on demonstration of efficacy of ectoparasiticides (7AE17a)*.

- Regulation (EU) 2019/6 promotes responsible use of antiparasitic products in order to minimise the occurrence of resistance, and considers the risk for antiparasitic resistance development as part of the benefit-risk balance to be assessed when making decisions for product approval. This should be taken into account when elaborating guidance on the benefit-risk assessment for VMPs.
- The availability of ectoparasiticides for limited markets should be promoted to reduce off-label use. The development of specific guidance for minor species should be pursued, in line with the requirements of Regulation (EU) 2019/6.

10.2. Responsibility of Member States

- Decisions on prescription status for ectoparasiticides are not within the responsibility of the CVMP for nationally authorised products. Nevertheless, National Competent Authorities (NCAs) should take decisions in that matter in line with the available CVMP guidance and, as far as possible, in a harmonised manner.
- NCAs may play a role in ensuring that appropriate pack sizes are made available.
- NCAs should facilitate and stimulate pharmacovigilance reporting. Veterinarians and other qualified individuals, as well as farmers and animal keepers, should be encouraged to report any lack of expected efficacy and this, as far as possible, in such a way that the underlying reasons can be identified.
- NCAs should be encouraged to control advertising for ectoparasiticide VMPs which are available without veterinary prescription to be sure that it is coherent with the summary of product characteristics and does not include information which could be misleading or lead to overconsumption of the product.

10.3. Other recommendations

The following topics fall outside the mandate of the CVMP and national regulatory agencies. However, they are of importance for understanding and monitoring the development of resistance in ectoparasites.

- Member States should be encouraged to establish systematic monitoring systems at national level or EU-wide to monitor resistance in ectoparasites, in particular parasites for which there is a particular concern.
- There is a need to continue research on resistance mechanisms and their genetic basis, and based on this, to develop reliable and practical tests for detection of resistance in different ectoparasite species, and markers that trace early stages of resistance development in an ectoparasitic population. A threshold to confirm resistance in different ectoparasite species needs to be established for each target animal species. The development of monitoring tools, e.g. user-friendly software/applications that could be routinely used (by farmers or veterinarians) should be supported. The availability of valid test methods should be ensured, e.g. by carrying out inter-laboratory ring tests as necessary.
- Continued research on management strategies that could reduce the need for ectoparasiticides is desirable. This includes continuing research on biological alternatives.

- It is necessary to educate and enhance awareness of resistance in ectoparasites amongst veterinarians and other persons qualified to prescribe veterinary medicinal products, in accordance with applicable national law, as well as animal owners.
- The benefits and risks in relation to resistance development associated with the use of multi-active ectoparasiticides should be further explored through appropriate scientific evaluation. More generally, further research and data are needed on the impact of all combination products on resistance development.

Definitions

ABC (ATP Binding Cassette) transporters: Transporter proteins which are expressed in all organisms and which are essential to several physiological processes (e.g. translocation of endogenous substances, cellular defence against xenobiotics).

Cross-resistance: Decreased susceptibility to more than one active substance within different chemical classes, due to the alteration of a common target site, or to another shared resistance mechanism.

Discriminating dose or concentration: The dose which is considered as fully effective against a susceptible test ectoparasites population. Individuals from field collected isolates which survive at this dose are by definition resistant.

Multi-active product: Product containing two or more substances with activity against the same target parasite but with a different mode of action.

Multiple resistance: Decreased susceptibility to more than one active substance within different chemical classes with no common target site, thus possibly involving several different resistance mechanisms.

Refugia: Parasite population purposely left unexposed (within untreated animals, or in the environment) in order to reduce the population under selection pressure and thereby, allow the dilution of alleles that confer resistance.

Resistance factor (RF): The ratio of the lethal concentration (for example LC50 or LC90, corresponding respectively to a RF₅₀ or RF₉₀) of a field population relative to the same benchmark dose for a susceptible reference strain.

Resistance ratio (RR): see RF.

Side-resistance: Decreased susceptibility to more than one active substance within the same chemical class.

Abbreviations

ABC transporter: ATP (Adenosine triphosphate) Binding Cassette transporter

AChE: acetylcholine esterase

BPU: benzoylphenyl urea

CDC: US Centers for Disease Control and Prevention

Cyt P450: Cytochrome(s) P450

DDT: dichlorodiphényltrichloroéthane

FAO: Food and Agricultural Organization

IPM: Integrated Pest Management

kdr: knockdown resistance

LC50, 90, 95: Lethal Concentration for 50%, 90%, 95% of the exposed parasites

NCA: National Competent Authority

IGR: Insect Growth Regulator

nAChR: nicotinic acetylcholine receptor

OP: organophosphate

PBO: piperonyl butoxide

R4P Network: Research and Reflection Ring on Pesticide Resistance <https://www.r4p-inra.fr/en/glossary/>

RF: Resistance Factor

RR: Resistance Ratio

VMP: Veterinary Medicinal Product

WHO: World Health Organization

γ-BHC: γ-benzene hexachloride

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