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Sabina Bajda-Wybouw

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Genetics and fitness costs of acaricide resistance in spider mites

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General introduction

1.1. The pest problem

In the Holocene epoch, the growing human population density initiated a shift from a hunting-gathering lifestyle to a sedentary agricultural way of living (Richerson et al., 2001). As human settlements grew, so did their demands for food resources. Hence, more and more organisms, once considered as harmless co-inhabitants of agricultural lands, rose to the status of pests. A pest is usually defined as any living organism that potentially poses a threat to humans or human interests, livestock, or construction. Currently, approximately 40% of the global pre-harvest agricultural production is lost due to pests including insects, mites, plant pathogens, and weeds (Pimentel and Peshin, 2014). Damage caused by arthropod herbivory mounts up to 18-26%, and translates in to an annual economic loss of US$ 470 billion (Oerke and Dehne, 2004; Pimentel and Peshin, 2014).

In their natural environment, plants and herbivorous arthropods have been interacting for over 400 million years, developing adaptations and counter-adaptations against each other (Labandeira, 2002, 2006). It has been hypothesized that the (co)evolution of interacting species leads to a state where the probability of extinction is fairly constant over time. In other words, the evolutionary dynamic relationships have kept populations of plants and herbivores in relative balance (Jones and Dangl, 2006; Labandeira, 2007; Van Valen, 1973). Hence, in natural ecosystems, damage due to herbivory has the tendency to be minor (Mattson and Addy, 1975; Soholt, 1973). This raises the question of why agricultural production is so vulnerable to damage by arthropods and other pest species. The answer may lie in the structure of modern agriculture. Below, I outline why agricultural intensification, crop domestication, and artificial selection of elite cultivars have made agro-ecosystems unstable and dependable on continuous human intervention.

Modern practices in agriculture tend to reduce agro-ecosystem species diversity through, e.g., practicing monoculture and decreasing crop rotation. Maintaining agricultural biodiversity is important for several reasons. For instance, it ensures appropriate fertility and structure of the soil and prevents soil erosion as well as increases the chances for crop pollination and efficient biological control (Pimentel et al., 1997). Expansion of farmlands comes at the expense of marginal habitats like hedges and grassy fields, which typically provide refuge for many arthropod species. The elimination of these refuge hot spots leads to less complex communities from which predatory and beneficial arthropods are typically lost. These communities shift to an increased abundance of specialist species feeding on the cultivated plants (Matson et al., 1997). Yield optimization leads to higher biomass accumulation, which, however, also speeds up the growth of unwanted arthropod communities. Additionally, lack of crop rotation supports pest community buildup over multiple cropping seasons, since life cycles of arthropods thriving on the crop are not interrupted (Bullock, 1992; Vitousek and Reiners, 1975).

A second major reason why modern agrosystems face an increasing pest problem is related to crop domestication and breeding practices that suits man's needs of high yield, large size, and high esthetic and nutritional quality. The process of plant domestication can be described as selecting heritable changes that increase the economic output of the plants. Unfortunately, it simultaneously decreases the genetic diversity of crop plants (Bevan et al., 2017; Chaudhary, 2013). Hence, these 'elite cultivars' can be less genetically equipped to resist phytopathogens and herbivorous arthropods, which in turn impedes the long-term sustainability of agricultural production. For instance, the wild apple *Malus sieversii*, a major progenitor of the cultivated *Malus domestica* (Velasco et al., 2010), displays high levels of both phenotypic and genetic diversity and has been found a desirable source for disease and pest resistance in cultivar breeding (Velasco et al., 2010; Yin et al., 2013). Similarly, it has been observed that *Helicoverpa armigera* shows a considerable reduction in larval performance and survival when it feeds on three wild relatives of chickpeas, *Cicer microphyllum, C. canariense,* and *C. macracanthus,* compared to the domesticated variety (Chaudhary, 2013).

Global warming presents yet another challenge to agriculture. Theoretical models predict that rises in temperature will reduce crop yield and at the same time induce the proliferation of pest species (Mathukumalli et al., 2016; Nelson et al., 2009). Furthermore, the dynamics of phytopathogens may also be altered by shifting their range of vectors, which in turn can compromise the immune response of crops (Garrett et al., 2006). Global warming will lead to shifts in the spatial and temporal distribution of arthropod species and will alter their phenology (Sharma, 2014). For instance, a large scale modeling study on coleopteran and lepidopteran pests in Swedish forests predicts a considerable increase in species' distribution areas, which may result in unprecedented outbreaks (Hof and Svahlin, 2016). In a study based on species prevalence, variability in environmental responses and modeling, Meynard et al. (2013) predict that global warming will shift the threat of the tomato red spider mite, *Tetranychus evansi,* invasion towards northern Europe and some other temperate regions in the Americas while tropical regions in Africa will be at lesser risk.

Currently, the most accessible and efficient ways of crop protection are achieved through application of pesticides/acaricides or in case of BT toxin, engineering artificial plant resistance. As a response, arthropod species typically evolve resistance to the applied control measures (Pimentel and Peshin, 2014; Van Leeuwen and Dermauw, 2016). Worse still, in several cases, pesticide application has led to the selection of so-called 'super pests', arthropod populations that developed resistance to virtually any chemical agent used for their control (TABLE 1.1). In SECTION 1.2.2, I elaborate further on chemical control of pests and resistance development.

1.2. ARTHROPOD HERBIVORES

Arthropods are invertebrate animals that own their name to characteristic jointed appendages (from the Greek arthron, 'joint' and pous, 'foot'). Arthropods are further characterized by a segmented body plan and a chitin-based cuticle. Since the chitinous exoskeleton is rigid, arthropods rely on periodic molting for growth. Although the majority of species has not been described yet, the phylum Arthropoda accounts for over 80% of the currently described 1,242,040 animal species (Zhang, 2011). First paleontological records of arthropod fossils date back to the early Cambrian, 541 million years ago (Edgecombe and Legg, 2014). Over hundreds of millions of years of co-evolution, plants and arthropods have developed a plethora of different relationships, of which herbivory, or feeding on living plant tissues, is by far the most abundant.

The fossil record dates arthropod herbivory of terrestrial vascular plants to the Silurian (420 million years ago) and shows that the herbivorous feeding mode developed relatively soon after plants colonized land (Labandeira, 2002, 2006). Interestingly, coprolites show that these ancient arthropods were mainly feeding on spores (Scott et al., 1992). Henceforth, interactions between plants and phytophagous arthropods intensified and diversified. Throughout the Paleozoic and early Mesozoic, arthropods presented mainly marginal uninterrupted feeding strategies (uninterrupted feeding at the edges of a leaf), while in the Cretaceous period they additionally developed interrupted-marginal (feeding at the edges of a leaf, interrupted and consequently initiated multiple times at different positions) or non-marginal feeding (indiscriminate feeding from the entire surface of a leaf) (Scott et al., 1992) strategies. These and other changes in feeding behavior are thought to have been driven by an ongoing evolutionary arms race, in which plants aim to reduce the herbivorous activity via development of physical (including trichomes and spines) and chemical defenses. The phytochemicals may serve to attract the natural enemies of the pest (Birkett et al., 2000), deter or kill the target herbivore, or reduce digestibility of the plant material in the arthropods' gut (Haruta et al., 2001). In turn, herbivorous arthropods develop behavioral and physiological counter-adaptations to overcome plant defenses. Co-evolution of plants and arthropods resulted in high species diversity, reflected for example, in a plethora of different food acquisition techniques. For instance, based on mode of feeding (mining, chewing, sucking), developmental stage (larvae, adult), feeding habit (internally, externally), and plant part or tissue that is consumed (leaves, flowers, fruits, xylem, phloem), Novotny et al. (2010) proposed no fewer than 24 different feeding guilds.

Monophagous arthropods are restricted to feed on one or a few related plant species. For example, the Yellow stem borer, *Scirpophaga incertulas*, is limited to feed almost solely on rice. Oligophagous arthropods are able to feed on a group of plants from the same family. For instance, the oligophagous cabbage butterfly, *Pieris rapae*, only infests species of the mustard family (Brassicaceae). Polyphagous arthropod herbivores reside on the other side of the diet breadth spectrum; they can feed on a variety of plant species from a number of (unrelated) families. Although true generalists are rare they can be found across the whole arthropod phylum. Indeed, the desert locust, *Schistocerca gregaria* (Insecta: Orthoptera), the sap sucking green peach aphid, *Myzus persicae* (Insecta: Hemiptera), the cotton ball worm, *H. armigera* (Insecta: Lepidoptera), and the two-spotted spider mite, *Tetranychus urticae* (Arachnida: Trombidiformes), all thrive on a wide range of plants from phylogenetically distinct families.

Herbivorous arthropods can be found in each of the four currently living arthropod subphyla: Insecta, Chelicerata, Myriapoda, and Crustacea. Herbivorous myriapods and crustaceans are generally represented by saprophagous species, feeding on decaying plant matter, and typically do not evoke serious cost to agriculture (David and Handa, 2010). In contrast, insects and chelicerates contain a profusion of species that feed on valuable crop plants, causing great economic losses. Therefore, below I will focus on these two lineages.

1.2.1. Chemical control and resistance of arthropod pests

The first scientific report on the development of insecticide resistance to a chemical control agent was published over 100 years ago by A.L. Melander, then an entomologist at the Washington Agricultural Experiment Station, who wrote the prophetic

TABLE 1.1 – Ranking of top 12 insecticide-resistant arthropods (Whalon et al., 2010-2017)

Species	Common name	Order	No. of compounds
Tetranychus urticae	Two-spotted spider mite	Trombidiformes	94
Plutella xylostella	Diamondback moth	Lepidoptera	92
Myzus persicae	Green peach aphid	Hemiptera	76
Musca domestica	House fly	Diptera	62
Bemisia tabaci	Sweet potato whitefly	Hemiptera	55
Leptinotarsa decemlineata	Colorado potato beetle	Coleoptera	55
Rhipicephalus microplus	Southern cattle tick	Ixodida	50
Aphis gossypii	Cotton aphid	Hemiptera	49
Panonychus ulmi	European red mite	Trombidiformes	48
Helicoverpa armigera	Cotton bollworm	Lepidoptera	48
Blattella germanica	German cockroach	Blattodea	42
Culex quinquefasciatus	Southern house mosquito	Diptera	40

words: *"That the San Jose scale should become acclimatized to a sulphur-lime environment is not altogether a strange thing"* (Melander, 1914).

In the 1940's, DDT and other synthetic organic pesticides were introduced into agriculture and successfully controlled infestations of various arthropod pest species. These new and effective compounds became immensely popular and were used on a broad scale. The large-scale introduction of organic pesticides, however, was soon followed by an increase in the number of reported resistance cases. By 1984, populations of at least 17 insect species were reported as resistant to all major classes of insecticides (Georghiou, 1986). By 1991, resistance to at least one insecticide had been noted for 504 species (Georghiou and Lagunes-Tejada, 1991; Wood and Mani, 1981). In 2010, the reported number of resistant insects and mites grew to 570 species (Whalon et al., 2010-2017). Current statistics show that *T. urticae* and the diamondback moth, *Plutella xylostella,* have over 400 cases of resistance each to more than 90 different compounds (Table 1.1; Van Leeuwen and Dermauw, 2016).

As evidenced by the data above, the evolution of pest resistance to pesticides is an increasingly urgent problem that threatens human food supply worldwide (Pimentel and Peshin, 2014). Since the development of new effective pesticides becomes more and more time consuming, laborious, and expensive (Sparks, 2013), it is essential to preserve the utility and efficiency of current and future pesticides as much as possible (Sparks and Nauen, 2015). In the early 1980's, the Insecticide Resistance Action Committee (IRAC) was established, which aimed at describing the different insecticide and acaricide modes of action (MoA) as well as updating the MoA classification. IRAC also offers an official definition of resistance: 'a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species' (http://www.irac-online.org/about/resistance/). The IRAC public database is fundamental for designing an optimal Insecticide Resistance Management (IRM) strategy to control agricultural pests and disease vectors (Sparks and Nauen, 2015). Currently, IRAC's classification scheme recognizes over 25 different MoA groups, and over 55 different chemical classes (Sparks and Nauen, 2015). However, as pointed out by Tabashnik et al. (2014), IRACs definition of resistance has its limitations in the timely detection and response to resistance in the field. The authors proposed the following alternative, more practical definition of resistance: *"field-evolved resistance that reduces pesticide efficacy and has practical consequences for pest control"*.

The world market for insecticides continues to grow, despite efforts to limit the use of chemical pest control agents (Pimentel and Peshin, 2014). The value of insecticide sales for 2013 was estimated at US$ 17,016 million (Sparks and Nauen, 2015). Among currently available insecticides and acaricides, the compounds that target neuronal and muscle tissues account for 85% of the total market value. Here, neonicotinoids (such as imidacloprid) and pyrethroids (such as deltamethrin) are the two most popular groups, with market shares of 27 and 16%, respectively. Chemicals that disrupt growth and affect energy metabolism account for, respectively, 9 and 4% of the total market value. Within the class of growth regulators, acylureas and tetronic acid, take an equal share of 3% of the market, whereas Mitochondrial Electron Transport Inhibitors (METIs I) are the most popular insecticides that target energy metabolism (Sparks and Nauen, 2015). Finally, midgut membrane disruptors (such as sprayable Bt) and insecticides of an unknown mode of action each take up 1% of the market (Sparks and Nauen, 2015).

The importance of identifying the MoA of insecticides and acaricides becomes especially apparent in the light of pesticide cross-resistance and multiple resistance. Cross-resistance occurs when selection for resistance to one insecticide leads to the development of resistance to a different compound to which the pest population has not yet been exposed. A recent example is the cross-resistance to cyflumetofen and cyenopyrafen, novel complex II inhibitors, which was detected in European field strains of *T. urticae* (Khalighi et al., 2014) despite the compounds have never been used in Europe. In such a scenario, effective IRM strongly advises against using these insecticides in alteration in order to prevent further spread of resistance. Multiple resistance (also called multi-resistance) occurs when, over the course of several generations, an arthropod population is exposed to a variety of different MoA pesticides and subsequently develops different mechanisms to counteract each MoA specifically (Metcalf, 1989). Multiple resistance poses a serious threat to resistance management, since it directly calls for the development of new control agents. An example of multiple resistance can be found in the peach potato aphid where populations have developed at least seven distinctive mechanisms of resistance to five different MoA insecticides (viz., organophosphates and carbamates, pyrethroids, cyclodienes, and neonicotinoids) (Bass et al., 2014). Similar findings have been reported for spider mite populations (Van Leeuwen et al., 2005).

