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ARTHROPODS AND MEDICAL ENTOMOLOGY - ORIGINAL PAPER

Insecticide resistance in stable flies (*Stomoxys calcitrans*) on dairy farms in Germany

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Abstract

Stable flies (*Stomoxys calcitrans* Linnaeus, 1758) can have a considerable negative impact on animal well-being, health, and productivity. Since insecticides constitute the mainstay for their control, this study aimed at assessing the occurrence of insecticide resistance in *S. calcitrans* on dairy farms in Brandenburg, Germany. First, the susceptibility of stable flies from 40 dairy farms to a deltamethrin-impregnated fabric was evaluated using the FlyBox® field test method. Then, *S. calcitrans* strains from 10 farms were reared in the laboratory, and the offspring was tested against the adulticides deltamethrin and azamethiphos and the larvicides cyromazine and pyriproxyfen. The FlyBox® method indicated 100% resistance in stable flies against deltamethrin. Later, to the offspring of those 10 established laboratory strains previously caught on suspected dairy farms, these field findings could be confirmed with mortalities well below 90% 24 h following topical application of the calculated LD_{95} of deltamethrin and azamethiphos. The ten strains could therefore be classified as resistant to the tested insecticides. In contrast, exposure to the insect growth regulators cyromazine and pyriproxyfen at their recommended concentrations demonstrated 100% efficacy. Both larvicides inhibited the moulting process of the stable fly larval stages completely, showing that the stable fly strains tested were susceptible to them. The intensive use of insecticides in recent decades has probably promoted the development of insecticide resistance. Systematic surveys in different livestock production systems and vigilance are therefore deemed necessary for estimating the risk of insecticide resistance development on a nationwide scale.

Keywords Stable fly · *Stomoxys calcitrans* · Insecticide resistance · Topical application · Deltamethrin · Azamethiphos

Introduction

Stable flies (*Stomoxys calcitrans* Linnaeus, 1758) are serious pests of livestock, companion animals, and humans. They are vectors of certain blood-borne pathogens (Baldacchino et al. 2013), and the nuisance and stress caused by their painful bites can reduce the productivity of livestock and lead to suffering in companion animals and humans. Livestock production systems like dairy farms act as ideal sources for the viability and reproduction cycle of flies. In cows, the pain caused by bites from stable flies can result in a decrease of pasture time by inducing defensive behaviour (Dougherty et al. 1993; Taylor et al. 2012). It may also lead to less time spent in repose and bunching (Berry et al. 1983). Even though bunching decreases the attack rate of stable flies on individual animals, it increases heat stress and reduces weight gains (Catangui et al. 1993). Therefore, insecticides represent the mainstay for stable fly control. The annual economic loss to the US cattle industry caused by *Stomoxys calcitrans* is estimated to be of about 2.2 billion dollars (Taylor et al. 2012).

Unfortunately, little is known about the efficacy of insecticides against stable flies in Germany. Thus, the aim of this study was to assess the use of different insecticides and to evaluate the occurrence of insecticide resistance in stable flies on dairy farms in the federal state of Brandenburg. Finally, its

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ultimate objectives were to raise the farmers' awareness on an adequate use of insecticides, to develop strategies for a better integrated pest management, and to minimize both the use of insecticides and the risk of resistance development. The study was based on previous surveys which indicated resistance in *Musca domestica* against several adulticides in husbandry systems in Germany (Hildebrand 2017; Jandowsky et al. 2010). Since, however, three forth of the flies in livestock production systems were observed to be stable flies (Sømme 1958), the assessment of the occurrence of insecticide resistance in stable flies in Germany seemed to be long overdue.

Insecticides that are no longer able to control livestock pests put an unnecessary strain on the environment and human health. Therefore, in 2018, the European Commission decided in cooperation with the European Food Safety Authority (EFSA) on an EU wide ban for the outdoor use of three neonicotinoid pesticides. This is anticipated to protect pollinating insect populations and, thus, to inhibit the ensuing drastic reduction of natural pollination (Kirchner and Liebrich 2017).

After a previously implemented questionnaire analysis on the frequency and mode of insecticide use, stable flies from 40 dairy farms were exposed to a deltamethrin-impregnated fabric using the FlyBox® method. For confirmatory tests, *S. calcitrans* strains from 10 farms were reared in the laboratory. There the susceptibility of the F_1, F_2, and F_3 generations was analyzed under controlled conditions against both the synthetic pyrethroid deltamethrin and the phosphoric acid azamethiphos, each by topical application of the calculated discriminating dose (LD_{95}) and multiples of it. In addition, the larvicidal efficacy of two commonly used insect growth regulators cyromazine and pyriproxyfen was assessed at different concentrations based on the manufacturers' recommendations.

