Detecting pyrethroid resistance in predatory mites inhabiting soil and litter: an *in vitro* test

Marine El Adouzi, a* Olivier Bonato b and Lise Roy a

**Abstract**

**BACKGROUND:** While resistance against insecticides is widely known in pest arthropods, it remains poorly known in non-target arthropods of the same agrosystems. This may be of crucial importance in the context of organic pest management or integrated pest management. First, stopping of pesticide pressure during farm conversion may lead to important rearrangements of non-target communities due to fitness cost of resistance in populations of some species. Second, resistant biological agents may be useful to farms with low synthetic pesticide use. Communities of mesostigmatid mites, encompassing numerous predatory species, are supposed to be involved in important ecological processes in both crop soils and animal litter/manure.

**RESULTS:** Here we provide a tarsal contact method for assessing resistance in different populations from various species of mesostigmatid mites. Analyses of data from repeated tests on three populations from different mesostigmatid families proved the method to be robust and able to generate consistent and reliable mortality percentages according to insecticide concentration.

**CONCLUSION:** Our bioassay system allows for both one-shot estimate of pyrethroid sensitivity in mite populations and estimation of how it changes over time, making possible survival analyses and assessment of recovery from knockdown. The rating system retained makes it possible to score response to insecticides in a consistent and standard way in species from different mesostigmatid families.

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Supporting information may be found in the online version of this article.

**Keywords:** resistance; bioassay; multi-species; Mesostigmata; pyrethroid

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1 **INTRODUCTION**

The massive and widespread use of insecticide/acaricide substances during the last decades has resulted in selection of diverse mechanisms of resistance in pest arthropod populations that have led to recurrent failure of pest treatments in crop and animal farming and in control of parasites and pathogens of humans. Adaptation to new environmental stress is often associated with an alteration of one or more life history traits. Consistently with the allocation principle,1 fitness costs of insecticide resistance in environments that are free of insecticides were recurrently reported in diverse arthropod groups.2–5 However, departures from this expectation (uncostly resistances) have also been recurrently reported.4,6,7 Absence of cost may result from a diversity of factors, such as lack of energy costs associated with insecticide resistance,7 functional compensation between two mutant alleles,4 complex effects of genetic background2 and interactions with environmental conditions (see e.g. Jensen et al.9). As a result, depending on the frequency of resistant alleles in the different constituent species of a given community as well as on the fitness costs associated with each allele, release from selective pressure on resistance can lead to alterations of the community structure. However, the magnitude of the alteration may be very difficult to predict. Where resistance imposes no cost, resistant genotypes may be fixed, remaining at high frequency in the absence of insecticides; where resistance is costly, resistant genotypes may have greatly reduced fitness. Their loss may be speeded up if immigrant populations of natural enemies of pests – of natural origin or introduced in the framework of biocontrol – introduce non-resistant individuals.

In both crop and animal farming, non-target soil or litter mite communities are known or suspected to play important roles in processes of soil functioning9 and in pest regulation via predation on a diversity of pests, such as other mites, nematodes and insects (see e.g. Halliday and Holm10). Studies of insecticide/acaricide resistance have focused mainly on arthropods of economic and/or sanitary interest.11–15 The few studies that have considered non-target organisms are exclusively dedicated to improving the effectiveness of exogenous biological control agents (arthropods) released in crops.16–18 However, resistance in non-target communities that spontaneously inhabit agrosystems is an important issue for identifying resistant populations of auxiliary organisms in the context of integrated pest management (i.e. where pesticides are allowed but used in reduced quantities) and for assessing the impact of the selective pressure release on...
population assemblages. In soil from a conventional crop farm, or in litter from a conventional animal farm in the process of conversion to organic agriculture, release from pesticide pressure could lead to considerable rearrangements of community composition if resistance is associated with strong fitness costs. The extent of such changes would depend on the relative importance of resistant populations in the community and on migration events, whether natural or human-induced. Predicting such rearrangements is important, since they could have effects (transitory or durable) on the ecosystem services provided by communities of non-target organisms.

Although plant-dwelling predatory mites are most frequently used as agents of biological control, soil- or litter-dwelling predatory mites are increasingly used to control soil insects (e.g. through predation on eggs, pupae), nematodes and other soil pests, either in combination with plant-dwelling phytoseiids or alone (Laelapidae, Macrochelidae, etc.). For instance, in France, five firms to date are allowed to sell non-indigenous laelapid (Stratiolaelaps miles, S. scimitus, Androlaelaps casalis, Gaeolaelaps aculeifer) and macrochelid (Macrocheles robustulus) species for biocontrol purposes (Annex 1 of the French ministerial order NOR: AGRG1502673A - https://www.legifrance.gouv.fr/eli/arrete/2015/2/26/AGR1502673A/jo/texte). As the imported individuals come from populations unexposed to pesticides, such migration events are likely to dilute mutant alleles conferring resistance in their species’ populations, or to establish populations of locally new species. If costly resistance is present, such introductions could lead to rapid collapse of some of the initial populations, thereby leading to changes in community structure.