Resistance evolves via natural selection of random mutation. Growth and reproduction of individuals that possess resistance genes is not, or less hindered by the repetitive pesticide application. Such individuals pass the resistance genes to their progeny, eventually resulting in a resistant population. Whether the resistance genes exist in the population prior to the pesticide application (pre-adaptive resistance) or arise as *de novo* mutations (post-adaptive resistance) has been a matter of an ongoing debate since Crow's review in 1957 (Crow, 1957; ffrench-Constant, 2007, 2013; ffrench-Constant and Bass, 2017). Taking advantage of museum specimens, the pre- vs post-adaptive resistance hypothesis was tested for some point mutations that confer malathion resistance in populations of the Australian sheep blowfly*, Lucilia cuprina*. Sequencing of pinned specimens collected between 1930 and 1949, revealed presence of the malathion-resistance mutation prior to the introduction of organophosphorus insecticides in Australia (Hartley et al., 2006). With an exception of the abovementioned case, the origin and history of nucleotide polymorphisms associated with resistance is, however, unknown.

Resistance to pesticides can depend on a single gene or on multiple genes (i.e., monogenic or polygenic resistance) (ffrench-Constant, 2013). Monogenic resistance is thought to be more challenging to control in the field than polygenic resistance, which may be lost when resistant individuals mate with susceptible genotypes (Roush and McKenzie, 1987). Another important factor contributing to the spread of the resistance and its phenotypic manifestation is the degree of dominance of the resistance trait. Recessive resistance will spread much slower than dominant resistance as heterozygotes are killed by the pesticide. The ability of an arthropod population to develop resistance may also heavily depend on its mode of reproduction. In ar-

rhenotokous arthropods, females are diploid whereas males are haploid. In such haplodiploid species, any adaptive change in the haploid male genome will be manifested in the phenotype, regardless of its dominance status. Due to natural selection, these characters can then quickly increase their frequency in the population (Denholm et al., 1998; Helle and Overmeer, 1973). Hence, for the traits that are recessive or partially recessive, the rate of resistance development in diploid arthropods is expected to be lower than in the haplodiploid species (Denholm et al., 1998).

1.2.2. Mechanisms of pesticide resistance

1.2.2.1. State-of-the-art

Phytophagous mites and insects adapt to pesticides through a multiplicity of strategies. According to the traditional classification based on their physiological manifestation, those strategies can be categorized as either toxicodynamic or toxicokinetic. Toxicodynamic modifications involve alterations of the pesticide target-site, whereas toxicokinetic modifications affect the metabolism, penetration, sequestration, efflux, and excretion of the pesticide (Feyereisen et al., 2015). Genomics and transcriptomics allow to discover yet another dimension of resistance, uncovering a broad spectrum of molecular genetic modifications that underlie the physiological changes. Recently, Feyereisen et al. (2015) argued to further categorize resistance mechanisms according to the exact underlying genotype and its phenotypic manifestation, in order to provide a better theoretical framework for resistance management. According to this classification, mutations involved in pesticide resistance may fall into three categories. The first category encompasses mutations that change the coding sequence of a gene, resulting in an altered structure of its product. The second and the third category include mutations that result in overexpression or knockout of gene expression. These mutations can be further categorized depending on whether they affect the whole structural gene [duplication, disruption or loss of open reading frame (ORF)], or just the regulatory elements of the gene (Feyereisen et al., 2015). In a single resistant population, the toxicodynamic and toxicokinetic mechanisms can be present alone or in different combinations. Hence, achieved levels of resistance may equal the sum of resistance reached by each of the mechanisms alone or they combine multiplicatively, which leads to resistance levels higher than the sum of both mechanisms (Bohannan et al., 1999; Raymond et al., 1989; Zhang et al., 2016; Zimmer et al., 2017). Antagonistic interactions between resistance loci may also occur (Brindley and Selim, 1984). In this section, I will introduce the main mechanisms governing pesticide resistance in insects. In Chelicerates, I will primarily focus on *T. urticae*, representing the first arthropod from this subphylum, with full genome sequence and ample amount of molecular data available (Grbić et al., 2011)

1.2.2.1.1. Toxicodynamic

There are many examples of target-site resistance to insecticides and acaricides with different MoAs. For instance, mite and insect species across the globe have developed high resistance levels to a class of pesticides called organophosphates (OPs) which inhibit normal nerve and muscle function. OPs were introduced in 1944 and became the most used class of insecticides by the mid-1960s. Unfortunately, in the 15 years that passed since their first introduction, OPs resistance had already been reported in 29 arthropod species of agricultural and sanitary importance (Brown, 1961). OPs are toxic because they interfere with the normal functioning of acetylcholinesterase (AChE). AChE is an enzyme essential for hydrolyzing acetylcholine and a few other choline neurotransmitters. By catalyzing hydrolysis, AChE plays a crucial role in the termination of synaptic transmission. OPs are acetylcholine analogs and act by binding to the AChE active site and thus block the enzyme (Aldridge, 1950). In 1991, OP resistance has been accounted for almost 50% of all resistance cases (Georghiou and Lagunes-Tejada, 1991). Remarkably, the first ever experimental proof that resistance to pesticides can evolve as result of target-site modification was obtained using an organophosphate-resistant population of *T. urticae* (Smissaert, 1964). Mutations in AChE causing organophosphate resistance have been particularly well studied, revealing complexity of the resistance mechanism. For instance, mutations may occur in different combinations, e.g., F331W with T280A/G328A/G119S. Some mutations may function as fitness modifiers, not contributing to the resistant phenotype themselves (Kwon et al., 2012). Furthermore, loss of catalytic activity due to the presence of a target-site mutation, can be restored via allele amplification and overexpression. For instance, some organophosphate-resistant strains of *T. urticae* carry multiple copies of wild-type AChE allele, thereby minimizing the deleterious effects of the mutated, resistant allele (Kwon et al., 2010b). An overview of the more than a dozen amino acid substitutions at different positions in the AChE sequence that are associated with OP resistance can be found in the ESTHER database (Feyereisen et al., 2015; Lenfant et al., 2013).

Another well-documented case of a neurotoxin is that of DDT and pyrethroid resistance caused by mutations in the *voltage sensitive sodium channel* (*VSSC*) gene. The knockdown resistance caused by *kdr* and *super-kdr* mutations in the highly conserved regions of the VSSC protein, were first reported in *M. domestica* populations in 1957 in Italy and then in 1964 in Japan (Milani and Travaglino, 1957; Tsukamoto, 1964; Williamson et al., 1993, 1996) and their impact has been thoroughly reviewed (Dong, 2007; Dong et al., 2014; Rinkevich et al., 2013). Until now, insensitivity to pyrethroids has been found in at least nine orders of insects and also in mites, with 51 species reported to carry one or multiple mutations in VSSC (Feyereisen et al., 2015). According to a recent count, the astonishing number of 61 different amino acid substitutions at different locations within VSSC, which are present in different combinations, have been identified (Dong et al., 2014; Feyereisen et al., 2015). Presumably due to differences in amino acid sequence within and at the periphery of the VGSC-binding pocket, the *kdr* and *super-kdr* mutations causing DDT and pyrethroid insensitivity in insects are not found in Tetranychoidea except for *T. evansi*, carrying the *super-kdr* mutation (Nyoni et al., 2011; Van Leeuwen and Dermauw, 2016). In *T. urticae,* F1538I in domain III L6 and L1024V in domain II L6 are located at unique positions and have been found at relatively high frequency in many regions of the world (Ilias et al., 2014; Kwon et al., 2010a). Their contribution to the resistant phenotype was mainly assessed through genotyping and association with phenotype. Additionally, mutation F1538I has been also studied via electrophysiological studies in *Xenopus laevis* oocytes, where its contribution to resistance to pyrethroids has been confirmed (Tan et al., 2002).

Cyclodiene and phenylpyrazole insecticides target the γ-aminobutyric acid (*GABA*)*-gated chloride channel gene* (*Rdl*) product, a receptor for the GABA neurotransmitter. Mutation A301S/G or N in the second membrane spanning domain, lining the ion channel pore, has been commonly found in association with resistance to cyclodienes: it was identified in 27 species belonging to six insect orders (Feyereisen et al., 2015). As research on cyclodiene and phenylpyrazole insecticides progressed, several other mutations were reported to occur together with A301, suggesting that prolonged selection by this group of insecticides can lead to accumulation of minor resistance traits in addition to the major, primary mutation (Feyereisen et al., 2015; ffrench-Constant, 2013). Three orthologues of *Rdl* genes coding for a 'mutated' subunit of GABA receptors, have been found in *T. urticae* genome. Whereas in insects, fipronil and cyclodiene resistance is caused by substitution of A301 to S, G or N, in *T. urticae* none of the orthologues has alanine at the conserved position 301, but instead the resistance associated serine (Tu_Rdl2 and Tu_Rdl3) or a histidine (Tu_Rdl1) (Dermauw et al., 2012). Mutations, T350A and T305L, previously found in a fipronil-resistant *Drosophila* and dieldrin-resistant cattle tick, *Rhipicephalus microplus,* respectively, were also identified in *T. urticae_Rdl1* (Dermauw et al., 2012; Hope et al., 2010; Le Goff et al., 2005). Function of the T350A and T305L was further confirmed with electrophysiological experiments on TuRdl1-expressing *Xenopus* oocytes, where inhibitory activity of fipronil was found to be very low (Asahi et al., 2015).

Other insecticides that affect nerve and muscle action and for which target-site insensitivity has been reported are neonicotinoids which operate as nicotinic acetylcholine receptor (nAChR) agonists (Bass et al., 2011), spinosyns which act as nAChR allosteric activators (Baxter et al., 2010; Hsu et al., 2012; Wang et al., 2016), avermectins which act on glutamate-gated chloride channels (GluCls) but also on GABA channels (Dermauw et al., 2012; Kwon et al., 2010c), and diamide insecticides which target ryanodine receptors (RyRs) (Douris et al., 2017). GluCls- and GABA-gated chloride channels are targets of macrocyclic lactones (e.g., avermectins and milbemycins), although GluCls are thought to be the main target. The mite *T. urticae* possesses six orthologous *GluCl* genes, which gives a clear contrast with insects, harboring only one *GluCl* copy. In *T. urticae,* a G314D substitution in GluCl1 was associated with resistance to abamectin (Kwon et al., 2010c). In a later study, authors took an advantage of the available *T. urticae* genome sequence to reveal the presence of an additional mutation: G326E in TuGluCl3 (Dermauw et al., 2012). Crossing experiments suggested that a combination of two mutations in TuGluCl1 and TuGluCl3 is needed for abamectin resistance (Dermauw et al., 2012). Recent *in vitro* work established that mutation G326E in a single receptor gene abolishes the antagonistic interactions of macrocyclic lactones (Mermans et al., 2017).

Among insecticides targeting growth and development, target-site resistance has been investigated for the keto-enol insecticides spiromesifen and spirotetramat, representing lipid synthesis inhibitors which act on acetyl CoA carboxylase (ACC), and for the chitin synthase inhibitors benzylureas and buprofezin. Karatolos et al. (2012) found a E645K substitution in strains of the white fly, *Trialeurodes vaporariorum,* characterized by moderate resistance to spiromesifen, and observed that repetitive insecticide selection produces populations with increased frequencies of the substitution. The E645K substitution, however, has not been found in any other insect or mite species and is not located in the CT-domain of the enzyme where interaction with inhibitors most likely occurs (Lümmen et al., 2014). Certain amino acid residues in the carboxyltransferase domains of plant ACCs are known to bind established herbicidal inhibitors. Mutating homologous residues of ACC in spider mites, however, did not result in inhibition of *T. urticae's* ACC by spirotetramat-enol, thus failing to identify the exact ACC's amino acids responsible for pesticide binding in spider mites (Lümmen et al., 2014). In case of chitin synthase inhibitors, CRISPR-Cas9-mediated genome editing functionally validated that mutations at homologous positions in *P. xylostella*, *C. pipiens,* and *T. urticae* confer high levels of resistance to benzoylureas, buprofezin, and the mite growth inhibitor etoxazole in *Drosophila melanogaster*, demonstrating that compounds share a MoA by directly interacting with chitin synthase (Douris et al., 2016a; Grigoraki et al., 2017).

Target-site resistance has been also detected for mite-specific compounds like the complex III inhibitors bifenazate an acequinocyl and the mite growth inhibitors etoxazole, hexythiazox, and clofentezine (Van Leeuwen and Dermauw, 2016). Indeed, resistance mutations helped to define the target-sites of these acaricides, which was possible thanks to comparative genomics and high-resolution genomic mapping. Resistance to bifenazate in *T. urticae* was known to be inherited exclusively maternally (Van Leeuwen et al., 2006a). This observation motivated sequencing of the whole mitochondrial genome of bifenazate-susceptible and -resistant strains. Comparative genomics revealed few polymorphisms between strains, all located at the cd1 (G126S, I136T, S141F, D161G) and ef (P262T) helices that form the binding pocket of cytochrome b (cytb) (Van Leeuwen et al., 2008; Van Nieuwenhuyse et al., 2009). Therefore, based on strong experimental evidence, initially launched as an acaricide-affecting respiratory target, bifenazate was reclassified as a METI-III, Qo inhibitor (IRAC). Resistance to bifenazate became and remains the only example of a mitochondrial genome-encoded resistance mechanism documented in arthropods.

Discovery of a target-site-resistance mutation (and therefore the target-site itself) for the mite growth inhibitors etoxazole, hexythiazox, and clofentezine, has been facilitated by high-throughput genome sequencing data and population-level bulked segregant analysis mapping (BSA; section 1.2.2.2.). The approach resulted in mapping of etoxazole resistance to a small region in the genome of *T. urticae*, while complementary experiments lead to identification of a I1017F substitution in chitin synthase 1 (CHS1) as the resistance mutation (Van Leeuwen et al., 2012a). A similar but improved mapping technique was subsequently used to map resistance loci in clofentezine- and hexythiazox-resistant strains, revealing that I1017F provides cross-resistance to all three compounds (Demaeght et al., 2014).