Materials and methods

Questionnaire analysis

To evaluate the local stable fly abundance and the use of insecticides and to gather data regarding the animal husbandry systems, a questionnaire analysis was performed on 52 respondent dairy farms in the federal state of Brandenburg, Germany. In total, 78 dairy farms had been selected. Sixty farms corresponded to farms of a prior house fly study in 2008 (Jandowsky et al. 2010), and eighteen dairy farms were recommended by local veterinarians. All of them had been derived from a list of the best dairy farms registered in Brandenburg based on milk yield and quality. In compliance with the farmers, dairy farms were included into the study on the condition that they had at least 50 dairy cows and more than five flies per cow.

Insecticides

The deltamethrin-impregnated fabric PermaNet® with a concentration of 55 mg/m^2 was manufactured by Vestergaard-Frandsen (Lausanne, Switzerland). The pure substances deltamethrin and azamethiphos were produced by PESTANAL® and purchased from Sigma-Aldrich (Steinheim, Germany). Cyromazine was acquired as the commercial product Neporex® 2SG 20 mg/g from Sigma-Aldrich (Steinheim, Germany). The commercial product Archer® which contained pyriproxyfen was produced by Syngenta® Crop Protection AG (Basel, Switzerland).

Determination of resistance

The degree of resistance was based on thresholds proposed by the WHO (2016) for mosquitoes. Thus, a mortality rate below 98% at the established discriminating concentration (DC) indicates possible resistance, while a mortality rate below 90% can be considered high resistance (WHO 2016). A degree of suspected (90–97% mortality) or high resistance (below 90% mortality) must be verified with five× and 10× the DC, respectively, and a mortality rate at or below 98%. The proposed resistance scheme of the WHO was slightly modified in this study: every established strain was evaluated in the laboratory at the LD_{95} (one fold of the discriminating concentration), the fourfold of the LD_{95} and the 16-fold of it according to previous studies on house flies (Hildebrand 2017; Jandowsky et al. 2010).

Reference strains

For the FlyBox® test, the *Stomoxys calcitrans* strain (2015) of the Federal Environment Agency (UBA) in Berlin, Germany, served as a sensitive reference for deltamethrin. The strain had originated from a stable fly strain, which had been isolated by Novartis Animal Health Inc. CRA St. Aubin at the Centre de recherche animale, St. Aubin, Switzerland. A second *Stomoxys calcitrans* strain kindly provided by MSD Animal Health Innovation GmbH (Schwabenheim, Germany) served as the MSD reference strain in each of the laboratory tests. This susceptible strain had been isolated by Hoechst AG in Frankfurt, Germany, and was subsequently transferred to MSD Animal Health in the 1990s.

Rearing method

The fly populations were kept at 25 °C and 50% relative humidity and under a 12-h light cycle. They were maintained in cages consisting of a 14 cm × 30 cm bottom panel and two wire bows (Ø = 28 cm) and were covered with gauze. The flies were fed daily with preheated citrated bovine blood collected from a nearby abattoir. Blood was collected weekly in

one-liter autoclaved glass bottles containing 102.4 ml sodium citrate (3.13%) to prevent coagulation. It was stored for up to 1 week at four °C. To induce oviposition in the five-to seven-day-old flies, a 500-ml plastic cup was half filled with medium and placed in the fly cage for three days. The medium consisted of 1.5 kg of lucerne meal, 1.5 kg of shredded wheat bran, 84 g of dry yeast, 208 g of sugar, and seven liters of tap water. It was stirred daily and fermented for three days at 25 °C room temperature.

FlyBox® test

The FlyBox® test method was developed by Dr. Burkhard Bauer and performed in accordance with previous studies (Hildebrand 2017; Jandowsky et al. 2010). The FlyBox® consists of a folded cardboard box of five cm × 18 cm × 8 cm (Co. FAPACK, Berlin). A small flap at one end of the box allows transferring insects with the aid of a test tube into the box. When opening the other larger end of the box, insects can be released. The inside of the box was lined with a net fabric by Vestergaard-Frandsen (Lausanne/Switzerland) containing 55 mg/m^2 deltamethrin. The impregnated polyester fabric was cut down to the size of the FlyBox® and attached to the box with staples. Since the number of stable flies varied among farms, between 10 and 90 flies were used per test. Stable flies were caught with a special scoop net that at the end had an evagination sewed on which facilitated the transfer of the flies into a test tube. With the assistance of the test tube, stable flies were released into the FlyBox®. Inside the dark, closed FlyBox®, the flies sat down upon the impregnated fabric and were exposed to the contact insecticide. After an exposure time of 10 or 30 s, the flies were released into an observation cage which was of the same size as the aforementioned breeding cages. It contained a water-soaked cotton pad to prevent the flies from dehydration. The mortality of the flies was recorded after one, five, 10, 15, and 60 minutes, and six and 24 h. Flies were considered dead when they were lying on their side or back.