Pyrethroids have long exerted – and continue to exert – selective pressure on many arthropods in both crop and animal farms, and it has been shown that many pest arthropods have developed resistances against this class of insecticides. It would not be surprising if soil- or litter-dwelling mesostigmatid mites, a non-target community living close to the soil surface and/or litter and commonly associated with pest arthropods in a diversity of agro systems, were shown to have also developed pyrethroid resistances. We already know that resistance against insecticides, including pyrethroids, commonly occurs in mites and that fitness costs may be associated with pyrethroid resistance in at least one predatory mesostigmatid mite species (Metaseiulus occidentalis (Mesostigmata, Phytoseiidae)); in a joint lab and field study, after a resistant population of M. occidentalis was released in an orchard where native susceptible mites were abundant, resistance was quickly lost, whilst when the same resistant population was released in an orchard after a pyrethroid application had eliminated the susceptible natives, the resulting population maintained its pyrethroid resistance for several generations. As a result, resistance against pyrethroids and associated fitness costs are likely to influence the community structure of mesostigmatid mites. To assess the longer-term effect of fitness cost of resistance on soil/litter mesostigmatid mite communities, we must first be able to assess the resistance phenotype of mesostigmatid mite communities without any a priori knowledge of the mechanisms involved in resistance. Attempts to detect resistance in monitored populations commonly compare L50 and in some cases L90, standard sensitivity values. Resistance phenotypes of populations are characterized by bioassays in which individuals are exposed to different concentrations and their responses recorded after a given interval. The FAO’s recommendation guidelines for resistance management, which are focused on the biomonitoring of single pest species, stipulate that resistance bioassays need to be simple, cheap, capable of generating results rapidly and sensitive enough to detect early emergence of resistance among pest populations in the field. In contrast, the present study was focused on multi-species communities. Its main goal was to develop a test to assess sensitivity to pyrethroids in different species of communities of mesostigmatid mites living in farm soils or litters as a tool to investigate the long- and short-term effects of insecticide use on ecological processes. This objective required exposing different mesostigmatid mites to fixed concentrations of pyrethroids and following their responses in an acceptable timeframe. Because considerable differences between species are expected in the expression of the response to toxic substances (delay from the start of exposure, observable symptoms of toxic effect) and because resistance may be associated with transient knockdown with pyrethroids, mites should be individually exposed to insecticides and kept individually isolated in order to observe changes in the response over time. In this study, we tested the sensitivity to deltamethrin of populations of three species belonging to three families of Mesostigmata (Macrochelidae, Laelapidae and Parasitidae). Preliminary observations identified a few observable symptoms that provided suitable criteria for mortality assessment. Different toxicity indices, including LC50, were estimated and survival analyses (Kaplan–Meier curves, Cox proportional hazards regression model) were performed. A mixed model was used to assess the robustness of the test by measuring the relative magnitude of fixed effects (deltamethrin concentration and exposure duration) and random effects associated with the experiment (different replicates, different working solutions).

2 MATERIALS AND METHODS

2.1 Mites
One population of each of two distantly related mesostigmatid species, namely Stratiolaelaps scimitus (Mesostigmata: Laelapidae) and Macrocheles robustulus (Mesostigmata: Macrochelidae), were used. These two species, kindly provided by A.I. Lacordaire (Koppert, France), are common soil/litter-dwelling predatory mites and are commercially distributed as biological agents to control thrips and sciarid flies (eggs, larvae and pupae). A population of a third species (Parasitidae sp.) collected from soil sampled in a conventional peach orchard near Nîmes (southern France) was also tested. The three species were mass reared in culture vessels (Microbox® (initially for plant experiments), Avamoplast, Lokeren, Belgium) to prevent the medium from drying out and maintained in a climate chamber set at 22 ± 2 °C. They were fed ad libitum on astigmatid mites (Astigmata) in a medium composed of equal parts of vermiculite and compost. For the purpose of standardization, only adult females of S. scimitus and M. robustulus were considered. Because field-sampled mites belonging to Parasitidae were almost solely deutonymphs, the tested stage for this population was a mix of adult females and deutonymphs.

2.2 In vitro bioassay protocol
Because the main way pyrethroids enter an organism’s body to exert their effects is by contact (penetration through the epidermis) and owing to the small size of the mites, the bioassay was based on tarsal contact. Note that our test does not attempt to mimic the likely complex modes of exposure undergone by mesostigmatid mites in their native substratum, but only to measure the sensitivity levels at a point in time. As a result, we
did not consider the other different possible mechanisms of exposure to pyrethroids (aerial exposure in vapour phase, ingestion), which are likely to be secondary.25

To follow mite behaviour over time, we used flat-bottomed transparent polystyrene 96-well microplates (Nunc, MicroWell™ 96-Well Plates, Thermo Fisher Scientific, Roskilde, Denmark), as already done by Nordenfors and Höglund26 for the poultry red mite Demanyssus gallinae (Parasitiformes: Mesostigmata: Demanysidae) and by Lovis et al.27 for the cattle tick Rhipicephalus (Boophilus) microplus (Parasitiformes: Ixodidae: Ixodidae).