1.2.2.1.2. Toxicokinetic

This section will focus on the evolution of resistance through changes in the metabolic detoxification of xenobiotics; for a description of reduced penetration and behavioral avoidance as a resistance mechanism, I refer to the review by Panini et al. (2016). Enzymatic detoxification of xenobiotics can be divided into three phases in which different enzyme families operate. Phase I involves the metabolism of the xenobiotic, while enzymes of phase II conjugate the xenobiotic with other compounds, in-

creasing their water solubility. Finally, enzymes of phase III translocate the toxin across cell membranes (Brattsten, 1988). Cytochrome P450 monooxygenases (CYPs) are phase I enzymes and play an extremely important role in the catabolism and anabolism of xenobiotics and endogenous compounds. Scott (1999) argued that monooxygenase-mediated pesticide resistance is probably the most common metabolism-based resistance type. Depending on the compound being metabolized, CYPs can act as isomerases, reductases, or oxidases. They are, however, best known for their monooxygenase activity, where they catalyze the transfer of one atom of molecular oxygen to a substrate and reduce the other atom to water (Feyereisen, 1999). Four major clans can be distinguished in the CYP gene family, namely CYP2, CYP3, CYP4, and M (mitochondrial CYP genes) (Feyereisen, 2012). In vertebrates, mitochondrial clan M CYPs are solely associated with steroid and vitamin D metabolism. In insects, the M clan comprises two groups that differ in their biological role. One group is involved in ecdysteroid metabolism, while the second group metabolizes a variety of xenobiotic compounds (Feyereisen, 2012). The number of CYP2 genes is relatively well conserved and several insect CYP2 genes are known to be involved in essential physiological functions. Insect CYP3 and CYP4 clans comprise the insect-specific families that are well known for their involvement in environmental response/detoxification functions against xenobiotics and phytotoxins (Feyereisen, 2012). The genome of *T. urticae* revealed 81 full length CYPs (Grbić et al., 2011). This number is similar to what is found in insects, but with an expansion of *T. urticae*-specific intronless genes of the CYP2 clan. Several members of the CYP2 clan were previously found to be upregulated in acaricide-resistant *T. urticae* strains or in a *T. urticae* strain adapted to a new challenging host plant (Demaeght et al., 2013; Dermauw et al., 2013b; Khalighi et al., 2016). To date, three CYP2 genes have been functionally expressed, viz., *CYP392A11, CYP392A16,* and *CYP392E10,* and found to metabolize METI acaricides cyenopyrafen (METI-II) and fenpyroximate (METI-I), abamectin (Chloride channels activator), and spirodiclofen (Acetyl CoA carboxylase inhibitors), respectively (Demaeght et al., 2013; Riga et al., 2014, 2015). Importance of P450-mediated detoxification has been additionally indicated by biochemical assays for a number of other cases, for example the pyrethroid bifenthrin and the mitochondrial uncoupler chlorfenapyr (Van Leeuwen et al., 2006b; Yang et al., 2002); however, specific genes involved in these detoxification processes have not yet been identified.

Carboxylcholine esterases (CCEs) are another important enzyme group of phase I shown to be involved in conferring metabolic resistance to some of the major classes of insecticides, including organophosphates (OPs), carbamates, and pyrethroids (Hemingway and Karunaratne, 1998; Oakeshott et al., 2005a; Wheelock et al., 2005). They detoxify toxins via hydrolyzing covalent ester bonds and their traditional classification is based on the interaction with organophosphate compounds and other inhibitors (Aldridge, 1953; Aldridge and Reiner, 1972). CCEs can also sequester toxic molecules away from their target-sites and into the fat body or hemolymph (Devonshire and Moores, 1982). Apart from their involvement in xenobiotic detoxification, carboxylesterases are known to paly other physiological functions. In a phylogenetically-based classification, insect CCE were grouped into 13 clades spread over three classes of different biological function (Oakeshott et al., 2005b). Clades A-C fulfil a role in the assimilation and detoxification of the diet. Enzymes of clades D-G and I-M are generally secreted enzymes, the former known as a hormone/semiochemical processing class and the latter operating in neurodevelopmental pathways (Claudianos et al., 2006; Oakeshott et al., 2005b). While the total numbers of CCEs in insects and *T. urticae* are similar, the neurodevelopmental class is expanded in *T. urticae* with two new subclades J' and J''. In contrast to insects, no *T. urticae* CCEs have been identified within the currently recognized classification of insect dietary CCEs (Grbić et al., 2011). Involvement of CCEs in pesticide metabolism has been best studied in aphids and mosquitos, where overexpression of genes coding for CCEs was usually attributed to a gene duplication or amplification (Bass and Field, 2011; Li et al., 2007; Wheelock et al., 2005). In *T. urticae*, duplication has been reported for AChE (Kwon et al., 2010b) and importance of CCE enzymes in the process of pesticides detoxification has been suggested in biochemical assays and differential expression studies (Demaeght et al., 2013; Feng et al., 2011; Sun et al., 2010). Demaeght (2015) managed to functionally express the CCE04 of *T. urticae*, previously implicated in the spirodiclofen metabolism. Study showed that indeed, the metabolic activity of CCE04 towards the model substrate p-nitrophenyl acetate (p-NA) was reduced in the presence of spirodiclofen (Demaeght, 2015).

Glutathione-S-transferases (GSTs) belong to a multifunctional enzyme family, known to play an important role in both phase I and phase II of the xenobiotic metabolism. In addition to pesticide detoxification by conjugation, insect GSTs have also been reported to play a role in attenuation of oxidative stress caused by pesticide exposure and sporadically in the direct metabolism of pesticides (Ortelli et al., 2003). Insects cytosolic GSTs can be divided into seven classes: delta (δ), epsilon (ε), omega (ω), sigma (σ), theta (θ), and zeta (ζ), with delta and epsilon GSTs well known for their role in detoxification of organophosphates and organochlorines (Enayati et al., 2005; Feyereisen et al., 2015). The cytosolic GSTs are more numerous in the *T. urticae* genome than in the genomes of insects, counting 31 genes belonging to four families: delta, mu, omega, and zeta. Interestingly, mu-class GSTs, previously believed to be vertebrate specific, apart from *T. urticae* genome, have also been identified in the genomic data of *Ixodes scapularis* (Reddy et al., 2011). Biochemical assays revealed the putative importance of GSTs in the metabolism of OP's (AChE inhibitors), bifenthrin (VGSC modulator), abamectin (Chloride channels activators), cyenopyrafen (METI II inhibitors), and Acetyl CoA carboxylase inhibitors (Ghadamyari and Sendi, 2008; Khalighi et al., 2016; Stumpf and Nauen, 2002; Yang et al., 2002). In insects, delta and epsilon GSTs are well known for their role in detoxification (Enayati et al., 2005). Similarly, delta GST, *TuGSTd14*, has been found upregulated in multiresistant strains of *T. urticae* (Dermauw et al., 2013a). The recombinant TuGSTd14, showed high affinity towards abamectin, indicating its possible involvement in abamectin detoxification (Pavlidi et al., 2015). In a recent study, Pavlidi et al. (2017) investigated the ability of TuGSTd05 to metabolize a newly introduced acaricide, the inhibitor of the complex II of mitochondrial electron transport chain cyflumetofen. Molecular docking suggested that both cyflumetofen and its de-esterified metabolite are potential substrates for conjugation by TuGSTd05, simultaneously identifying the residues most likely involved in the enzyme- acaricide interaction (Y107 and N103). To further investigate the interaction, the wild type TuGSTd05

and the TuGSTd05, with mutated Y107 and N103 residues, were recombinantly expressed and kinetically characterized. Cyflumetofen was found not to be a strong inhibitor, but its de-esterified metabolite showed strong affinity for TuGSTd05. Subsequent HPLC-MS analysis suggested that TuGSTd05 catalyzes the conjugation of ionized glutathione to cyflumetofen and/or its de-esterified metabolite. The resulting metabolite and the site of attack were identified (Pavlidi et al., 2017).

In addition to enzymes, membrane-binding transporters play a prominent role in xenobiotic detoxification. The ATP-Binding Cassette (ABC) protein family is a large and ubiquitous family of proteins. Most members of this family use ATP to transport substrates across lipid membranes, and hence are referred to as ABC transporters. Based on the sequence similarity of their nucleotide binding domain (NBD, the domain that binds ATP), the ABC protein family can be divided into eight subfamilies (A to H). Although extensively characterized for their involvement in drug resistance in vertebrates and bacteria (Choi, 2005; Dawson and Locher, 2006; Lage, 2003; Luckenbach et al., 2014; Stieger and Higgins, 2007), the role of ABC transporters in arthropod xenobiotic resistance is less known. The genome of *T. urticae* has revealed 103 ABC coding genes. This is the highest number discovered in a metazoan species to date. Involvement of ABCs in the xenobiotic metabolism in *T. urticae* has been inferred from increased expression (RNA-seq) upon transfer of multi-pesticide-resistant mite strains from their preferred host (bean) to a more challenging host (tomato) (Dermauw and Van Leeuwen, 2014; Dermauw et al., 2013b; Grbić et al., 2011).

MFS transporters are another example of membrane transporters. Along with ABCs, they are the most numerous transporter family described. MFS transporters are single-polypeptide carriers that work as a symport-antyport system (Reddy et al., 2012). Their role in the transport of toxic substances has been mainly described for bacteria and fungi (Kretschmer et al., 2009; Massoud et al., 2006). However, recent data obtained for *T. urticae* indicate that MFS transporters may play a prominent role in host plant adaptation and detoxification of pesticides (Dermauw et al., 2013b).

1.2.2.2. Molecular techniques for studying resistance mechanisms

Many studies have explored the genetic architecture of pesticide resistance using a plethora of different biochemical, genetic, and, more recently, genomic and transcriptomic approaches. Prior to the genomic revolution at the end of the 90s', genetic and physical mapping delivered the biggest contribution to research on the genetic basis of pesticide resistance. Genetic mapping relies on linkage maps that use genetic markers along chromosomes to indicate the position and relative distances between markers. Three types of genetic markers have been developed for arthropods: 1. morphological markers, which produce a distinguishable phenotype, 2. biochemical markers, represented by allozymes, enzymes of different allelic variants, and 3. molecular markers, which show polymorphisms in the DNA sequence and offer the greatest sensitivity and precision of mapping (Jones et al., 1997). By genetic mapping techniques, the chromosomal location of quantitative trait loci (QTL) associated with phenotypes of interest can be inferred. Here, QTL mapping depends on the marker segregation by chromosome crossing-over during meiosis in a sexually reproducing organism. The frequency of recombination between markers seen in the progeny is then used to calculate the genetic distance between markers: the lower the recombination rate between two markers, the shorter is the distance between them on the chromosome (Collard et al., 2005). The major shortcoming of genetic mapping is its accuracy, which is highly dependent on the number of crossovers, their randomness and the size of the population used in the mapping study. The limitations of genetic mapping can be overcome with physical mapping.

Physical mapping uses a set of overlapping DNA clones, that cover complete chromosomes and is able to show the real physical distance between loci. Among the different approaches used to create physical maps are fluorescent in situ hybridization (FISH), bacterial artificial chromosomes (BACs), yeast artificial chromosomes (YACs), P1 artificial chromosomes (PACs), and mapping with sequence tagged site (STS). STSs are unique sequences in the genome with known exact chromosomal locations, used to generate a physical map. This PCR-based approach proved a landmark development and greatly increased mapping precision and robustness, and significantly simplified methodology compared to clone-based approaches (Green and Green, 1991). Expressed sequence tags (ESTs) are short STSs that are identified with cDNA libraries. ESTs are dedicated for rapid and efficient profiling of tissue- and cell-specific gene expression (Adams et al., 1993; Rudd, 2003). A single sequence length polymorphism (SSLP) is another kind of STS, used to link genetic and physical maps.

The breakthrough technological discovery of Sanger et al. (1977) and more recently developed 'Next-Generation Sequencing' (NGS) technologies allow to sequence and analyze complete genomes. Because of the agricultural and medical importance of arthropods, the 'i5k' sequencing project and other projects aim to produce a vast genomic databank of 5,000 arthropod species and populations and use this genomic resource to answer both fundamental and applied research questions.

Statistically powerful genome-wide association (GWA) approaches have been developed to examine a genome-wide set of genetic variants in different individuals, to see if any variant is associated with a specific phenotype. Bulk segregant analysis (BSA) is a modification of GWA and relies on recombination between two phenotypically different populations. Phenotype-based selection results in a population where the markers adjacent to the targeted gene will be in linkage disequilibrium, allowing identification of the locus of interest within a window of 600 kb in length (Bryon et al., 2017a; Michelmore et al., 1991; Quarrie et al., 1999; Van Leeuwen et al., 2012b).

The clustered regularly interspaced short palindromic repeats (CRISPR) is a form of acquired immune response in bacteria but has been co-opted as an RNA-mediated nuclease for use in targeted genome engineering (Cong et al., 2013; Deltcheva et al., 2011; Garneau et al., 2010). As a tool for manipulation of genomic DNA, it is characterized by ease of use and efficiency. CRISPR has been widely and successfully used for editing bacterial, yeast, nematode, zebrafish, mice, rat, human, and also insect genomes (Cui et al., 2017; Douris et al., 2016b; Gilles et al., 2015; Kistler et al., 2015; Li et al., 2013; Singh et al., 2015; Somers et al., 2015). The principle of CRISPR-Cas9-mediated gene disruption is as follows: A single guide RNA (sgRNA), consisting of a crRNA that is identical to the DNA target, and a tracrRNA that interacts with the Cas9 protein, binds to a Cas9 DNA endonuclease. The resulting complex leads to target-specific dou-

ble-stranded DNA cleavage. The cleavage site will be repaired by non-homologous end joining (NHEJ) unless a template for homologous recombination is provided.

Additional molecular genetic technologies further facilitate the study of arthropod biology on the molecular level by quantifying the transcriptome. For instance, gene-expression microarrays monitor expression of tens of thousands of genes in a single assay (Hughes et al., 2001). Here, oligonucleotide probes are deposited at specific locations on a glass slide (a 'chip') and are complementary to the gene transcripts of a particular organism. Gene transcripts are labelled with different fluorescent dyes and spotted on the chip. Due to hybridization of the labelled RNA with the probes, each spot on the chip has a certain fluorescent intensity which reflects a certain gene-expression level. As it is becoming increasingly less costly, researchers are now turning to RNA sequencing (RNAseq) to quantify the transcriptome. This technology possesses several significant advantages over the microarray technology, since it does not rely on DNA or RNA sequences of existing genomes. Moreover, it directly determines the sequence of cDNA, is more sensitive than microarrays, and lacks the background signal (Malone and Oliver, 2011; Ozsolak and Milos, 2011; Wang et al., 2009). In a typical RNAseq experiment, RNA or DNA fragments are converted into a cDNA library. Subsequently, sequencing adaptors are added to each cDNA fragment which is then sequenced using high-throughput sequencing technology (Wang et al., 2009). The resulting sequence reads are then aligned to a reference genome. If a reference genome is not available, first a *de novo* transcriptome must be assembled using a library that contains all sequenced transcripts (Oppenheim et al., 2015). In parallel with the above-mentioned genome sequencing project 'i5k', the '1kite'-project aims to generate assembled transcriptomes of over 1000 insect species (www.1kite.org).