Topical application

Based on the outcome of the FlyBox® tests, *Stomoxys* populations from 10 farms were caught and reared in the laboratory. In the questionnaire, seven of the selected dairy farms had claimed to have a high fly abundance and to apply insecticides regularly. One farm was associated with the Demeter-Wirtschaftsverbund, which precludes the application of insecticides, and two of the selected farms had not used insecticides in the past three years by choice.

Further investigations were performed in the laboratory with the progeny (F_1 to F_3 generations) of the established field strains. The insecticidal activities of deltamethrin and azamethiphos were determined by topical application using the electronic dispensing system EDOS 5222 (Eppendorf GmbH, Wesseling–Berzdorf, Germany), with which 1 μl of acetone-diluted insecticide was applied to the dorsal surface of the stable flies' thorax. Solvent only was applied as a control. In comparison with the FlyBox® test, the forced contact with the insecticide provided absorption of a known amount of active ingredient. Five- to 10-day-old stable flies were removed from the breeding cages by letting them crawl or fly into a test tube and immobilized by placing those tubes on ice for five minutes. The test group was comprised of five female and five male flies. After their treatment was applied, flies were placed in 250-ml plastic cups. The cups contained a 10% water-sugar solution in small test tubes sealed with dental swabs to prevent the flies from dehydration. Fly mortality was recorded after 24 and 48 h.

Calculation of the LD_{95}

To detect resistance in the flies receiving topical applications, mortality values of the field strains were compared with the LD_{95} of the sensitive laboratory "MSD" strain.

Moyses and Gfeller had estimated LD_{95} values using the topical application method for deltamethrin and azamethiphos based on two times 10 stable flies for each serial dilution (Moyses and Gfeller, unpublished). This number of tested flies was, however, not conclusive. Since other significant data based on a reliable number of flies was not available, the LD_{95} was newly defined through a dose-range-finding test against both active ingredients with the established laboratory strain of MSD Animal Health Innovation GmbH. As a rough orientation, first, a serial dilution by a factor of 10 was prepared around the reported LD_{95} values by Moyses and Gfeller. To adjust the LD_{95} as precisely as possible, in a second step, another serial dilution around the potential LD_{95} concentrations was set up. The generated data were then evaluated with the statistics software GraphPad Prism® (GraphPad Software Inc., CA, USA). Generated data were logarithmically transformed first. Then, they were analyzed logarithmically by a dose/effect regression model. The R^2 (coefficient of determination) was used for depicting the proportion of variance in the dependent variable that can be derived from the independent variable. The LD_{95} of both deltamethrin and azamethiphos was established to be 2.34 ng/μl and 4.92 ng/μl, respectively. All ten stable fly field strains were topically treated with the LD_{95}, a fourfold of the LD_{95}, 9.36 ng/μl and 19.7 ng/μl, respectively, and a 16-fold the LD_{95}, 37.5 ng/μl and 78.6 ng/μl. Each concentration was tested three times with ten stable flies at a time.

Larvicide tests

The susceptibility of the stable fly test populations to the triazine derivative cyromazine and pyriproxyfen, a derivative of juvenile hormone analogue fenoxycarb, was assessed by larvicide tests. The serial dilution of the test concentrations was

based on the dose recommended by the manufacturer, which is 5 μg and 0.027 μg per gramme of rearing medium for cyromazine and pyriproxyfen, respectively. Four cyromazine medium dilutions (1.25 μg/g, 5 μg/g, 20 μg/g, 80 μg/g) and five pyriproxyfen medium dilutions (0.005 μg/g, 0.01 μg/g, 0.05 μg/g, 0.1 μg/g, 1 μg/g) were examined. To obtain the serial dilutions in the rearing medium, a tenfold stock solution of the highest concentration was prepared in water and subsequently diluted with water at the decided ratio according to the scheduled concentrations. Finally, 30 ml of each concentration was mixed with 270 g of the larval medium (ratio 1:10). Then, three 500-ml plastic cups were filled with 75 g of larval medium. For each concentration, 30 to 50 *Stomoxys* eggs were put onto a 1.5 × 1.5 cm piece of filter paper which then was placed in the medium in each cup. The sensitive "MSD" strain served as a control for each test. Two days after treatment, the number of hatched eggs was determined under a stereoscope. Then, the larval medium in each cup was covered with one-centimeter thick layer of sawdust to prevent desiccation. The cups were kept at 25 °C at 50% relative humidity. Three to five weeks later, the number of developed imagoes was counted and the efficacy of the larvicides was calculated.