The protocol for microplate preparation was roughly similar to that described by Lovis et al.27 A 400 μL aliquot of deltamethrin/ethanol solution was deposited in each well of the microplate. The solution was allowed to evaporate for at least 48 h under a fume hood. On each plate, series of eight wells (columns) were filled with different deltamethrin concentrations. For the control, series of eight wells were filled with ethanol only. Between three and eight microplates (replicates) were used for each mite species. To standardize the method, the replicates were processed in the same laboratory conditions.

The microplates were used 5 days after impregnation. After checking that there was no residual fine layer of ethanol condensation, a disc of agarose (10 g L⁻¹) (see explanation below) was introduced into each well (stuck onto wall) and the microplate was sealed with stretched paraffin film (Parafilm®). One tiny opening was made on each well using a sharpened scalpel for introducing mites. The mites were carefully transferred into the well using a flexible thin brush. Once all mites were introduced into wells of a whole microplate line, these wells were covered again with Parafilm® to prevent the mites from escaping (and so on).

Records were performed by individual observation of mites from the bottom of each well using a stereomicroscope with 40× magnification. This was repeated four times at fixed intervals following the beginning of insecticide exposure, corresponding to four different exposure durations: 24, 48, 78 and 168 h (1 week).

2.2.1 Moisture conditions adaptation

Because survival of arthropods may be strongly influenced by the relative humidity of the environment, it is important to determine whether the species under test can survive without any source of moisture in the test conditions.28 For this purpose, we first compared the survival of M. robustulus and S. scimitus with that of the previously tested closely related species D. gallinae in the test conditions without deltamethrin. Without any additional moisture source in the 96-well microplates sealed with either film, no individuals of either M. robustulus (n = 22) or S. scimitus (n = 23) were found alive after 24 h (virtually no reactive mites after 12 h, most of them visibly dehydrated). In contrast, D. gallinae was able to survive several weeks under similar conditions. To provide a moisture source suitable for keeping soil/litter species alive and permitting their observation in polystyrene wells, we conducted preliminary experiments comparing mite behaviours in film-sealed microplates with different moisture conditions, namely without any source of moisture (room hygrometry) or with three different increased moisture conditions: (1) microplates sealed with Hydrofil® (permeable to both air and water vapour) and maintained in a water-saturated atmosphere (in a 20 cm x 30 cm closed plastic box filled with ca 0.25 L of water); (2) microplates sealed with Parafilm® (permeable to air but not to water vapour) and containing one small moist vermiculite pellet in each well; (3) microplates sealed with Parafilm® and containing a slice of transparent gel (10 g L⁻¹ agarose, thickness 1 mm, diameter 2 mm) in each well. We then conducted all subsequent experiments using the conditions found to be most suitable (see results).

2.2.2 Deltamethrin concentrations

Technical-grade deltamethrin (≥98%, Sigma-Aldrich Saint-Quentin Fallavier, France) was used in this study. To prepare the stock solution, deltamethrin was dissolved in ethanol (96%, Sigma-Aldrich Saint-Quentin Fallavier, France) and submitted to ultrasonic bath treatment for 3 min until completely dissolved. The reference concentration was the mean authorized concentration for use in France in peach orchards against different insect pest species, i.e. 1 g h⁻¹ (mean authorized amount per hectare: 1000 L).

Seventeen different concentrations allowing to impregnate the wells’ internal walls with deltamethrin amounts ranging from 4.00 to 6.55 x 10⁻³ times the reference amount according to surface were tested (Table 1). As recommended by Miller et al.,28 the number of runs was increased at concentrations close to the estimated LC₅₀ in order to increase the accuracy with which this index is estimated (Table 1). Then additional tests on higher or lower concentrations were performed to be able to estimate lethal concentrations from LC₁₀ to LC₉₀. For each test, two ranges of concentrations were tested in order to increase replication (replicated work solution).

2.2.3 Mite response

Defining clear criteria for mortality assessment requires some attention.28 Most published studies rely on the reaction of

<table>
<thead>
<tr>
<th>Coded concentration</th>
<th>M. robustulus</th>
<th>S. scimitus</th>
<th>Parasitidae sp.</th>
<th>Ratio work concentration / field use</th>
</tr>
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<tbody>
<tr>
<td>C1</td>
<td>4</td>
<td>1</td>
<td>NT</td>
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<td>NT</td>
</tr>
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<td>3</td>
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<tr>
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<td>1</td>
<td>NT</td>
</tr>
<tr>
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<td>3.906 x 10⁻³</td>
<td>7</td>
<td>3</td>
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<tr>
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</tr>
<tr>
<td>C15</td>
<td>3.051 x 10⁻⁵</td>
<td>6</td>
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<td>4</td>
</tr>
<tr>
<td>C16</td>
<td>1.525 x 10⁻⁵</td>
<td>7</td>
<td>2</td>
<td>7</td>
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</table>

The reference concentration or mean use concentration is C₃ = 1 g h⁻¹. The corresponding effective work solution was 6.83 g h⁻¹, which allowed to reach a concentration/surface ratio similar to field uses with 410 μL per well. The number of replicates per concentration is detailed per species. For each run, there were between one and three replicates. NT, non-tested concentration for the considered species.