The RNA interference (RNAi) technologies is a new and promising reverse genetics tool, aiming at assigning function to unknown genes. The RNAi relies on a delivery of dsRNA into a cell. This dsRNA is complementary to the target sequence and once in the cell, it is processed by Dicer enzymes into primary siRNAs. In turn, siRNAs direct the endonuclease Argonaute (Ago) to their target mRNAs, which undergo digestion after recognition by siRNA and binding (Gordon and Waterhouse, 2007).

1.2.3. Understanding population dynamics in aid of pest control

The rate at which pesticide resistance evolves is influenced by three factors; the genetic architecture underpinning resistance, the precise control measures in the field, and the biology/ecology of the pest (see Table 1.2 for an overview of parameters that influence selection for resistance to insecticides in field populations) (Georghiou and Taylor, 1977). The latter factor is of particular importance in the light of relatively recently developed integrated pest management (IPM) strategies (Cate and Hinkle, 1994). IPM deploys a range of methods, considered carefully within the given environment and the population dynamics of a pest, and aims to reduce pesticide use in agriculture and horticulture. Such control methods not only include biological control and host-plant resistance breeding but also exploitation of potential fitness costs associated with pesticide resistance, by creating refuges for susceptible individuals (ffrench-Constant and Bass, 2017; Nylin, 2001; Pimentel and Peshin, 2014; Thomas, 1999).

Based on their life history strategies, animals can be classified on a scale from r to K. Generally speaking, r species display a high reproductive rate, high food uptake, and flourish for a relatively short time in an ephemeral environment. In contrast, K strategists maintain a steady population size over time and do not deplete their habitat's resources (Southwood, 1977). The majority of crop pests are r-strategist, however, the degree to which they actually pose a threat to agriculture often depends on the feedback they receive from the environment. Alien species reside outside of the framework of biotic and abiotic conditions of their natural habitat, which typically limit their uncontrolled growth. Once an alien species with high reproductive potential is established in a new favorable environment, it can become a main driver of biodiversity decline leading to reduced ecosystem productivity (Assessment, 2005). Notable examples of arthropods pest alien in Europe include the Argentine ant, *Linepithema humile,* known to have detrimental effect on biodiversity of native ants communities but also other arthropods, and birds and lizards (Lach, 2008). Other examples include the species complex of the whitefly, *Bemisia tabaci*, imported to Europe through infested potted plants (Oliveira et al., 2001) and originating from South-East Asia, the Asian tiger mosquito, *Aedes albopictus*, a vector for many tropical diseases (Enserink, 2008). Hence, the indigenous vs alien and invasive status of some arthropods may be important to understand the origin of pest outbreaks (Blackburn et al., 2011).

In most cases where arthropods are involved (with the notable exception of horizontally transferred bacterial or viral infections), pest problems are the result of population sizes that increase uncontrollably. The potential of an individual population to increase can be estimated using rm, the intrinsic rate of natural increase, as defined by Birch (1948). The intrinsic rate of natural increase is described as 'the rate of increase per head under specified physical conditions, in an un-limited environment where the effects of increasing density do not need to be

Table 1.2 – Parameters influencing selection for resistance to insecticides in field populations (according to Georghiou and Taylor, 1977)

Genetic	Biological	Operational
Frequency of R alleles	Generation turnover	Chemical nature of pesticide
Number of R alleles	Offspring per generation	Relationship to earlier-used chemicals
Dominance of R alleles	Monogamy/polygamy, parthenogenesis	Persistence of residues
Penetrance, expressivity, interactions of R alleles	Isolation, mobility, migration	Application threshold
Past selection by other chemicals	Monophagy/polyphagy	Selection threshold
Extent of integration of R genome with fitness factors	Fortuitous survival, refuge	Life stage(s) selected
		Mode of application
		Space-limited selection
		Alternating selection

considered' (Birch, 1948). The rm parameter for every species is determined by reproductive parameters such as fecundity, hatchability, length of oviposition, longevity, rate of development, and survivorship but also by extrinsic factors such as temperature, humidity, predation, competition, and host plant species or cultivar. The two life history parameters that affect the rm the most, are developmental time and fecundity (Snell, 1978). However, Cole (1954) demonstrated that the age of first reproduction has a more pronounced impact on rm than does fecundity; this was further analytically proven by Caswell and Hastings (1980).

The ability of arthropods to disperse also has its impact on agriculture. Firstly, immigration of susceptible individuals into populations resistant to acaricides/pesticides may slow down the development of a full blown resistance (Tabashnik and Croft, 1982; Taylor and Georghiou, 1982). Secondly, better understanding of dispersal may help to explain the spatial and temporal dynamics between pest arthropods and their predators. Thirdly, dispersal also facilitates spread of invasive species to new environments, with new hot spots of outbreak as a result. Dispersal is triggered by decline in food quality, overcrowding, predator avoidance, or simply due to plant yearly senescence (Price, 1984) but may also take place, although to a lesser degree, when none of the mentioned factors applies (Lawson et al., 1996). In fact, modeling and experimental studies imply that dispersal is beneficial to arthropods, regardless if they reside in stable or unstable habitats, affecting the spatial distribution of genetic diversity and increasing fitness of fugitive colonies (Bitume et al., 2011; Bonte et al., 2014; Hamilton and May, 1977; Van Petegem et al., 2017). Herbivorous arthropods of minute size like mites may disperse passively, drifting on air currents (Fleschner et al., 1956; Wanibuchi and Saitô, 1983). Active dispersal occurs by swimming, walking, or flying. Walking is a viable option for chelicerates, while insects stand alone in their ability to fly.

Many herbivorous arthropods that live in a climatic zone characterized with a seasonal pattern, have developed specific behavioral or physiological adaptations in order to endure the changing weather conditions. For instance the green peach aphid, *Myzus persicae,* developed a complex reproductive adaptation in which one sexual generation on the primary host, peach, *Prunus persica* L. (Rosaceae), produces overwintering eggs in autumn. A single sexual generation is then followed by many asexual generations during spring and summer, which reside on so-called secondary hosts, which are usually herbaceous plants (Blackman and Eastop, 2000, 2007). Several authors investigated the importance of the secondary host in the context of IPM (Rubiano-Rodríguez et al., 2014; Rubio-Meléndez et al., 2017). In principle, weedy and grassy field/orchard borders are not subjected to chemical pest control, hence they may provide refuge for individuals escaping pesticide selection pressure. In autumn, winged females travel back to the primary host for sexual reproduction. Those individuals may provide an important reservoir of susceptible alleles, consequently slowing down the development of pesticide resistance (see SECTION 1.2.1).

Seasonality of climate induces specific biochemical and physiological changes in insects and mites, including diapause. Such changes have been found to differently influence survival of certain pesticide-susceptible and pesticide-resistant populations. For example, overwintering populations of diazinon-resistant *L. cuprina* flies have lower survival than their susceptible counterparts (McKenzie and Clarke, 1988). Similar observations have been made for dieldrin-resistant (i.e., resistant to dieldrin or *Rdl*) blowflies (Mckenzie, 1990).

1.2.3.1. Pleiotropic effects of resistance mutations

As described in SECTION 1.2.1, insecticide resistance is mediated by (sets of) genes that allow the arthropod pest to survive and reproduce despite exposure to the pesticide. The development of insecticide resistance in a certain pest arthropod population can either rely on standing genetic variation, present in the population prior to pesticide exposure, or on newly arisen genotypes (see also SECTION 1.2.1). Based on Fisher's model of adaptation (Fisher, 1999), ffrench-Constant (2007) and ffrench-Constant & Bass (2017) discuss the impact of the two evolutionary scenarios on potential fitness costs of insecticide resistance in detail. Briefly, the evolution of resistance based on standing genetic variation, or pre-adaptive resistance, is less likely to carry a fitness cost, since the causal polymorphisms have already been incorporated in a matching genotypic background by natural selection. In contrast, a *de novo* mutation that underlies resistance, or post-adaptive resistance, has not been subjected to natural selection and may thus have other, negative pleiotropic effects on the arthropod's biology (Fisher, 1999).

In the absence of the pesticide, genotypes that carry resistance mutations with associated fitness costs are expected to be less competitive than susceptible conspecifics. This reduction in fitness of resistant individuals is of interest to pest management strategies and a reliable quantification of such a fitness cost in the field can aid in designing pesticide spraying schemes (Dennehy et al., 1990). However, experimental verification and quantification whether certain genetic mechanisms of insecticide resistance result in pleiotropic fitness costs is not straight-forward and previous studies on resistance-related fitness costs suffer greatly from erroneous experimental methodologies (Kliot and Ghanim, 2012; Roush and McKenzie, 1987). ffrench-Constant and Bass (2017) address this issue in a recent review and propose an ideal experimental design that future studies on resistance-related fitness costs in arthropods should aim to follow. Although an in-depth outline of the underlying rationale goes beyond the scope of this general introduction, I list below the most important guidelines. For more information, I refer to the review of ffrench-Constant and Bass (2017) and CHAPTERS 5 and 6 of this thesis.

- The pesticide resistance should not have evolved under laboratory conditions, but in the field from an ancestral susceptible genotype.
- Genetic markers, preferably targeting the causal allele, should be known to reliably differentiate between the related resistant and susceptible genotypes.
- Studies should be based on near-isogenic resistant and susceptible lines that only differ in the resistance allele (see CHAPTERS 3, 4 and 5 of this thesis)
- If the study system allows, experiments should be performed in the field; if not, environmental conditions in the laboratory should mimic natural field conditions as best as possible.
- Field studies should place the resistant genotype in direct competition with the ancestral susceptible genotype. By tracking the genotype frequencies within the mixed population over time, resistance-associated fitness costs can be reliably quantified.

The sheep blowfly *L. cuprina* has been a pioneering study system in the quantification of resistance-related fitness costs. For instance, McKenzie (1994) examined the fitness costs of a resistance gene in genetically related diazinon-susceptible and diazinon-resistant *L. cuprina* populations and showed that the ability to successfully overwinter is significantly reduced in flies of a resistant genotype. Moreover, McKenzie identified a 'modifier' locus, which can restore the fitness in the resistant population in the absence of the pesticide. With this study, McKenzie provided experimental evidence that natural selection will accommodate the resistance mutation in the genome, by giving rise to additional mutations alleviating the negative fitness effects of the resistance allele in a pesticide-free environment (McKenzie, 1994).

Due to various methodological constraints, in particular, uncontrolled dispersal rates, the majority of studies on resistance-associated fitness costs have been carried out in controlled laboratory settings. These laboratory studies have the benefit that life history parameters can now be measured and the exact pleiotropic effect of pesticide resistance on the pest biology can be better understood (Roush and Daly, 1990).

1.3. TETRANYCHIDAE, A FAMILY OF PLANT FEEDING SPIDER MITE PESTS

Within the class of the Arachnida, the orders Sarcoptiformes and Trombidiformes are known to contain obligate herbivorous species. In the Trombidiformes, obligate herbivory has been a more successful strategy in terms of species diversity and is associated with three species-rich mite lineages, viz., Eriophyoidea, Tetranychoidea, and Tarsonemidae (Krantz and Lindquist, 1979; Lindquist, 1998). Tetranychidae are a family within the Tetranychoidea and hold over 1,250 species, including one of the most notorious agricultural pest worldwide (Van Leeuwen et al., 2015; Zhang, 2003), the two-spotted spider mite, *T. urticae*. Phytophagous mites are controlled primarily by acaricides, and acaricide treatment in 2013 was estimated to cost € 900 million (US$ 1.1 billion) (Van Leeuwen and Dermauw, 2016). Due to their ability to produce silk-like webbing, tetranychid species are often called spider mites (Migeon and Dorkeld, 2006-2017).

As is true of all Acari, the tetranychid body plan consists of only one segment, which can be artificially divided into the idiosoma and the gnathosoma with the latter holding the mouth parts and two pairs of eyes. Male adult spider mites can easily be recognized since males are typically smaller, leaner, and tapered towards the hind end compared to the oval-shaped female spider mite adults. Spider mites display an extensive intra- and inter-species variety in body color ranging from yellowish green to red, brown, and deep orange. Biochemical and genomic studies on pigment mutants have shown that spider mite body color is primarily determined by carotenoids which spider mites seem to synthesize *de novo* (Bryon et al., 2017a, 2013; Veerman, 1972).

The feeding apparatus of phytophagus mites consists of modified chelicerae, which form a hallow tube, commonly called a 'stylet', (Evans, 1992; Jeppson et al., 1975). Spider mites use their stylet to puncture spongy parenchyma cells and ingest protruding cell fluid by a lacerate-and-flush mechanism. Spider mite feeding typically results in chlorotic spots in the green foliage (Evans, 1992; Jeppson et al., 1975). Overexploitation of plants eventually leads to necrosis and complete defoliation (Devine et al., 2001). Within Tetranychidae, species have evolved that display all levels of host plant specialization, from specialist to generalist (see also SECTION 1.3.1). *Tetranychus lintearius* and the recently identified *Bryobia abyssiniae* feed exclusively on gorse (*Ulex europaeus*) and *Lobelia rhynchopetalum*, respectively (Fashing et al., 2016; Hill and O'Donnell, 1991). *T. evansi*, the red tomato spider mite, is a worldwide pest of solanaceous crops (DeMoraes et al., 1987), while *Panonychus ulmi* thrives on at least 38 different plant families, including quite a few economically important fruit trees (Migeon and Dorkeld, 2006-2017).