Data analysis

The mortality of the field strains was adjusted with Abbott's formula (Abbott 1925). The efficacy was calculated and classified according to both the formula and definition of resistance as recommended by the WHO for malaria mosquitoes (WHO 2016). The mortalities of the field and the MSD strains were statistically examined with the software SPSS version 22, IBM. Results with a p value below 0.05 were categorized as statistically significant.

Results and discussion

Questionnaire analysis

Of the 52 dairy farms included in the questionnaire analysis, 78.9% had employed insecticides against flies and 34.6% had applied physical control methods like sticky tapes or calcium hydroxide. Eleven percent of the farmers had not used any type of control measure in the past 10 years. None of the farm managers used potential biological control measures such as the pteromalid wasp (Geden and Moon 2009; Skovgård and Jespersen 1999) or *Bacillus thuringiensis* (Lysyk et al. 2010). The collection of data turned out to be quite problematic since most of the employees did not have an overview over the applied insecticides, nor did they know the exact time span between two subsequent applications. Only 29 out of the 52 (55.8%) dairy farmers were able to name the insecticides that had been used. Since it is vital for an active ingredient to be applied at the lethal dosage to prevent resistance development, it would have been of interest which products at what kind of dosages had been used. On 25 of the 29 dairy farms (86%) where there was some knowledge about the kind of products that had been employed, pyrethroids had been applied. According to the questionnaire analysis, the application of the larvicide cyromazine was limited to one out of 29 farms (3%). Three of the 29 farms (10%) used organophosphates. Spinosyns and neonicotinoids, both feed-through biocidal insecticides, were applied by one (3%) and two (7%) of the farms, respectively. Within the insecticide class of pyrethroids, deltamethrin was the most frequently used pyrethroid at a percentage of 41%. Thirty-one percent of the 29 farmers claimed to have used two or more chemical compounds against flies at the same time. Thirty-eight percent had changed the product during the past three years. In 30% of those cases, however, the products belonged to the same class of insecticides. Only three out of the 52 farms interviewed (5.8%) claimed to make use of a combination of an adulticide and a larvicide. Thirty-one percent of the dairy farmers employed insecticides three to five times a summer, 34% used insecticides whenever needed, and six % of the farmers applied insecticides daily. Only 15% employed them once a summer. Eleven farmers (21%) were not satisfied with the result of the control measure.

Seventy-nine percent of the cows on the dairy farms were kept in free stall housing systems with open cubicles. Nineteen percent were reared in deep litter stables, and one of the 52 dairy farms still practised the tethered housing system. However, the chi-squared test could not discover any statistically significant difference between the fly occurrence in open cubicle–type stables with slatted floor and deep litter stables (X^2, $p = 0.152$). Even when comparing the fly abundance in stables based on the predominant flooring conditions, the chi-squared test revealed no significant difference between stables where there was litter in several areas and stables where there was litter only in the calving and calf rearing areas (X^2, $p = 0.294$). Accordingly, neither did the dung storing system appear to be significantly related to the degree of stable fly abundance (X^2, $p = 0.473$). This suggests that regardless of the housing conditions, flooring systems, or dung storage arrangement, any management system can promote the reproduction cycle of *Stomoxys calcitrans*. All systems visited, however, were relatively similar: they were never entirely litterless and the slurry was never kept away. The frequency of using insecticides as was claimed by the famers was not in accordance with the information on the package leaflet of the veterinary medicinal products they had indicated to have applied. Additionally, when employing wall sprays, they never wore skin or mouth protection during the application, which also indicates that they were not always aware of the recommendations given by the respective safety data sheet concerning the safe use of the insecticide for the operator, the animals, and

the environment. In conclusion, farmers as well as veterinarians need to be more sensitized to the correct use of insecticides, to the specific danger for the environment, and to the concept of integrated pest management.

FlyBox® test

Following studies on house flies (Hildebrand 2017; Jandowsky et al. 2010), field tests using the FlyBox® test were performed (see "FlyBox® test") in order to get a first estimation concerning the susceptibility of the stable fly farm populations to deltamethrin. The FlyBox® is a self-dosing test, similar to the tests suggested by the WHO (2016) for malaria mosquitoes. The practicability of this test was extensively proven by Hildebrand (2017) and Jandowsky et al. (2010). As aforementioned, the inside of the FlyBox® was lined with a bed net impregnated with 55 mg deltamethrin per square metres. However, information regarding the discriminating dose and time of exposure for stable flies to pyrethroids as contact insecticide varies in literature. Cruz-Vázquez et al. (2005) successfully employed permethrin at a concentration of 0.026 mg/m^2 (LD_{99}) on a glass vial for a two hour contact against stable flies. Salem et al. (2012) determined a LD_{90} of 264.3 mg deltamethrin per square metres for a resistant field population and 28.1 mg deltamethrin per square metres for a sensitive stable fly population after contact with treated filter papers for one hour.