Table 1. Table of concentrations used in the present study: concentration codes and ratio to the mean authorized concentration for use in peach fields (C3)

The reference concentration or mean use concentration is C₃ = 1 g h⁻¹. The corresponding effective work solution was 6.83 g h⁻¹, which allowed to reach a concentration/surface ratio similar to field uses with 410 μL per well. The number of replicates per concentration is detailed per species. For each run, there were between one and three replicates. NT, non-tested concentration for the considered species.
individuals to some direct action such as overthrow or gentle hit. When using sealed microplates, there is a need to identify unambiguous symptoms observable without any direct contact. Lovis et al. combined the observation of the motility of individuals when the microplate is warmed by holding it in the hand (heat commonly activates hematophagous arthropods) and their general appearance in terms of dryness. Neither of these criteria is applicable to predatory mesostigmatid mites in our experiments (their response to heat is usually different and the experiment involved the addition of a source of moisture, preventing the drying out of dead mites). Our assessment of mite condition was based on observation of mite motility after a mechanical stimulus (produced by hitting the microplate) to activate the mite, followed by observation of the mite’s reaction during 10 s.

In order to be able to propose the most accurate and standard rating system for a satisfying sensitivity assessment in mite populations, we first observed mites under test and defined the three following conditions: not affected, affected, not active. At hit stimulation, the reaction of the mite was observed during 10 s for deciding its condition.

Not active. Mite does not move. Not affected. Mites with at least one leg moving (movements of pedipalps and chelicerae were not considered). Walking mites (before or after the hit stimulus) were systematically recorded as not affected. Affected. An intermediate posture was also considered: a mite lying on its back, moving one or more legs after stimulation, but unable to walk. Mites lying on their back were considered either as affected or not active.

2.3 Statistical analysis

Data were processed and analysed using the statistical software R.

Selection of a binary rating system. In order to generate suitable data for sensitivity assessment, a binary rating is most appropriate to discriminate between impacted and not impacted mites and thereby assess mortality percentages from which toxicity values can be calculated. From the above-described three mite conditions, the following two binary rating systems were drawn: (1) ‘NA’ rating (moving, i.e. Not Affected + Affected, versus Not Active mites); (2) ‘back’ rating (active mites on legs, i.e. Not Affected, versus mites lying on their back, independently of whether they were reactive or not, i.e. Not Active + Affected). The resulting data set included both NA and back rates for each individual, at each of the four different exposure durations, making possible paired tests. McNemar paired tests were performed to compare results from the two rating systems (see 2.3.4).

2.3.1 Survival analysis

A standard survival analysis using the Kaplan–Meier method was performed to calculate an LT50 (lethal time 50%, or median life duration) using the R package ‘Survival’. As described in Holbrook et al., data on time to mortality were used to construct proportional hazards models (Cox regression models) containing coefficients from comparisons between a reference population and the two other ones. Exponentiation of the coefficients yielded hazard ratios (HR), which quantified the instantaneous probability of death for individuals of the population under test as compared with those of the reference population. Since the exact time of a mite’s death was unknown, but occurred within a certain interval of time, an exact likelihood method was used to estimate regression coefficients. The decision threshold for rejecting all null hypotheses was \( \alpha = 0.05 \).

2.3.2 Lethal concentrations

Estimated values as well as 95% confidence intervals (CIs) of concentrations lethal to 10, 30, 50, 70 and 90% of the population were calculated with the function dose.p available in the MASS library.

2.3.3 Random effects

In order to estimate robustness of the test, a mixed model was used to describe the corrected mortality rate (calculated as recommended by Abbott) with respect to the fixed effects (deltamethrin concentration, exposure duration) and the random effects (replicates - microplate + run effect- and working solutions of deltamethrin).

2.3.4 Awakening events

An individual was considered as having awakened when it was rated ‘on its back’ or ‘unreactive’ in one or more records (T + 24h, T + 48h, T + 72h) and found ‘on legs’ and ‘reactive’ in a subsequent record (T + 48h, T + 72h, T + 168h). Using formulae in Excel, awakening events that occurred at T + 48h, T + 72h and T + 168h were scored. Based on these, the frequency of awakening events recorded from control individuals was compared with that from exposed individuals (tests using any deltamethrin concentrations) to see whether they occurred significantly more in test than in control and so detect recovery from the knockdown effect of pyrethroid exposure. To estimate the level of discordance between pairs with the ‘NA’/ ‘back’ ratings (pairs of rating for each individual), McNemar paired tests were conducted.