Spider mites generally reproduce through arrhenotokous parthogenesis: males are haploid and develop from unfertilized eggs, whereas females are diploid and develop from fertilized eggs. Virgin females produce only males, mated females can produce both female and male progeny. The complete life cycle of spider mites consists of an egg stage and a sequence of four active-feeding stages: six-legged larvae, protonymph, deutonymph, and adult. Larvae, protonymph, and deutonymph are followed by transitional quiescent stages, protochrysalis, deutochrysalis, and teleiochrysalis, respectively (Helle and Sabelis, 1985). Usually, males eclose earlier than females and remain close to teleiochrysalid females, showing a so-called guarding behavior. Copulation occurs immediately after female eclosion (Mitchell, 1973; Potter et al., 1976). The life cycle of tetranychid pests of economic importance is typically very short and can take less than two weeks. In addition to a short life cycle, mite pests also have a high fecundity. These two parameters are known to vary extensively and heavily depend on various inherent (genetic diversity, density of population, fertilization status etc.) and external factors (temperature, humidity, light, host plant, predation level etc.), with temperature as one of the most important ones. TABLE 1.3 lists values of several reproductive characteristics measured for *T. urticae* at a temperature range between 15 and

TABLE 1.3 – Total (mean ± SEM) development time, sex ratio, longevity, oviposition rate, and selected life table parameters for *Tetranychus urticae* investigated on strawberry leaf discs, at four constant temperatures (Bounfour and Tanigoshi, 2001)

Life stage/table parameter			Temperature (°C)			
			15	20	25	30
	Total development time (egg to adult) (d)	Female	25.3 ± 0.6	16.0 ± 0.4	13.9 ± 0.4	7.4 ± 0.3
		Male	24.9 ± 0.6	15.9 ± 0.5	10.7 ± 0.45	6.0 ± 0.3
	Sex ratio (% females)		65	63	65	63
Longevity and oviposition	Female longevity (d)		22.0 ± 1.4	26.9 ± 0.8	20.9 ± 1.1	17.1 ± 1.6
	Eggs/female/day		1.7 ± 1.1	4.6 ± 0.1	4.4 ± 0.2	7.1 ± 0.2
	Total eggs/female		38.1 ± 2.9	124.7 ± 4.6	92.8 ± 6.4	121.2 ± 12.4
Life table parameters	Net reproductive rate R0		24.7	81.0	54.9	86.0
	Intrinsic rate of increase rm (d^{-1})		0.1	0.2	0.2	0.3
	Mean generation time T (d)		38.3	26.5	21.3	13.9

Figure 1.1 – Plant-feeding spider mites on a bean leaf. A: *Panonychus ulmi*, B: *Tetranychus urticae* – green morph, C: *Tetranychus urticae* – red morph. Photos: Jan van Arkel.

30°C (Bounfour and Tanigoshi, 2001). Often, an increase in temperature is related to shorter development time and higher oviposition rate, until the physiological limits of the mites are reached (Helle and Sabelis, 1985). In poly- and oligophagous mites, reproductive characteristics can be also affected by the host plant species and even its varieties (Gotoh et al., 1993; Jeppson et al., 1975; Taj et al., 2016).

1.3.1. A tale of two mite pests

Tetranychus urticae and *P. ulmi* are two tetranychid species that have evolved into extremely successful pests of economically important plants. *Tetranychus urticae* females can easily be recognized in the field by the two characteristic lateral black spots that are formed due to food accumulation in the mite digestive tract; the species is therefore appropriately called the two-spotted spider mite. *Tetranychus urticae* occurs as a red or green morph. Although green and red morphs are now considered to belong to the same species, older studies erroneously regarded the red morph as a separate species (*Tetranychus cinnabarinus*) (Auger et al., 2013). Immature stages of *P. ulmi* have a dark green coloration while the body of adult females is dark red colored, with white setae and white spots at their base (Figure 1.1). Adult males of *P. ulmi* are yellowish with a hint of redness. In contrast to the circular transparent-dark orange *T. urtica* eggs, eggs of *P. ulmi* are reddish and lenticular in shape with a spike at the top.

Both *T. urticae* and *P. ulmi* rank high on the insecticide/acaricide resistance list in arthropods (Table 1.1). Currently, *P. ulmi* takes the 9th place in the ranking, with resistance to 48 different compounds, while *T. urticae* scores at the top of the list, with 94 compounds (Table 1.1). Indeed, both species are considered to be important agricultural pests and their control via chemical compounds is becoming increasingly challenging (Van Leeuwen and Dermauw, 2016). However, based on data from 2008, of the total acaricide market value spent on spider mite control, 62% is dedicated to control *Tetranychus* spp., predominantly *T. urticae*. *Panonychus ulmi* together with *P. citri* have a market share of only 16% (Van Leeuwen and Dermauw, 2016; Van Leeuwen et al., 2015). As these data show, *T. urticae* represents a bigger threat to agricultural production than *P. ulmi* and has been the subject of a high number of studies. As a result, the biology, ecology, and the mechanisms that lead to pesticide resistance of *T. urticae* are by far the best understood of all the Tetranychidae (Bonte et al., 2014; Bounfour and Tanigoshi, 2001; Bryon et al., 2017a, 2013; Helle and Sabelis, 1985). In 2011, the 90 Mb genome of *T. urticae* has been sequenced using Sanger technology and its study has greatly increased our understanding of the mechanisms that underlie resistance in this mite pest on the molecular level (Grbić et al., 2011). In sharp contrast, how *P. ulmi* develops resistance remains largely unknown [with the exception of the studies by Grosscurt et al. (1994) and Kramer and Nauen (2011)] and a great need has arisen for next-generation sequencing data that can serve as a resource for future studies.

Despite both being devastating pests, *T. urticae* and *P. ulmi* differ in their reproductive and feeding strategies which, at least partially, may be the result of different selection pressures imposed by their host plants. The following paragraphs further introduce the two mite species and describe their biological, behavioral, and demographic characteristics in light of their adaptive advantages that led to the successful colonization of so many annual and perennial plant species.

1.3.1.1. Overwintering (diapause) strategies

Tetranychus urticae survives harsh winter conditions as diapausing adult females. Diapausing *T. urticae* females do not lay eggs and accumulate higher levels of keto-carotenoids, which typically leads to a bright orange body color. In *T. urticae*, the transition into a diapausing state is associated with extensive rearrangements in the transcriptome and proteome, affecting a diverse set of physiological pathways including digestion, detoxification, and carotenoid biosynthesis (Bryon et al., 2017b, 2013). Surprisingly, diapausing females were found to produce an excess of unique antifreeze proteins which are thought to additionally protect a mite body from low temperatures (Bryon et al., 2013). Diapausing females typically require a period of low temperatures to initiate diapause termination. The length of that period, however, largely varies (reviewed in Helle and Sabelis, 1985).

Panonychus ulmi survives winter conditions as diapausing eggs. While summer eggs are generally deposited on the underside of infested leaves, winter eggs tend to be laid in crevices that are formed by the branches of their host plant. Winter eggs are typically larger than summer eggs, protected with an outer wax layer and characterized by a more intense red coloration (Newcomer and Yothers, 1929). How and when *P. ulmi* winter eggs are produced and how the diapause behavior is terminated remains largely unknown. Studies have, however, already indicated that geographical location, prolonged periods of low temperature (0-5°C) and day-length can induce diapause termination, while humidity seems to have a limited impact (Helle and Sabelis, 1985; Hueck, 1951; Koveos and Broufas, 1999).

1.3.1.2. Host plants, population dynamics, and dispersal

Panonychus ulmi is native to Europe but can now be found in almost every commercial fruit-growing region of the world due to trade globalization (Jeppson et al., 1975). In addition to fruit

tree species that include apple, peach, cherry, and pear, *P. ulmi* also feeds from elm, several herbaceous plants (Blair and Groves, 1952; Jeppson et al., 1975), dwarf bamboo (Ehara and Gotoh, 1991; Gotoh, 1987), fig, hibiscus, tomato, apricot, ivy, and mulberry (Dar et al., 2015; Kumar and Bhalla, 1993). *Panonychus ulmi* feeding leads to characteristic browning of host plant leaves, and in case of heavy infestation, induces defoliation (Newcomer and Yothers, 1929). *Panonychus ulmi* mites do not typically feed on fruits, but heavy leaf infestation has been indirectly implicated in decreased fruit size and number in prune and apple orchards (Van de Vrie, 1956). *Tetranychus urticae*, most likely originating from Eurasia, currently has a vast world-wide distribution and has become a major pest of greenhouse crops and ornamentals (Hemingway and Karunaratne, 1998). *Tetranychus urticae* is undeniably the most polyphagous spider mite (Jeppson et al., 1975) and is able to feed on over 1,000 plants from more than 140 plant families (Jeppson et al., 1975; Migeon and Dorkeld, 2006-2017). Feeding of *T. uricae* typically results in white or yellowish foliar spots, which often merge to form dried-out silver-white patches when damage intensifies.

Carey (1982) demonstrated that under natural conditions, populations of *Tetranychus* and *Panonychus* are close to a stable age distribution, meaning that they grow exponentially, which may explain their potential for causing outbreaks. Although the intrinsic rate of natural increase (rm, SECTION 1.2.3) may vary considerably depending on host plant species, cultivar, temperature, and geographical origin of mite strain, it typically reaches higher values for *T. urticae* than for *P. ulmi*. This pattern may be explained by the preference of *P. ulmi* populations to live on perennial plants. *Panonychus ulmi* populations thus enjoy a more stable habitat and are expected to be under a reduced selection pressure for high rm values than *Tetranychus* populations, which do not show this preference (Saito, 1979; Carey, 1982; Devine et al., 2001) (FIGURE 1.2). As discussed in SECTION 1.2.3., rm highly depends on fecundity and developmental rate (expressed as time^{-1} from egg to adult). FIGURE 1.2 shows an overview for those two characteristics for *P. ulmi* and *T. urticae* populations that were fed on two and six different host plant species, respectively (additionally, different cultivars were also tested), within a range of temperature of 15-30°C. There is little experimental data on *P. ulmi* in comparison to *T. urticae*, but the observed tendencies suggest that (1) regardless the developmental rate, total fecundity of *P. ulmi* scarcely exceeds 50 eggs/female, (2) total fecundity of *T. urticae* increases with the developmental rate, until physiological limits are reached, at approx. 0.14 d^{-1}, (3) total fecundity of *T. urticae* may reach approx. 125 eggs/female, and (4) within a given temperature interval, *T. urticae* has the potential to reach a higher developmental rate than *P. ulmi*.

According to Saito (1979), efficiency of dispersal is, like rm, related to the stability of the mite environment. To survive under unstable conditions, *T. urticae* must have evolved a reliable and effective dispersal strategy (Saito, 1979). However, a recent study of Van Petegem et al. (2017), investigating factors promoting fast and long distance dispersal in *T. urticae*, concluded that dispersal is mainly driven by kin competition, where mites migrate to avoid inbreeding, thus allowing their kin to have more food and shelter and reproduce more efficiently (Van Petegem et al., 2017). In *Panonychus ulmi,* the selective pressure to disperse is less, since its host is long-lived and usually has enough resources to regenerate after defoliation (Saito, 1979). In both mite species,

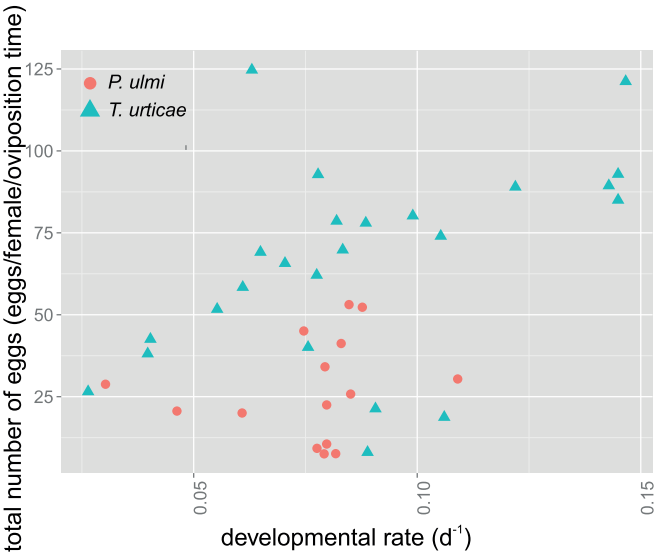

FIGURE 1.2 – The relationship between the mean egg-to-adult developmental rate (d^{-1}) and mean total oviposition. The data were obtained from experiments carried out at temperatures between 15 and 30°C, with adult female mites. Data for *Panonychus ulmi* were obtained from tests on apple (Gotoh et al., 2003; Rabbinge, 1976; Yin et al., 2013) and mulberry (Dar et al., 2016) leaves, while *Tetranychus urticae* were tested on cucumber (*Cucumis sativus*), pepper (*Capsicum annuum*), and common bean (*Phaseoleus vulgaris*) (Praslicka and Huszar, 2004), peach (*Prunus persica*) (Riahi et al., 2013), persimmon (*Diospyros virginiana*) (El-Halawany and El-Wahed, 2013), and raspberry (*Rubus* spec.) (Bounfour and Tanigoshi, 2001) leaves.

increasing density triggers an increasing proportion of dispersing animals (Lawson et al., 1996). However, Lawson et al. (1996) hypothesize that *P. ulmi* commits some individuals to disperse, regardless of the mite densities on the foliage. The extraordinary ability of *T. urticae* to disperse and successfully colonize plants is thought to be facilitated by its wide host range, behavioral adaptations, and abundant web production. *Tetranychus urticae* spins long threads between the leaves, which aid their intra-plant movement. Aerial dispersal in *T. urticae* is mostly attributed to a sophisticated behavioral adaptation, when mites lift up their front legs and face the wind to be carried away (Helle and Sabelis, 1985; Lawson et al., 1996).

1.4. GENERAL OUTLINE OF THE THESIS

The main goal of this thesis was to investigate and validate the molecular mechanisms that govern acaricide resistance in *P. ulmi* and *T. urticae* and how these mechanisms influence the fitness of resistant populations. In CHAPTER 2, I provided extensive genomic resources for *P. ulmi*, a major pest in European fruit orchards. Both the raw reads as well as the assembled transcriptomes were submitted to public databases to maximize their impact and usability by the scientific community. In addition, I provided an assembled transcriptome of *P. citri*, based on submitted raw reads of a previous study (Liu et al., 2011; Niu et al., 2012), as no assembled transcriptome was present in public databases. Furthermore, I annotated all major target-sites of acaricides in *P. ulmi*, performed a phylogenetic analysis of annotated major detoxification gene families, and searched for and annotated horizontally transferred genes in *P. ulmi* and *P. citri*. This has allowed to partially interpret the specific proliferation of *T. urticae* described in the initial genome paper (Grbić et al., 2011),

but also revealed specific expansions for the genus *Panonychus*. Last, I have used the replicated RNAseq data to determine differentially expressed genes between susceptible and spirodiclofen-resistant strains. Transcriptome profiling has identified a number of genes that may be associated with spirodiclofen resistance, supporting the results of earlier biochemical assays.