Thus, in comparison with other FlyBox® field surveys on *Musca domestica* where the deltamethrin concentration had been 100 mg/m^2 and 280 mg/m^2 (Hildebrand 2017; Jandowsky et al. 2010), the choice of a lower net concentration was deemed to be better suited in this study. In contrast to other self-dosing tests on stable flies (Cruz-Vázquez et al. 2005; Salem et al. 2012), where the time of exposure had been one or two hours, in this study, the flies were exposed for only 10 or 30 s. A one- or two hour time of exposure could lead to physical and physiological stresses, which could then result in a higher mortality rate or in a decrease of other physiological functions. Furthermore, due to the irritating effect caused by the insecticide, under natural circumstances, a time of exposure which exceeds one or two minutes is not expected.

When assessing a 55-mg/m^2 impregnated fabric against the susceptible laboratory stable fly strain "UBA" of the Federal Environmental Agency in Berlin, the concentration turned out to be effective. Nevertheless, for future testing, the strength as well as the time of exposure of deltamethrin should be standardized to obtain comparable results. For each of the two exposure times, 10 and 30 s, four tests were performed on the susceptible strain "UBA". To verify the effectivity of the attached impregnated fabric, the test was repeated after two and six months. A mortality rate of 100 ± 0% was reached after 15 minutes. After six h, the medium mortality rate was 99 ± 1.5% and after 24 h, it had decreased to 95 ± 5.7%. This may be caused by a reversibility of the knockdown effect of deltamethrin. When 24 h had passed, a medium of 5 ± 5.7% stable flies were no longer paralyzed. Very similar results were detected when exposing stable flies for 30 s to deltamethrin. When flies are paralyzed after six h of observation, they are considered dead according to the definition of the WHO (WHO 2016). Thus, the concentration of the impregnated fabric and the times of exposure of 10 and 30 s could be considered adequate.

The results of the 40 field populations differed strikingly from those of the susceptible "UBA" strain. At all times of exposure as well as times of observation, the sensitive laboratory strain was significantly more susceptible to the tested insecticide, deltamethrin, than the field strains (the Mann-Whitney U test, $p < 0.001$).

After 10 s of exposure and 15 minutes of observation, the field stable fly strains showed a mortality rate of 32.8 ± 21.1% on average. After one hour, the mortality rate was at 39.4 ± 24%. After six h, it was at 37.2 ± 23% and after 24 h, it was at 36.7 ± 23.7%. None of the tested field strains was susceptible to the deltamethrin-impregnated polyester fabric (Fig. 1). Thus, according to WHO (2016), after six h of observation, 100% of the strains were classified as resistant at a mortality rate below 90%. Forty-nine percent even displayed a mortality rate below 40%.

Comparable results were observed after an exposure time of 30 s. The mortality rates after 10- or 30-s exposure time did not differ significantly from each other (the Mann-Whitney U test, $p = 0.617$). Thus, all the 40 tested populations (100%) were classified as resistant in the FlyBox® test. Eighty-six percent of the farmers had employed a pyrethroid with deltamethrin being the most frequently used active ingredient within this group (41%). Hence, it is not surprising that 100% of the 40 tested fly populations were resistant against the pyrethroid in the FlyBox® test. In other studies, side resistance against the synthetic class of pyrethroids was demonstrated (DeVries and Georghiou 1980; Jandowsky et al. 2010). Consequently, following the results of the FlyBox® test, the class of the synthetic pyrethroids should no longer be considered effective means of control.

The staff of ten farms had claimed that no insecticide had been used during the past 10 years. Nevertheless, stable fly strains from seven of those farms were still categorized as "resistant" according to the FlyBox® test. This indicates that resistance development is not a local problem; it can rather be spread either from the neighbouring agricultural crop land or from conventional animal husbandry to organic farms. Resistant fly material from adjoining livestock can be moved from one farm to another by flight and wind drift or as developmental stages in manure.