3 RESULTS

3.1 Validation of experimental set-up

3.1.1 Moisture conditions

Results of preliminary trials (30 individuals per condition and per species) showed that a source of moisture was required to perform bioassays on non-hematophagous mesostigmatid mites. In contrast to the hematophagous mesostigmatid mite D. gallinae (100% survival at room hygrometry for up to at least 2 weeks), individuals of both S. scimitus and M. robustus began dying (unmoving dried bodies) as early as 24 h after the start of the experiment in the absence of any source of moisture, as well as in the presence of moist vermiculite + Paraflim® (increased moisture condition 2). In the presence of saturated atmosphere and Hydrofilm® or of agarose gel and Paraflim® (increased moisture conditions 1 and 3 respectively), however, the survival rate was 100% for up to at least 1 week.

With Paraflim® as the sealing film (increased moisture condition 3), mite individuals of both species were systematically observed to run swiftly and for long distances on the walls of the polystyrene well, immediately after being introduced into the well. We were thus confident that they systematically made tarsal contact with the insecticide-impregnated walls. In contrast, with the Hydrofilm® (increased moisture condition 1), owing to the adhesiveness of the film, mites were unable to immediately run over the walls’ walls and usually needed several seconds or minutes to get unstuck from the film. Mites were repeatedly observed to remain stuck on the film, though they remained active. Although the Hydrofilm® + saturated atmosphere condition was the less
time-consuming of the two moisture-satisfying conditions, it did not ensure tarsal contact with the molecule tested. Because we were unable to find any more suitable film (i.e. permeable to water vapour but not adhesive), we retained the Parafilm® + agarose condition. The transparency of the agarose gel allowed easy observation of mites through the microplate. All subsequent experiments were performed using this protocol.

3.1.2 Mortality in controls

In the control conditions, no mortality was reported at T + 24h in any of the three species tested with any of the two rating systems (unreactive mites/mites lying on their back). At T + 72h, mortality was <3% in all three species. At T + 168h, it was slightly increased in M. robustulus and S. scimitus (~8% with the ‘back’ rating) and substantially increased in Parasitidae sp. (26%).

When the Cox proportional hazards regression model was applied to the control data set (Table 2), the probability of death (or hazard rate HR) of M. robustulus appeared to be intermediate compared with that for S. scimitus (HR lower by 0.323, P = 0.0275) and Parasitidae sp. (HR higher by 3.334, P = 0.0002) (likelihood ratio test (LRT) = 32, degrees of freedom (DF) = 2, P = 1.1 × 10⁻², n = 385, failure events = 44). When analysing the data set excluding the fourth record (T + 168h), the Cox proportional hazards regression model resulted in non-significant coefficient values (LRT = 0.6, DF = 2, P = 0.74, n = 385, failure events = 7). The survival probability as estimated using the Kaplan–Meier curves was closest to 1, with narrow 95% CI, up to T + 72h for the three species and up to T + 168h for M. robustulus and S. scimitus (Table 2). Two of the three mite species maintained high survival up to 168 h in the agarose-filled microplates, and this system was suitable for testing all three species, with an acceptable mortality rate in the absence of pesticide (control) during up to 72 h of experiment.

3.1.3 Mite response rating

The ‘back’ rating was much easier to apply than the ‘NA’ rating, as it required less time and attention and was systematically observable. In contrast, in applying the ‘NA’ rating, we were sometimes unable to assess with certainty the reactive condition of some individuals lying on their back (some minor leg movements are difficult to separate with certainty from significant movements). In control conditions, fewer than 2% of individuals were recorded as ambiguous (mites reactive, but lying on their back; n = 154, 161 and 70 in S. scimitus, M. robustulus and Parasitidae sp., respectively), all of which were unreactive at T + 72h except for one M. robustulus individual. Given the difficulties associated with the ‘NA’ rating and the quasi-absence of ambiguous individuals when using the ‘back’ rating in individuals not exposed to insecticide, the ‘back’ rating may be considered as reporting an unambiguous response to insecticide exposure. The two different rating systems yielded similar estimated values of lethal concentrations at the retained exposure duration T + 72h (see below).

3.1.4 Awakening events

When considering exposed mites (in the test conditions), awakening events were recurrently recorded in mites that were exposed to pesticides, for all three species tested (Table 3). In two of the three species tested, significantly fewer awakening events were recorded using the ‘NA’ rating than when the ‘back’ rating was used (Table 3, McNemar test, P < 0.0001 in M. robustulus and S. scimitus, P = 0.2482 in Parasitidae sp.). This strongly suggests that careful observation of mites’ leg movements may allow discriminating between definitive collapse and transient knockdown in mites lying on their back and confirms that an individual follow-up of the mite position (on back/on legs) over time after insecticide exposure is a good indicator for estimating the frequency of post-knockdown awakening events.