In chapter 3, I report on the discovery of a single point mutation (H92R) in the highly conserved region of the PSST subunit of complex I in METI-resistant *T. urticae* strains. This is the first report of a potential target-site based resistance mechanisms for any insecticidal inhibitor targeting complex I in Metazoa. The involvement of the mutation in resistance was supported by modeling, selection experiments, and genetic mapping (QTL analysis). In order to assess the relative phenotypic effect of the mutation, we introduced it into a susceptible *T. urticae* line by marker assisted back crossing. Finally, using CRISPR-Cas9 technology, I introduced the *T. urticae* complex I mutation in the *Drosophila melanogaster* PSST homologue and found it to be lethal to the fruit fly.

In chapter 4, I investigate the relative contribution of nine known target-site mutations found in *T. urticae*, conferring resistance to abamectin, pyrethroids, bifenazate, and mite growth inhibitors. I adopted the method of back crossing, used in chapter 3, and succeeded in generating 30 near-isogenic resistant and susceptible lines of *T. urticae*. When a combination of mutations in homologous genes was reported, the phenotypic levels of resistance were examined for both single mutations and their combination.

In chapter 5, I took advantage of the near-isogenic lines generated in chapter 4 to perform a comprehensive and detailed comparison of five fertility life table parameters and various life history traits to evaluate potential fitness costs associated with resistance conferring target-site mutations. In addition, I monitored temporary resistance dynamics of populations with different starting frequencies of the mite growth inhibitor resistance allele, to further support my findings. Results of the study contribute to the understanding of the pleiotropic effects of acaricide target-site mutations and help to explain the occurrence and spread of pesticide resistance within the context of integrated pest management.

Lastly in chapter 6, I integrated the findings of the four experimental chapters and discussed them in the light of possible methodological improvements, considering future research directions.

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2

Transcriptome profiling of a spirodiclofen susceptible and resistant strain of the European red mite *Panonychus ulmi* using strand-specific RNA-seq

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Abstract. The European red mite, *Panonychus ulmi*, is among the most important mite pests in fruit orchards, where it is controlled primarily by acaricide application. However, the species rapidly develops pesticide resistance, and the elucidation of resistance mechanisms for *P. ulmi* has not kept pace with insects or with the closely related spider mite *Tetranychus urticae*. The main reason for this lack of knowledge has been the absence of genomic resources needed to investigate the molecular biology of resistance mechanisms. Here, we provide a comprehensive strand-specific RNA-seq based transcriptome resource for *P. ulmi* derived from strains susceptible and resistant to the widely used acaricide spirodiclofen. From a *de novo* assembly of the *P. ulmi* transcriptome, we manually annotated detoxification enzyme families, target-sites of commonly used acaricides, and horizontally transferred genes implicated in plant-mite interactions and pesticide resistance. In a comparative analysis that incorporated sequences available for *Panonychus citri*, *T. urticae*, and insects, we identified radiations for detoxification gene families following the divergence of *Panonychus* and *Tetranychus* genera. Finally, we used the replicated RNA-seq data from the spirodiclofen susceptible and resistant strains to describe gene expression changes associated with resistance. A cytochrome P450 monooxygenase, as well as multiple carboxylcholinesterases, were differentially expressed between the susceptible and resistant strains, and provide a molecular entry point for understanding resistance to spirodiclofen, widely used to control *P. ulmi* populations. The new genomic resources and data that we present in this study for *P. ulmi* will substantially facilitate molecular studies of underlying mechanisms involved in acaricide resistance.

2.1. INTRODUCTION

The European red mite, *Panonychus ulmi,* is a member of the family Tetranychidae (Arthropoda: Chelicerata: Arachnida: Acari). Tetranychidae, or spider mites, use their stylet-like chelicerae to puncture the leaf mesophyll-cells to suck out cell contents. This results in chlorotic spots and necrosis and ultimately, leaf abscission (Jeppson et al., 1975). As a result, among mite pests, spider mites are economically important with 80% of the acaricide market used for their control (Van Leeuwen et al., 2015). Nonetheless, a major problem associated with spider mite control is the rapid development of acaricide resistance. Factors accelerating resistance evolution include not only the frequent use of acaricides, but also high fecundity rates, arrhenotokous reproduction (on haploid males selection is highly effective) and a short life cycle (Van Leeuwen et al., 2010). As a result, the two-spotted spider mite (*Tetranychus urticae*) and *P. ulmi* have developed resistance to nearly all acaricide classes (Whalon, 2014) and rank among the top 10 of most resistant arthropod species based on the number of unique active ingredients for which resistance has been reported (Van Leeuwen et al., 2015).

The acaricides spirodiclofen and spiromesifen belong, together with the insecticide spirotetramat, to the family of spirocyclic tetronic acids (keto-enols) and interrupt lipid biosynthesis by direct inhibition of acetyl coenzyme A carboxylase (ACCase) (Bretschneider et al., 2003; Lummen et al., 2014). Introduced relatively recently to the market (Elbert et al., 2002), spirodiclofen has been shown to be an effective tool in resistance management of tetranychid mites. It shows good to excellent residual control (De Maeyer and Geerinck, 2009; Wachendorff et al., 2002) and is effective against spider mites resistant to different acaricide classes (Ilias et al., 2012; Nauen et al., 2000; Pree et al., 2005; Rauch and Nauen, 2002; Thiel, 2006; Van Leeuwen et al., 2004, 2005). Although high levels of spirodiclofen resistance have not yet been reported in field populations, artificial selection studies have shown that spider mites have the potential to develop resistance to spirodiclofen comparatively rapid in the laboratory (Kramer and Nauen, 2011; Van Pottelberge et al., 2009). Selection for resistance was undertaken with *T. urticae*, *P. ulmi* and *Panonychus citri* and resistance in all species was synergized by piperonylbutoxide (PBO), suggesting the involvement of cytochrome P450s (Kramer and Nauen, 2011; Van Pottelberge et al., 2009; Yu et al., 2011). Strikingly, for all three tetranychid species spirodiclofen resistance was age dependent. The concentration of spirodiclofen causing 50% lethality (LC_{50}) in the resistant strains was much lower in eggs then in mobile stages, a finding Demaeght et al. (2013) suggested could be due to lower expression of detoxification genes in eggs.

In 2011, the high quality genome sequence of the spider mite *Tetranychus urticae* became available (Grbić et al., 2011), and afforded new resources and tools to investigate the molecular mechanisms underlying acaricide resistance (Demaeght et al., 2013, 2014; Dermauw et al., 2012; Ilias et al., 2014; Van Leeuwen et al., 2012). For example, although biochemical studies had shown that elevated P450 and esterase levels were associated with resistance to spirodiclofen in *T. urticae* (see above), studies with a genome-wide gene expression microarray allowed the identification of the genes coding for the detoxification enzymes associated with these activities (*CYP392E10* and *TuCCE-04*) (Demaeght et al., 2013). It is clear that our understanding of resistance to acaricides in phytophagous mites improved with the advent of the *T. urticae* genome, however, such genomic resources are scarce for other key tetranychid mites, particularly *P. ulmi*. With only 49 nucleotide sequences available in the NCBI public database (accessed May 2015), *P. ulmi* resistance research at the molecular level has hardly been possible.

For organisms without a sequenced genome, *de novo* transcriptome assemblies provide an important starting point for genomic analyses, and recent advances in high-throughput transcript sequencing have greatly contributed to our understanding of gene expression and structure (Mortazavi et al., 2008; Nagalakshmi et al., 2008; Vivancos et al., 2010; Wilhelm et al., 2008). This is especially true in agricultural pest management, where the majority of pest species do not have a reference genome and where the elucidation of resistance mechanisms of insecticides is vital (Cabrera et al., 2011; Karatolos et al., 2011; Liu et al., 2011; Niu et al., 2012; Pereira Firmino et al., 2013).

In this study we used the Illumina HiSeq2000 technology to generate deep paired-end, strand-specific RNA-seq reads for two strains of *P. ulmi*. The first strain (HS) is susceptible to most currently used acaricides, while the second strain (PSR-TK) was field collected and shown to be resistant to a number of acaricides, including hexythiazox and clofentezine. The strain also showed decreased susceptibility to spirodiclofen, and was further selected in the laboratory until high levels of spirodiclofen resistance (resistance ratio of 7000) were reached (Kramer and Nauen, 2011). Paired-end RNAseq reads were used to assemble a *P. ulmi* transcriptome, and with the same assembly methodology we constructed two *P. citri* transcriptomes based on previously published RNA-seq data available as single end reads (Liu et al., 2011; Niu et al., 2012). We then performed a phylogenetic classification of *Panonychus* gene families involved in detoxification, such as cytochrome P450 monooxygenases (CYPs), carboxylcholinesterases (CCEs), glutathione-S-transferases (GSTs), UDP-glycosyl transferases (UGTs) and ABC transporters (ABCs), and searched for horizontally transferred genes that were previously uncovered in the genome sequence of *T. urtic*ae (Ahn et al., 2014; Bryon et al., 2013; Dermauw et al., 2013b; Grbić et al., 2011; Wybouw et al., 2012, 2014). Moreover, a differential expression analysis between the acaricide susceptible and resistant *P. ulmi* strain allowed us to identify genes encoding proteins that are candidates for involvement in acaricide resistance. In addition, we identified and compared *P. ulmi* expressed sequences for known acaricide target-sites. The assembled annotated transcriptome of *P. ulmi* provides an important resource that should substantially facilitate molecular studies in this species, including the elucidation of detoxification mechanisms of xenobiotics.

2.2. MATERIALS AND METHODS

2.2.1. Mite strains

Mites from the three strains (HS, PSR-TK and Ge 16/09) used in this study were all identified morphologically as *P. ulmi* by Johan Witters (ILVO, Belgium) but also on the basis of cytochrome b (cytB) sequence similarity (cytB sequences of the HS and PSR-TK *P. ulmi* strain (FILE S2.1) had their best BLASTx hit (E-value $<1E^{-100}$) with *P. ulmi* cytochrome b (YP_002808558.1) in the NCBI database). The HS strain was originally collected in 1990 from an apple orchard in Burscheid, Germany and is susceptible to most currently used acaricides, including spirodiclofen (Kramer and Nauen, 2011). The Ge 16/09 is a field strain,

collected in 2009 from an orchard in Heidenfahrt-Heidesheim, Germany, and characterised by high resistance levels to mite growth inhibitors hexythiazox and clofentezine (extrapolated resistance ratios in comparison with the susceptible HS strain were 20000 and 3900, respectively) and moderate resistant to spirodiclofen with a reported spirodiclofen resistance ratio of 59 compared to the HS strain. The PSR-TK strain is a spirodiclofen-selected laboratory strain derived from Ge 16/09 and with a resistance ratio of more than 7000 compared to the HS strain (Kramer and Nauen, 2011). All mite strains were reared under identical conditions in climatically controlled chambers at 24 ± 1°C, 60% relative humidity and a 16:8 light:dark period for more than 10 generations on domestic plum trees (*Prunus domestica* L. var. Brompton). The PSR-TK strain was maintained under constant selection pressure by rearing on plum trees sprayed with 1000 mg a.i./L spirodiclofen until runoff.

2.2.2. RNA extraction, library construction, sequencing and assembly of the *P. ulmi* transcriptome

Total RNA was extracted from 200 1-3 day old adult female *P. ulmi* mites from the PSR-TK and HS strains using the RNEasy mini kit (Qiagen, Belgium) with four-fold biological replication (i.e., four replicates each for PSR-TK and HS). Each RNA sample was processed for sequencing by the sequencing service company Fasteris SA (Switzerland) according to the 'HiSeq Service Stranded Standard Protocol'. The resulting library was sequenced by Fasteris SA (Switzerland) using the HiSeq 2000 Illumina technology generating strand-specific paired-reads of 2x 100 bp. Adapter sequences were removed from the obtained 2x 100 bp reads by Fasteris SA and adapter trimmed reads were used for further analysis. Previously it was shown that a representative transcriptome assembly can be generated from a sub-sample of reads (Francis et al., 2013). Hence, to reduce computation time, a subset (the first 12 million paired-reads/sample, 48 million reads/strain, 96 million paired-reads in total) of the total number of reads was used for *P. ulmi* transcriptome assembly using the CLC Genomics Workbench (CLC) software version 6.5.1 and default settings (mapping mode = fast, automatic bubble size = yes, minimum contig length = 200 nt, automatic word size = yes, perform scaffolding = yes, auto-detect paired distances = yes). Using the same subset of reads we also used an alternative approach to assemble the *P. ulmi* transcriptome. Reads were first trimmed using Sickle (Joshi and Fass, 2011) with quality-cutoff set at 30 and length cutoff set at 90. Trimmed reads were assembled using the Velvet/Oases package (Schulz et al., 2012) and with the following settings for Oases: --kmin=59 --kmax=61 --kstep=2 --merge=61 –d '-shortPaired -strand_specific' -p 'ins_length=200'. Transcripts with maximum sequence length (30,044 transcripts) were filtered from the resulting Velvet/Oases assembly (44,903 transcripts) and used for further analysis. To compare the quality of both assemblies all RNA-seq reads (4 replicates/strain) were mapped using Bowtie version 2.1.0 (Langmead and Salzberg, 2012) against either the CLC or Velvet/Oases assembly (see SECTION 2.2.7 for mapping procedures).

Raw reads have been submitted to the NCBI Short Read Archive [SRA; experiment accession number SRX833872 (HS) and SRX833917 (PSR-TK)]. The CLC assembly was submitted to the NCBI Transcriptome Shotgun Assembly (TSA) Sequence Database and deposited at DDBJ/EMBL/GenBank under the accession GCAC00000000. The version described in this paper is the first version, GCAC01000000. *Panonychus ulmi* contigs containing more than 10% of unassigned nucleotides (N) or more than 14 Ns in a row (1,630 sequences) and contigs that were shorter than 200 nt after removal of vector contamination sequences (2 contigs) were not uploaded to the TSA Database following database submission procedures. After uploading, an NCBI contamination screen indicated that 227 out of the 26,145 uploaded sequences should be excluded as they showed very high identity with either rRNA of Bacteria, genes of *Prunus* sp. (host plant of *P. ulmi* strains) or *Wolbachia* sp. sequences. These sequences were removed from the TSA assembly and, finally, 25,918 contigs were uploaded. The complete collection of the CLC assembly (27,777 contigs) was added to this manuscript in FILE S2.2. The (alternative) Velvet/Oases *P. ulmi* assembly was added as FILE S2.3 to this manuscript.