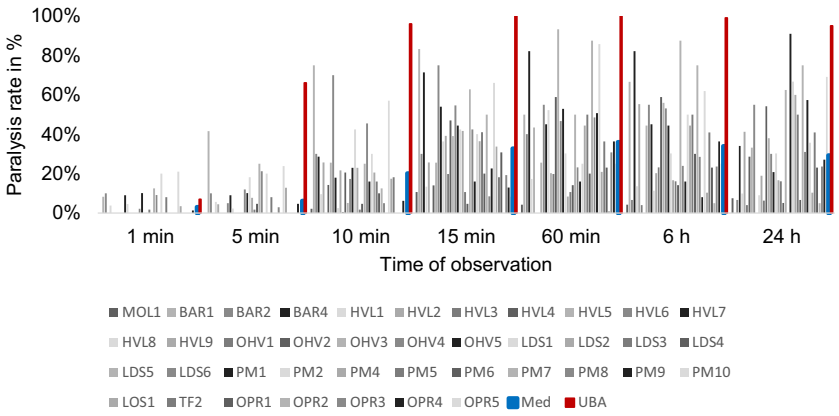

Fig. 1 Results of the FlyBox® tests. FlyBox® tests with a deltamethrin (55 mg/m^2) bed net after 10 s of exposure according to Abbott (1925); grey = mortality rates of the field strains, blue ("Med") = mean mortality rates of the 40 field strains, red ("UBA") = mean mortality rates of the sensitive "UBA" strain, GraphPad Prism 7

Topical application

Determination of the LD_{95}

When evaluating the topical application tests on the susceptible "MSD" strain for azamethiphos, the LD_{95} value was calculated to be 4.92 ng/μl (Fig. 2). The mortality rates did not differ much from each other. Hence, the coefficient-of-determination values (R^2) for the times of observation of 24 and 48 h were at 0.97 and 0.95, respectively, which can be interpreted as good.

However, evaluating the LD_{95} of deltamethrin with the "MSD" strain turned out to be rather challenging. The mortality rates varied by a power of 10 between 0.234 ng/μl and 2.34 ng/μl. In addition, five % of the susceptible laboratory flies recovered from the knockdown effect after 48 h of observation. Unlike azamethiphos, which inhibits the cholinesterase activity in the synapses of the neurons, deltamethrin prevents the sodium channels of the neurons from closing. This initially leads to agitation followed by paralysis. The latter is, however, known to be potentially reversible (Sfara et al. 2006). The R^2 values for an observation time of 24 and 48 h resulted in 0.61 and 0.69, respectively, and can therefore be categorized as moderately good. After correcting the mortality rates according to Abbott (1925), the LD_{95} was at 1.00 ng/μl for an observation time of 24 h and at 1.45 ng/μl for an observation time of 48 h (Fig. 2). The determination of the LD_{95} of deltamethrin was repeated later with the laboratory reference strain from the Federal Office of Environment "UBA". Again, the mortality rates varied as already observed with the "MSD" reference strain. For the "UBA" reference strain, a LD_{95} value of 2.36 ng/μl at 24 h and a LD_{95} of 6.65 ng/μl at 48 h were determined. The somewhat differing susceptibility against insecticides within the two laboratory strains, "MSD" and "UBA", can, however, be attributed to the occurrence of natural variations (Kristensen et al. 2001; Schaub et al. 2002; Scott et al. 2000) and appears to be common within stable fly populations independently from previous selection pressure through insecticides (Marçon et al. 1997). Since the mortality rates fluctuated very strongly, the LD_{95} value of 2.34 ng/μl determined by Moyses and Gfeller (unpublished) was used in the laboratory tests with the field strains. However, the aforementioned LD_{95} values, that were determined in the laboratory with the "MSD" and "UBA" strains, can be considered to be approximately of the same magnitude. Contrary to the sensitive *Musca domestica* strain "WHO", there is, at present, no international reference strain available for stable flies. Since in this study, LD_{95} values have been evaluated for both the "UBA" and "MSD" stable fly strains, both could serve as susceptible references for future insecticide testing.

In future studies, it would be more adequate to calculate resistance factors for each field population tested. This would make data even more comparable throughout the world.

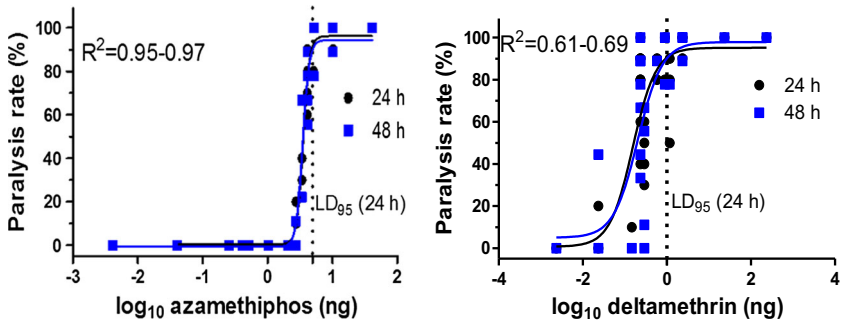

Fig. 2 Determination of the LD_{95}. Determination of the LD_{95} of azamethiphos and deltamethrin after topical application with the sensitive reference stable fly strain "MSD" at 24 h with an R^2 value of 0.95 to 0.97 for azamethiphos and an R^2 value of 0.61 to 0.69 for deltamethrin, Excel 2013