Using the ‘back’ rating, in any of the deltamethrin concentrations tested, the percentage of awakening individuals differed slightly among species, close to 15% in S. scimitus and below 10% in the two other species, suggesting different levels of transient knockdown effects in the three populations under test.

The outputs of the mixed models performed to analyse the random effects of experimental protocol (replicates, working solutions) relative to the fixed effects under test here (deltamethrin concentration and duration of exposure) are provided for all three species as supporting information (Tables A–C). For the three mite species, deltamethrin concentration and exposure duration showed significant effects on mortality, while effects of replicates and working solutions were not significant. Compared with T + 48h, exposure duration had a significant effect at T + 72h for S. scimitus and at T + 168h for both M. robustulus and S. scimitus. No significant effect of exposure duration was observed at either T + 48h or T + 72h for Parasitidae sp. (For this species, T + 168h was not considered in the analysis; see above.) These results confirmed that an accurate estimation of mite condition was reached at T + 72h.

<table>
<thead>
<tr>
<th>Table 3. Percentage of awakening individuals among deltamethrin-exposed (any concentrations) individuals having had at least one failure record with the ‘back’ rating from T + 24h to T + 72h</th>
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</thead>
<tbody>
<tr>
<td>Rating system</td>
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<tr>
<td></td>
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<tr>
<td>'Back' rating</td>
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<td>'NA' rating</td>
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<tr>
<th>Table 2. Kaplan–Meier survival estimates in control conditions (‘on back’ notation for failure events)</th>
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<tr>
<td>Species</td>
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<tr>
<td>------------------</td>
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<tr>
<td>M. robustulus</td>
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<td>Parasitidae sp.</td>
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<td>S. scimitus</td>
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### 3.2 Response to deltamethrin exposure

Because of the above-described decreased survival in Parasitidae sp. at T + 168 h, the response to deltamethrin exposure was only estimated for the three exposure durations 24, 48 and 72 h for all three species. We only analysed the data based on the ‘back’ rating.

**Estimates of lethal concentrations.** Considerable differences in sensitivity were observed among the three populations under test (Table 4). To maximize accuracy, more tests were conducted with concentrations surrounding the estimated LC₅₀ for each species. As a result, concentrations that were tested with the largest number of replicates differed among species. The following concentrations were tested: C5 – C16 in *M. robustulus* and *S. scimitus*; C2 – C16 (except C11, C13 and C14) in Parasitidae sp.

Because most awakening events were recorded during the second records (T + 48 h; as could be expected owing to possible transient knockdown) and because mortality in control conditions remained near zero up to T + 72 h, we decided to estimate lethal concentrations at T + 72 h (Fig. 1). We found that the estimated lethal concentrations were very similar for the two ratings used, ‘back’ rating and ‘NA’ rating.

**Survival analyses.** At C10, the only concentration for which numbers of tested individuals and replicates were comparable in all three species, the estimated LT₅₀ based on Kaplan–Meier curves was much lower in *M. robustulus* (between mar 0 and 24 h) than in *S. scimitus* (≥72 h) and Parasitidae sp. (≥72 h, not estimable with the data set, with records at T + 72 h) at that concentration above 70% survival) (Fig. 2). Using proportional hazards modelling, an LRT showed that regression coefficients representing the three species significantly improved the fit of the model (LRT = 17.7, DF = 2, n = 189, number of events = 114, P < 0.001). Each coefficient for species or for concentrations was, in itself, significant (Table 5). With *M. robustulus* as the sensitive reference, the hazard ratios associated with *S. scimitus* and Parasitidae sp. evaluated here were smaller than one in magnitude (Table 5), and none of their 95% CIs encompassed this value. This result provided strong evidence that both *S. scimitus* and Parasitidae sp. were dying at a significantly slower rate than *M. robustulus*. The instantaneous probabilities of death of an individual of *S. scimitus* or Parasitidae sp. were approximately one-fifth and one-half respectively of that for *M. robustulus*.

For the different concentrations under test in each species, with C10 as reference, LRTs showed that regression coefficients representing the different concentrations significantly improved the fit of the model in each species (*M. robustulus*: LRT = 532, DF = 12, n = 961, number of failure events = 440; *S. scimitus*: LRT = 549, DF = 12, n = 951, number of failure events = 371; Parasitidae sp.: LRT = 172, DF = 13, n = 429, number of failure events = 199; P < 0.001 in all three cases). In *M. robustulus* and *S. scimitus*, all individual coefficients were significant (P < 0.05 or P < 0.001), except for C11 (in both species) and C13 (in *M. robustulus*), with HR > 1 in concentrations > C10 (C5 – C9) and HR < 1 in concentrations < C10 (C11 – C16). In contrast, in Parasitidae sp., only high concentrations had individually significant coefficients (C7 – C2, with 0.05 < P < 0.001) and HR values > 1 (and low 95% CI bounds of HR values). No significant coefficients were recorded with C7 – C16 in Parasitidae sp., a finding that is likely explained by very low