2.2.3. Blast homology searches and sequence annotation

All *P. ulmi* contigs (27,777) were used for BLASTx searches against the NCBI non-redundant (nr) protein database (version of September 3rd, 2014; E-value cut-off of $1E^{-5}$) using Standalone BLAST 2.2.30+ (Shiryev et al., 2007). Sequences that did not yield BLASTx hits were subsequently searched against the non-redundant nucleotide database using BLASTn and with an e-value cut-off of $1E^{-5}$. As *T. urticae* is not yet included in the NCBI nr protein database, *P. ulmi* contigs were also used as query in BLASTx against the *T. urticae* proteome (version March 2014, http://bioinformatics.psb.ugent.be/orcae/overview/Tetur). All BLAST results were imported in Blast2GO (version 2.8) to assign gene ontology (GO) terms to sequences retrieved by BLAST search (Conesa et al., 2005). Further annotation was done with InterPro, where protein motifs were directly queried at the InterProScan web service and consequently merged to the existing annotation (Hunter et al., 2012). The annotation results were further fine-tuned with (1) the Annex function in order to augment the annotation through the inference of biological process and cellular component terms from molecular function annotations (Myhre et al., 2006), and (2) the generic GO slim reduction to summarize the functional information of the transcriptome dataset. Finally, to enable better visualisation of the results, Gene Ontology relationships and enzyme codes (ECs) were highlighted on the Kyoto Encyclopedia of Genes and Genomes (KEGG) maps.

2.2.4. Analysis of genes related to xenobiotic detoxification

Contigs encoding putative *P. ulmi* CYPs, CCEs, GSTs, UGTs and ABC proteins were retrieved from the *P. ulmi* transcriptome (CLC assembly) by a tBLASTn search and using protein sequences of *T. urticae* CYPs, CCEs, GSTs, UGTs and ABCs as query (Ahn et al., 2014; Dermauw et al., 2013a; Grbić et al., 2011). tBLASTn searches were performed with NCBI Standalone BLAST 2.2.30+ (Shiryev et al., 2007) and a cut-off E-value $<1E^{-5}$. Open reading frames (ORFs) of *P. ulmi* contigs encoding detoxification enzymes and ABC proteins were identified using 'EMBOSS 6.3.1 getorf' integrated in the Mobyle portal framework (http://mobyle.pasteur.fr/). *P. ulmi* ORFs were aligned against each other using BLASTn. In cases where two ORFs showed more than 94% identity at the nucleotide level, they were considered as allelic variants and the longest ORF was retained for phylogenetic analysis. ORFs with identical overlapping sequences were considered to be part of the same gene and

merged using BioEdit version (Hall, 1999), except if the merged ORF misaligned when blasted to the *T. urticae* protein database.

Two previously published *P. citri* transcriptomes were also mined for transcripts encoding detoxification enzymes and ABC transporters. For both published *P. citri* transcriptomes, the cDNA library was constructed using a mixture of RNA extracted from different developmental stages and subsequently sequenced using Illumina HiSeq 2000 technology with a read-length of 90 bp (Liu et al., 2011; Niu et al., 2012). However, as the *P. citri* transcriptome sequence assemblies (TSAs) have not been made publicly available, we assembled these transcriptomes *de novo* using a similar approach as for *P. ulmi* and using all reads in the sequence read archives (SRAs) ERR044692-ERR044695 and SRR341928 provided by the studies of Liu et al. (2011) and Niu et al. (2012), respectively. *P. citri* ORFs encoding CYPs, CCEs, GSTs, UGTs, and ABC proteins were identified using a similar approach as for *P. ulmi*. Likewise, these *P. citri* ORFs were aligned against each other using BLASTn. In case two *P. citri* contigs showed at least 94% identity they were considered as allelic variant and the longest ORF was retained for phylogenetic analysis.

A final selection of *Panonychus* ORFs encoding CYPs, GSTs UGTs and ABCs was translated into protein sequences and aligned with their homologues in *T. urticae* using MUSCLE version 3.8.31 (Edgar, 2004). Alignments were inspected by eye and for each alignment only protein sequences showing no misalignment and having a minimum ORF length (CYPs: 450 nt, CCEs: 450 nt, GSTs: 300 nt, UGTs: 450 nt) were retained for the final alignment. Two phylogenetic analyses were performed for the CYP gene family: one with all tetranychid CYPs and one restricted to tetranychid and *D. melanogaster* mitochondrial CYPs (see Good et al., 2014). *Panonychus* mt CYPs were identified based on the phylogenetic analysis of all tetranychid CYPs. For the phylogenetic analysis of CCEs, a reference set of arthropod CCE protein sequences was also included in the alignment (TABLE S2.1). As N- and C termini of CCEs are highly variable, the alignment of CCE protein sequences was trimmed as previously described (Claudianos et al., 2006). Only the nucleotide binding domain (NBD) of ABC proteins encoded by *Panonychu*s ORFs was used for phylogenetic analysis of ABC proteins. NBDs were extracted using ScanProsite (http://prosite.expasy.org/scanprosite/) and the PROSITE profile PS5089. Next to *T. urticae* ABC transporter NBDs, C- and N terminal NBDs of *D. melanogaster* ABC transporters were also included in phylogenetic analysis of tetranychid ABC transporter NBDs (Dermauw et al., 2013a). Model selection was done with ProtTest 2.4 and according to the Akaike information criterion WAG+G+F, LG+I+G+F, LG+I+G+F, LG+I+G, WAG+G+F and LG+G+F were optimal for the phylogenetic reconstruction of CYPs, mitochondrial CYPs, UGTs, GSTs, CCEs and ABC proteins, respectively. Finally, for each alignment a maximum likelihood analysis was performed using Treefinder (version 2011) (Jobb et al., 2004) bootstrapping with 1000 pseudoreplicates (LR-ELW). The resulting trees were midpoint rooted and edited with MEGA 6.0 software (Tamura et al., 2013).

2.2.5. Annotation of horizontally transferred genes (excluding UGTs)

Annotation of *P. ulmi* and *P. citri* contigs encoding horizontally transferred genes (HTGs) was done in a similar way as for genes involved in xenobiotic detoxification. Model selection and phylogenetic analysis of IDRCD genes was done in a similar way as for genes involved in xenobiotic detoxification. According to the Akaike information criterion WAG+I+G+F was optimal for phylogenetic reconstruction of tetranychid IDRCDs.

2.2.6. Identification of *P. ulmi* target-site sequences

To obtain *P. ulmi* target-site sequences as full length as possible we mined both *P. ulmi* transcriptomes (CLC and Velvet/Oases, see SECTION 2.2.2) for contigs encoding target-sites of acaricides using tBLASTn (E-value cutoff $<1E^{-5}$) and *T. urticae* target-site protein sequences as query. *P. ulmi* contigs encoding target-sites [from both transcriptomes (CLC and Velvet/Oases)] were aligned against each other and those contigs having identical overlapping regions were merged. The resulting contigs were manually assembled to obtain *P. ulmi* target-site sequences as full length as possible. All reads were then mapped against the *P. ulmi* target-site sequences as for the differential expression analysis (see SECTION 2.2.7) with this difference that resulting BAM files were merged for each strain using the SAMtools package (Li et al., 2009). For both the spirodiclofen susceptible and the resistant *P. ulmi* strain, a consensus sequence was then derived using a perl script (gene_extractor.pl) written by Rutger Vos and available at https://github.com/naturalis/fastq-simple-tools/tree/master/script. The obtained *P. ulmi* target-site sequences were screened for all target-site mutations previously described in the literature (Feyereisen et al., 2015).

2.2.7. Differential expression analysis

To assess differential gene expression between the HS and PSR-TK strains, we first took the reverse complement of each *P. ulmi* contig where the majority of its BLASTx hits mapped on the reverse strand. *P. ulmi* contigs were subsequently concatenated into a single sequence with spacers ('Ns') of length 100 added between contigs using a custom python script. For each RNA-seq replicate (4 per strain, 8 in total), reads were mapped against this concatenated sequence using Bowtie version 2.1.0 (Langmead and Salzberg, 2012) with the preset option '--very-sensitive-local' and with the maximum fragment length for valid paired-end alignments set to 1000 (-X 1000). Mapped reads were counted using htseq-count that is included in the HTSeq package (Anders et al., 2013). Forward strand reads were counted using the '--stranded=reverse' option while the '--stranded=yes' option was used to count reverse strand reads. Those *P. ulmi* contigs of which the proportion between the number of reads mapping to one strand and the total number of reads mapping to either the forward or the reverse strand was more than or equal to 0.95 were considered to have strand-specific reads (20,069 contigs, 72.3% of all contigs). Read counts of *P. ulmi* contigs with (1) a BLAST hit, (2) strand-specific reads and (3) not considered contamination (see above) were used for differential expression analysis. Differentially expressed contigs between the resistant (PSR-TK) and susceptible (HS) *P. ulmi* strain were determined using the DESeq2 (version 1.6.3; Anders et al., 2013) and Bioconductor (http://bioconductor.org/) R-packages. The 'unfiltered DESeq2 results' settings [dds <- DESeq(dds, minReplicatesForReplace=Inf) and res <- results(dds, cooksCutoff=FALSE, independent Filtering=FALSE)] were used for differential expression analysis. *P. ulmi* contigs with a fold change higher than two and a Benjamini-Hochberg adjusted *p*-value less than or equal to 0.05 were considered differentially expressed.

2.2.8. RT-qPCR

A set of eight differentially expressed contigs were evaluated by RT-qPCR. This set included 3 down- and 5 upregulated *P. ulmi* contigs. *P. ulmi* contig_01010 and contig_00741 showed best BLASTx hits with *T. urticae* RP49 (*tetur18g03590*) and ubiquitin (*tetur06g00900*), E-value of $4E^{-70}$ and $5E^{-59}$, respectively, and were used as reference genes for RT-qPCR. Primers were designed using Primer3 v.0.4.0 (Rozen and Skaletsky, 2000) and are listed in TABLE S2.2. RNA was extracted in triplicate from each strain as described above and 2µg was reverse transcribed using the Maxima First Strand cDNA synthesis kit for RT-qPCR (Fermentas Life Sciences). All qPCR reactions were conducted with a thermal cycler Mx3005P (Stratagene). Reactions were prepared with Maxima SYBR Green qPCR/Master Mix following the manufacturer's instructions (Fermentas Life Sciences) and run in two technical and three biological replicates. Non-template-controls were also included to eliminate potential contamination of the samples. The qPCR run protocol was as follows: initial denaturation at 95°C for 10s, 35 cycles of 95°C for 15s, 55°C for 30s, 72°C for 30s. To exclude the possibility of nonspecific amplification, each PCR was followed by a melting curve step (ramping from 95 to 55 °C by 1 °C every 2s) to confirm a single amplicon. Amplification efficiency of each primer pair was calculated from the standard curve, prepared of cDNA of all samples tested, with a dilution range from 0.4 ng to 50 ng RNA. The Ct values for *P. ulmi* contig_01010 (RP49) and contig_00741 (ubiquitin) were used for normalization. Significant differences in the relative expression values of the target genes were tested with pairwise fixed reallocation randomization (Pfaffl, 2001; Pfaffl et al., 2002).

2.3. RESULTS AND DISCUSSION

2.3.1. De novo sequence assembly

We performed high-throughput Illumina sequencing of RNA extracted from adult females from acaricide susceptible (HS) and resistant *P. ulmi* strains (PSR-TK). With the Illumina HiSeq platform we generated 66,308,047 and 95,412,934 strand-specific paired-end reads for the HS and PSR-TK *P. ulmi* strains, respectively. Two approaches were used to assemble the *P. ulmi* transcriptome (see SECTION 2.2 for details). The assembly created with CLC Genomics Workbench (CLC) contains 27,777 unique *P. ulmi* contigs longer than 200 bp, totalling 27.6 Mb of sequence with an overall GC content of 33.6%. The average contig size was 993 bp and the N50 was 2,087 bp. The Velvet/Oases assembly, on the other hand, consists of 30,044 transcripts totalling 18.6 Mb of sequence with a GC content of 34.5% and an average contig size/N50 of 619 bp/871 bp. Mapping all RNA-seq reads against both transcriptomes (CLC or Velvet/Oases) revealed that the overall mapping success rate, as a measure for assembly quality, was significantly lower for the Velvet/Oases assembly compared to the CLC assembly (see TABLE S2.3), with an average overall alignment rate of 91.7 and 73.4% for the CLC and Velvet/Oases assembly, respectively). Hence, unless otherwise stated, the CLC assembly was used for all further analyses described in this study. All raw reads and the CLC assembly were submitted to NCBI under BioProject PRJNA271858.

As the transcriptome assemblies of two previously published *P. citri* transcriptomes (Liu et al., 2011; Niu et al., 2012) were not publicly available, and to generate assemblies with similar methodology, we assembled these transcriptomes in a similar way (CLC) as for *P. ulmi* (FILES S2.4 and S2.5). *Panonychus citri* assembly statistics were in line with those of *P. ulmi,* with 25,529 contigs and an average contig size/N50 of 708 bp/1057 bp for the Liu et al. (2011) *P. citri* transcriptome and 32,362 contigs and an average contig size/N50 of 646 bp/969 bp for the Niu et al. (2012) *P. citri* transcriptome.

2.3.2. Homology searches, species distribution and Gene Ontology (GO) analysis

A total of 9190 sequences (33.1% of all contigs) had a BLAST hit in the NCBI non-redundant (nr) database, with 9109 sequences having a BLASTx hit against the nr protein database and 81 sequences having a BLASTn hit against the nr nucleotide database. As *T. urticae* gene annotations have not yet been uploaded to the NCBI database, we also performed a local BLASTx search against the *T. urticae* proteome. We found that 11,250 *P. ulmi* contigs showed a BLASTx hit with the predicted *T. urticae* proteome, including 2,483 that had no BLASTx-hit in the NCBI nr database. In total, 11,673 *P. ulmi* contigs (42.0% of all contigs) had a BLAST-hit in at least one analysis (TABLE S2.4). Although 42.0% of contigs with a BLAST-hit may appear to be limited, we found that this value does not significantly differ from other *de novo* transcriptome studies with non-model arthropod species [e.g., in Francis et al. (2013), Sparks and Nauen (2015)]. In the Niu et al. (2012) transcriptome study of the related *P. citri*, the percentage of contigs was also of similar magnitude (50.7%). Such a low percentage can be caused by multiple reasons. The majority of *de novo* transcriptomes contains a considerable amount of short contigs (<240 bp), containing incomplete (<80 AA) ORFs that in many cases will not BLAST at a given E-value threshold. On the other hand some contigs may be non-coding RNAs which do not BLAST with the non-redundant protein/nucleotide database (or just UTR fragments of otherwise coding genes).