Field strains

The topical application of 4.92 ng of the pure substance azamethiphos dissolved in 1 μl of acetone on the ten stable fly field populations resulted in a mean mortality rate of 16.37 ± 15.9% after 24 h (Fig. 3). This can be classified as highly resistant according to the WHO (2016). A fourfold increase in the initial azamethiphos concentration to 19.66 ng/μl resulted in a mean mortality rate of 81.13 ± 15.0% after 24 h, confirming the classification as highly resistant. In the highest concentration tested (a 16-fold of the "MSD" strain's LD_{95}), a mean mortality rate of 95.8 ± 5.2% was observed after 24 h. Since this value is below 98%, a high resistance was confirmed according to the WHO classification system (WHO 2016). Mortality rates at both points post application, 24 and 48 h, did not differ significantly from each other (the Mann-Whitney *U* test, $p = 0.631$). Considering each one of the ten populations 24 as well as 48 h after the topical application of the LD_{95} of 4.92 ng azamethiphos per microliters, the mortality rates were below 90% and, thus, in the WHO range of resistance. Accordingly (WHO 2016), it could be concluded that all these populations individually, and not only their mean, were resistant to the tested insecticide at the LD_{95}.

The topical application of 2.34 ng of technical deltamethrin dissolved in 1 μl of acetone (LD_{95}) revealed a mean mortality rate of 22.7 ± 12.7% after 24 h (Fig. 4). A fourfold increase of the LD_{95} to 9.36 ng/μl resulted in a mean mortality rate of 55.3 ± 14.5% after 24 h of observation. The topical application with a 16-fold increase of the LD_{95} to 37.49 ng/μl enhanced the mean mortality rates to 87.9 ± 11.6% ng/μl after 24 h. According to WHO (2016), this indicates high resistance in all populations tested. Comparing the mortality rates of the topical application of deltamethrin after 24- and 48-h observation time, no significant differences were detected (the Mann-Whitney *U* test, $p = 0.853$). Due to the mortality rates of all ten tested field populations being below 90% at both time points, a resistance against deltamethrin at the LD_{95} was evident (WHO 2016). These findings supported the previous results of resistance against deltamethrin in the field adapted FlyBox® test. A substantial agreement of the results of the FlyBox® test and the topical application showed Cohen's kappa coefficient ($k = 0.615$, $p = 0.035$), suggesting that the same qualitative results can be expected with both test methods.

All stable fly strains tested could be classified as resistant against azamethiphos and deltamethrin. For deltamethrin, the results were largely in line with the extensive use of synthetic pyrethroids on the ten farms tested. For azamethiphos, however, the results must be considered unexpected since none of the ten farms included in the study had mentioned the use of organophosphates in the past. This can be attributed either to the lack of knowledge of the interviewed farmers or to cross-resistance to other more frequently used insecticides (Bisset et al. 1997; Devonshire and Moores 1982; Liu and Yue 2000).

Larvicide tests

The results of the larvicide tests differed from those of the adulticides. When testing the larvae on medium mixed with cyromazine, all tested concentrations including the dose recommended by the manufacturer (5 μg/g) inhibited the development to stable fly imagoes completely (100 ± 0.7%). Previous studies in Germany had shown similar results for cyromazine on house flies (Hildebrand 2017; Jandowsky et al. 2010). However, several other authors found flies to be resistant against cyromazine. It should be noted, however, that these results occurred primarily in countries where cyromazine had been used as a feed additive on chicken farms (Acevedo et al. 2009; Bell et al. 2010; Pinto and Prado 2001).

With regard to pyriproxyfen, the inhibition rate of two out of the ten field populations tested (20%) at a concentration of 0.005 mg/kg was calculated to be 93.3 and 96.7%, respectively. At this concentration in the remaining test populations, the inhibition rates of pupa hatching were 100 ± 0%. At a dosage of 0.01 μg/g, the inhibition rate of pupa hatching was 96.7% in one stable fly population and 100% in all the other populations. Concentrations above the recommended dosage of 0.027 μg/g caused 99% inhibition for one and 100 ± 0% for the remaining field populations at 0.1 μg pyriproxyfen per gramme of rearing medium. Since pyriproxyfen is presently not available as an insecticide for livestock in Germany, those