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**Table 4.** Estimated LC₅₀ (g hL⁻¹) and confidence interval 95% for the three species under test at T + 24 h, T + 48 h and T + 72 h

<table>
<thead>
<tr>
<th>Species</th>
<th>T</th>
<th>LC₅₀</th>
<th>CI 95%</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. robustulus</em></td>
<td>T + 24 h</td>
<td>2.95 x 10⁻⁴</td>
<td>[2.79 x 10⁻⁴ ; 3.1 x 10⁻⁴]</td>
</tr>
<tr>
<td></td>
<td>T + 48 h</td>
<td>3.05 x 10⁻⁴</td>
<td>[2.88 x 10⁻⁴ ; 3.21 x 10⁻⁴]</td>
</tr>
<tr>
<td></td>
<td>T + 72 h</td>
<td>2.96 x 10⁻⁴</td>
<td>[2.79 x 10⁻⁴ ; 3.12 x 10⁻⁴]</td>
</tr>
<tr>
<td></td>
<td>T + 24 h</td>
<td>5.54 x 10⁻⁴</td>
<td>[5.38 x 10⁻⁴ ; 5.7 x 10⁻⁴]</td>
</tr>
<tr>
<td><em>S. scimitus</em></td>
<td>T + 48 h</td>
<td>5.88 x 10⁻⁴</td>
<td>[5.71 x 10⁻⁴ ; 6.04 x 10⁻⁴]</td>
</tr>
<tr>
<td></td>
<td>T + 72 h</td>
<td>5.98 x 10⁻⁴</td>
<td>[5.81 x 10⁻⁴ ; 6.14 x 10⁻⁴]</td>
</tr>
<tr>
<td></td>
<td>T + 24 h</td>
<td>0.08</td>
<td>[0.01 ; 0.32]</td>
</tr>
<tr>
<td>Parasitidae sp.</td>
<td>T + 48 h</td>
<td>0.04</td>
<td>[0 ; 0.23]</td>
</tr>
<tr>
<td></td>
<td>T + 72 h</td>
<td>1.74 x 10⁻²</td>
<td>[1.59 x 10⁻² ; 1.92 x 10⁻²]</td>
</tr>
</tbody>
</table>

---

**Figure 1.** Lethal concentration estimated for the three species under test at T + 72 h: M. rob, *Macrocheles robustulus*; Para, Parasitidae sp.; S. sci, *Stratiolaelaps scimitus*.

**Figure 2.** Kaplan–Meier survival curves as estimated from the three-record data set at the C10 concentration: green, *Macrocheles robustulus*; red, *Stratiolaelaps scimitus*; black, Parasitidae sp.
sensitivity (or even absence of sensitivity) to these concentrations in this species/population.

4 DISCUSSION

Our results showed that flat-bottomed 96-well microplates are appropriate for bioassays with mesostigmatid species living in farm soil or litter, as Nordenfors and Höglund\textsuperscript{26} and Lovis \textit{et al.}\textsuperscript{27} had already shown with other Acari species. However, important modifications were required and our study generated key information for adapting the system for application to these mites. First, a source of moisture had to be added for these moisture-requiring species to avoid their rapid dehydration. Soil- and litter-dwelling mites typically live in high-humidity environments. Although the easiest way, a priori, of providing a source of moisture (saturation of ambient atmosphere) was not feasible in the present study, the insertion of agarose slices in wells was shown to provide suitable conditions. With the agarose slices, records from control conditions showed that the system provided optimal moisture conditions up to 72 h after the beginning of the bioassay (mite mortality close to 0% in three different populations from three different families).

The process of impregnating the microplates was satisfactory for concentrations at least up to 1 g hL\textsuperscript{-1} (C3), as evidenced by the consistent and sigma-shaped curve of mortality percentage as a function of deltamethrin concentration. With concentrations greater than C3, some visible crystallization over the wall of the well was observed, making it difficult to score mite condition and determine with certainty the effective level of exposure. Therefore we recommend limiting the maximum concentration in assays to C3 and switching to another type of test in cases where mites are revealed to be highly resistant and there is a specific need to estimate median lethal concentrations.