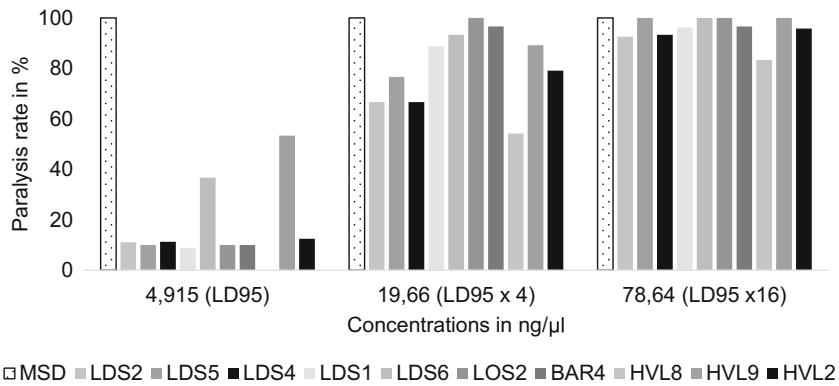

Fig. 3 Laboratory results of azamethiphos. Results of the topical application of azamethiphos after 24 h corrected according to Abbott (1925), a comparison of the results of the sensitive stable fly "MSD" strain (dotted) with those of the 10 field populations (grey), October–November 2015, Excel 2013

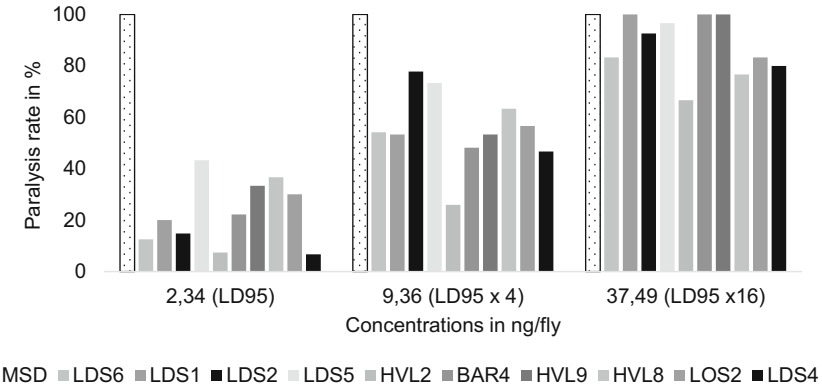

Fig. 4 Laboratory results of deltamethrin. Results of the topical application of deltamethrin after 24 h corrected according to Abbott (1925), a comparison of the results of the sensitive stable fly "MSD" strain (dotted) with those of the 10 field populations (grey), October 2015, Excel 2013

results confirmed the expected good efficacy of the IGR. Other authors (Pospischil et al. 1996; Shah et al. 2015a), however, did observe resistances in house flies against pyriproxyfen. Among them, Pospischil et al. (1996) described a resistance factor of 53 in a *Musca domestica* strain in Germany that had never been in contact with IGRs. Additionally, resistances against IGRs can also be artificially generated by serial selection as demonstrated by Keiding (1999) and Shah et al. (2015b) under laboratory test conditions. Thus, it could be expected that the constant exposure of the flies to IGRs would promote the selection of resistant populations as already observed in adulticides.

Conclusions

It has been shown that there is a widespread resistance of insect target species against many insecticides that are currently in use. This is particularly the case for the insecticide class of pyrethroids. At present, our knowledge is, however, limited to different livestock production systems in Brandenburg (Jandowsky et al. 2010) and Schleswig-Holstein (Hildebrand 2017). Further surveys in different production systems covering all federal states of Germany would therefore constitute a first and indispensable step of enhancing our knowledge. So far, we have ample evidence of an indiscriminate and non-strategic use of insecticides. If insecticides are to be applied, any application should be based on a sound knowledge of behaviour, biology, and insect physiology, which is apparently missing. Another problem appears to be the residual efficacy of most commercially available products. Especially pyrethroid wall sprays inside stables are protected from ultraviolet radiation and can therefore be effective for more than six months. The resulting long-term exposure to those insecticides is expected to enhance the development of resistances. Instead of relying on the use of residual pyrethroids, emphasis should be rather put on applying short-lived pyrethrins in combination with synergistic chemicals, for instance, piperonyl-butoxide (PBO). This strategy has been adopted in Denmark with quite some success (Kristensen, pers. communication). As we have observed that resistance of target insects against residual pyrethroids appears to be non-reversible, there is need to focus on the strategic and targeted applications of those insecticides and biocides that have shown to be effective. However, in a first step, it is recommended to rely on proper dung management and hygiene that can go a long way of achieving measurable suppression. Elsewhere, it has been reported that the release of sterile male stable flies (Patterson et al. 1981) or susceptible flies (Imai 1987) helped to substantially reduce target fly populations. Ultimately, insecticides should therefore, if ever, be considered a last resort for control.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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