The experiments described here allowed detection of significant differences in sensitivity levels among populations belonging to three distantly related species of mesostigmatid mites, with the most sensitive population being the commercially mass-reared population of \textit{M. robustulus} and the least one being the field-sampled population of a Parasitidae sp. The level of pyrethroid tolerance in the Parasitidae population was 68-fold higher than in the ‘intermediate’ population of \textit{S. scimitus}, based on LC\textsubscript{50} values at 48 h. Given that the two commercially mass-reared populations (\textit{M. robustulus} and \textit{S. scimitus}) stem from populations that have been protected from exposure to insecticides for hundreds of generations and that the Parasitidae population was sampled from a conventionally managed peach orchard, a crop that is among the most intensively treated with synthetic acaricides in Europe\textsuperscript{34} and in which high-level resistance against pyrethroids is widespread in populations of pest species (e.g. \textit{Myzus persicae} in Panini \textit{et al.}\textsuperscript{25}), it is not surprising that individuals of this population were much less sensitive than in the two others. However, this information must be interpreted with caution, since we could not test the same stage in this species (where deutonymphs were tested) and in the other two (adult females). The most appropriate stage for standardized tests on mesostigmatid mites is the adult female stage, because it is the most frequently sampled sex and the only stage unambiguously distinguishable using live mites (observed using a stereomicroscope). Owing to limitations in the sample we obtained for the Parasitidae sp., we were unable to test, for this species, any stage other than the largest available stage. We checked the sex and stage \textit{a posteriori} in the test microplates for this species (microscopic observation on slide-mounted individuals). The conspicuous difference in the sensitivity of this field population compared with the two lab populations is suggestive, but could also result from a stage-associated bias. It is important to keep in mind that the experiments presented here were only dedicated to the development of a protocol for bioassays and were not designed to be an extensive study.

Second, our results allowed us to propose a simple and accurate standard notation for mite response to pyrethroid insecticide. Careful records of mites’ posture and movements followed by paired comparisons of two different binary notations allowed us to show that simple discrimination between mites lying on their back and mites standing on their legs not only is less time-consuming than recording the response to mechanical stimulation, but also is a consistent indicator of the positive response to pyrethroid exposure. Similarly, other authors have preferred to consider a specific posture—as an expression of paralysis—as an indicator of positive response to pyrethroids instead of the general ability to move. Bloomquist and Miller\textsuperscript{36}, for instance, considered the inability to perform a stereotypic curling movement when probed with a warm needle as a response of pyrethroid-treated insect larvae to treatment.

Third, our \textit{in vitro} test was developed in such a way as to allow individual follow-up of exposed mites over time. This has at least two advantages: the individual monitoring (1) maximizes the accuracy of estimation of the effect of the pesticide to be tested, which is especially valuable when working with different species, and (2) allows recording awakening events, making it possible to measure the frequency of transient knockdowns, which may occur in pyrethroid resistance. Deltamethrin, like all other pyrethroids, acts by delaying the closure of the voltage-gated sodium channel. This leads to a paralysis called ‘knockdown’, described as a rapid loss of coordination of posture and locomotion.\textsuperscript{36} In a sensitive individual, death occurs after knockdown. The length of the knockdown effect may be a valuable indicator for assessing pyrethroid resistance, because a major resistance mechanism selected for by pyrethroid application on arthropods is the knockdown resistance (KDR). The strongly reduced neuronal sensitivity to this class of pesticide in knockdown-resistant (KDR) arthropods is due to amino acid substitution(s) in the target protein that cause a reduction or a loss of binding affinity with pyrethroid compounds.\textsuperscript{10} Individuals possessing the KDR allele either do not suffer this knockdown effect or may undergo a momentary paralysis, with subsequent full recovery of locomotion ability.\textsuperscript{33,37,38} Of course, in the present

\begin{table}
\centering
\begin{tabular}{|l|c|c|c|c|c|c|}
\hline
Species & Regression coefficient, b & SE of b & z & P value & HR = e\textsuperscript{b} & 95% CI of HR \\
\hline
Parasitidae sp. & −1.645 & 0.517 & −3.180 & 0.0015 & 0.193 & 0.070–0.532 \\
S. scimitus & −0.441 & 0.194 & −2.273 & 0.0230 & 0.643 & 0.439–0.941 \\
\hline
\end{tabular}
\caption{Proportional hazards model on time–mortality data (Macrocheles robustulus as reference species)\hspace{1cm}}
\end{table}
study, we did not specifically focus on such recovery, since the shortest exposure duration recorded was 24 h, while recovery may occur much earlier in the case of KDR arthropods (a few minutes in some cases). Nevertheless, such explorations are possible in mesostigmatid mites with the kinds of tests presented here, provided that intervals between records are adapted to the specific objectives of the study.

The main objective of this study was to validate a bioassay system to study insecticide resistance in different species of mesostigmatid mites inhabiting soils and litter/manure in agrosystems. The bioassay system was successfully developed and validated, and the study allowed us to identify an easily assessable response common to distantly related species of mesostigmatid mites. The generality of our results suggests that the same protocol could be used for diverse mite species, as proposed by Hammond et al., for amphibians. Lastly, while the bioassay system was developed using a pyrethroid molecule, it should be applicable in bioassays of other neurotoxic insecticides that act by contact.

To explore possible effects on mite community structure of the release from selective pressure for resistance, we plan in the longer term to compare the fitness, in the absence of pesticides, of resistant populations of species belonging to soil/litter mite communities in agrosystems into which mesostigmatid mites have been artificially introduced and communities free of such introductions. Comparison of community structure between ‘conventional’ farms and others that have recently converted to organic agriculture should allow progressing the question of possible adverse effects of pesticide pressure release.

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SUPPORTING INFORMATION
Supporting information may be found in the online version of this article.

REFERENCES