Out of the contigs having a BLAST-hit, 94.1% (10,989 contigs) of the top BLAST hits belonged to metazoan species, 2.9% to bacteria (342 contigs), 1.6% (181 contigs) to plants, 0.6% (75 contigs) to fungi and 0.7% (86 contigs) to other organisms. The highest homologies for the majority of the metazoan BLAST hits belonged to the Chelicerata (55.7%, 6,498 contigs) and Arthropoda (20.6%, 2,404 contigs) (FIGURE S2.1). A detailed analysis of all chelicerate BLAST hits revealed a high number of top BLAST hits with *T. urticae* (21.9%, 2,553 contigs), followed by the social velvet spider *Stegodyphus mimosarum* (17.8%, 2,080 contigs), the tick *Ixodes scapularis* (8.5%, 987 contigs) and the predatory mite *Metaseiulus occidentalis* (4.7%, 545 contigs).

We performed a Gene Ontology (GO) analysis of the *P. ulmi* assembled sequences after excluding contigs (227) that were considered as foreign contamination and consisted mainly of sequences belonging to *Wolbachia* sp. (111 contigs) and the host plant (*Prunus* sp.) of the *P. ulmi* HS and PSR-TK strains (79 contigs) (see SECTION 2.2). GO terms were assigned to 5,833 unique contigs (21.2% of 27,550 contigs) and in most cases multiple GO terms were assigned to the same *P. ulmi* contig. We found that 17,161 (54.8%), 6,623 (21.2%) and 7,534 (24.1%) GO terms emerged for biological process, cellular component and molecular function categories, respectively. Those were further categorized into 16 biological process, 9 molecular function and 7 cellular component sub-categories (FIGURE 2.1). Among the 16

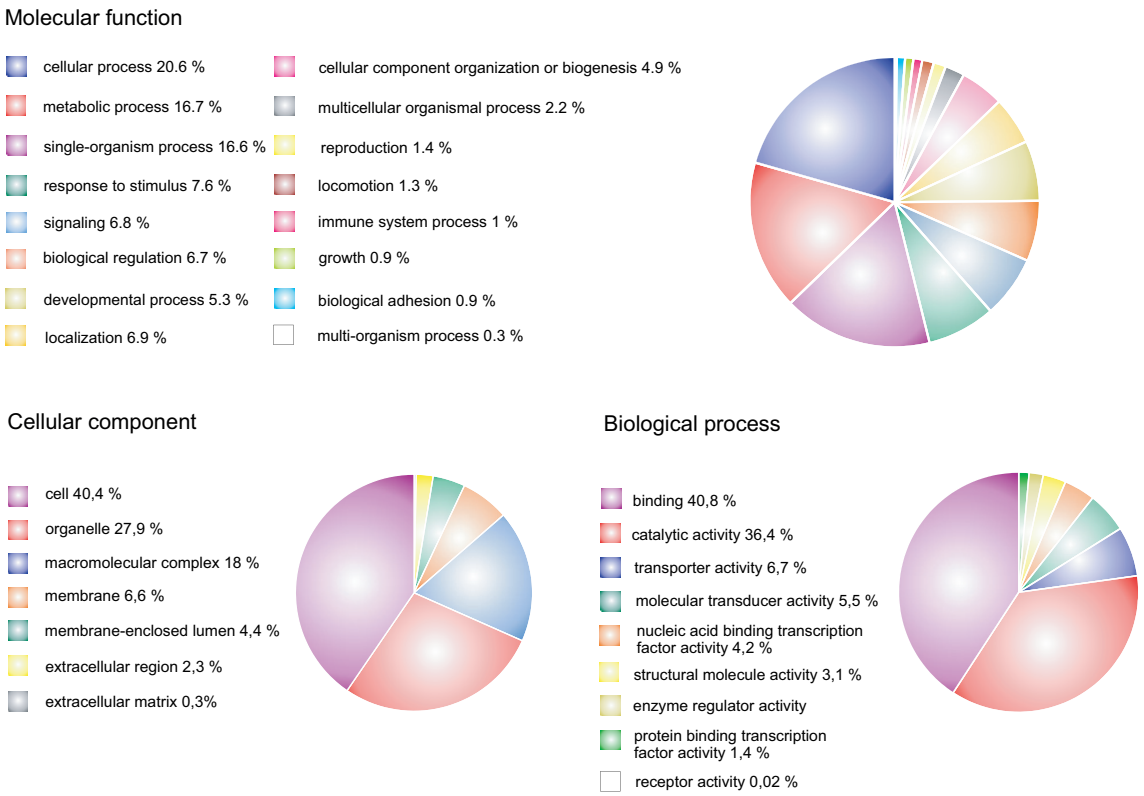

FIGURE 2.1 – Gene ontology annotation and classification (level 2) of the *Panonychus ulmi* transcriptome. Results are summarized into three main categories: molecular function, biological process and cellular component. In total GO terms were assigned to 5,833 *P. ulmi* contigs.

sub-categories of biological process, the cellular process occupied the highest number (3,535, 20.6%), followed by the metabolic process (2,858, 16.7%), single organism process (2,853, 16.6%) and response to stimulus (1,308, 7.6%) (FIGURE 2.1). The major sub-categories of the Molecular function category included binding (2,705, 40.8%), followed by catalytic activity (2,409, 36.4%) and transporter activity (445, 6.7%) (FIGURE 2.1). In the Cellular component domain, the majority of the GO terms were shown to be specific for the cell (3,047, 40.4%), followed by organelle (2101, 27.9%) and macromolecular complex (1,355, 18,0%) (FIGURE 2.1). To further increase the annotation functionality, all contigs were mapped to the reference canonical pathways in the Kyoto Encyclopedia of Genes and Genomes (KEGG). KEGG mapping resulted in 861 contigs that were assigned to 117 KEGG pathways, with 35.4% of *P. ulmi* KEGG enzymes identified as transferases, 25.2% as hydrolases, 22.4% as oxidoreductases, 8.1% as ligases, 6.2% as lyases, while isomerases constituted for 2.7% of annotated enzymes (FIGURE S2.2). Our GO and KEGG analyses of the *P. ulmi* transcriptome are in line with previous GO analyses of *P. citri* transcriptomes where cellular process, binding and cell were the three major sub-categories (Liu et al., 2011; Niu et al., 2012) and indicates that our approach using the Illumina HiSeq platform provided an extensive representation of the *P. ulmi* transcriptome.

2.3.3. Analysis of *Panonychus* genes involved in xenobiotic resistance

In general, arthropods have developed two types of mechanisms to cope with xenobiotic compounds, both of which can contribute to the development of resistance: mechanisms that decrease exposure due to quantitative or qualitative changes in major detoxification enzymes and transporters (pharmacokinetic mechanisms) and mechanisms that decrease sensitivity due to changes in target-site sensitivity caused by point mutations (pharmacodynamic mechanisms) (Despres et al., 2007; Feyereisen et al., 2015; Van Leeuwen et al., 2015). The pharmacokinetic mechanism can be subdivided into three phases (I-III). In phase I detoxification enzymes such as cytochrome P450 monooxygenases (CYPs) and carboxylcholinesterases (CCEs) incorporate a nucleophilic functional group (a hydroxyl, carboxyl or amine group) into the toxic compound, resulting in a more reactive and water soluble compound. During phase II, enzymes such as UDP-glycosyltransferases (UGTs) and glutathione-S-transferases (GSTs) further increase the water solubility of the phase I metabolite by conjugation with endogenous molecules like sugars and glutathione, respectively. In Phase III, conjugates are transported out of the cell by cellular transporters, e.g., ABC transporters (ABCs).

In this study we mined the *P. ulmi* and *P. citri* transcriptomes for genes encoding known target-sites and major detoxification enzymes and transporters (CYPs, CCEs, UGTs, GSTs and ABCs) and compared them with those of *T. urticae,* for which a high-quality genomic assembly and annotation is available (Grbić et al., 2011). In addition, we also performed a phylogenetic analysis for all major detoxification gene families. It should be noted, however, that as transcriptomic data for *Panonychus* sp. is compared to genomic data for *T. urticae*, care should be taken when comparing numbers and ortho/homologues of *Panonychus* detoxification genes as recent gene duplications and genes with very low expression levels might be missed. In addition, expression of detoxification genes might also be stage-dependent. Hence, gene number differences between both *Panonychus* species should also be carefully interpreted as both *P. citri* transcriptomes were assembled using RNA-seq data from mixed stages (Liu et

al., 2011; Niu et al., 2012) while the *P. ulmi* transcriptome was based solely on RNA-seq data of adult females (see SECTION 2.2).

2.3.3.1. Cytochrome P450 monooxygenases

Cytochrome P450 monooxygenases (P450s) are heme-containing enzymes with a diverse range of functions. Many P450s are important phase I detoxification enzymes with a crucial role in detoxification of plant secondary metabolites and in metabolizing insecticides/acaricides to less toxic compounds. Four major clans can be distinguished in the CYP gene family, namely the CYP2, CYP3, CYP4 and M (mitochondrial CYP genes) (Feyereisen, 2012). The advent of the *T. urticae* genome allowed a first insight into the CYP gene family of a phytophagous mite, revealing 81 full length CYPs. This number is similar to what is found in insects, but with an expansion of *T. urticae* specific intronless genes of the CYP2 clan (Grbić et al., 2011). A total of 63 *P. ulmi* and 118 *P. citri* CYP non-allelic ORFs could be identified (TABLES 2.1 and S2.5). Only those CYPs that did not misalign in the final alignment and had a minimum ORF length of 450 nt were included in a phylogenetic analysis (41 *P. ulmi* and 49 *P. citri* ORFs) (FIGURE 2.2). Based on this analysis or based on its best BLASTx hit with a *T. urticae* CYP, all *P. ulmi and P. citri* CYPs could be assigned to one of the four CYP clans (FIGURE 2.2, TABLES 2.1 and S2.5) (Grbić et al., 2011).

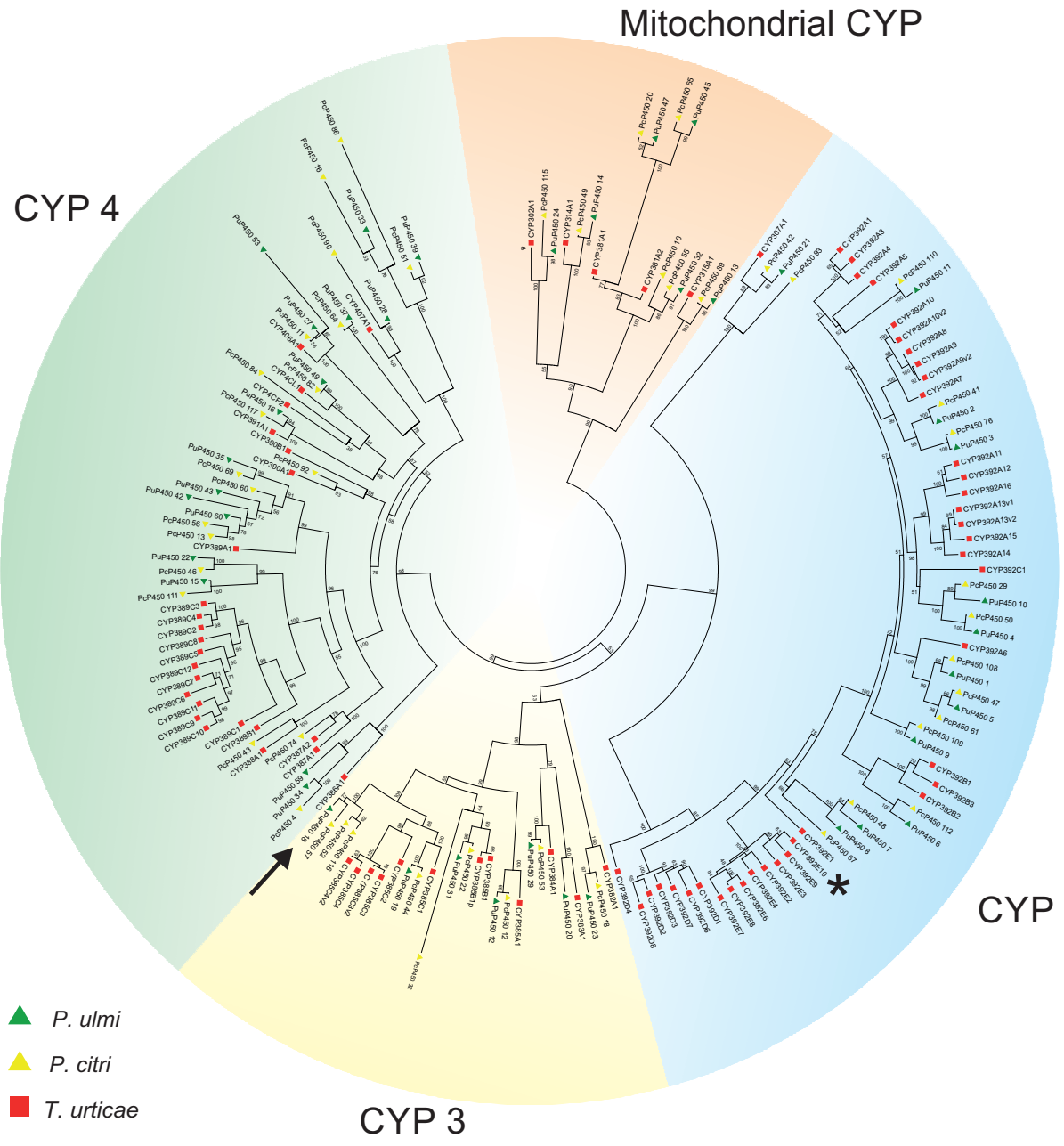

FIGURE 2.2 – Phylogenetic analysis of *Panonychus ulmi* putative CYPs. Maximum likelihood tree of *P. ulmi, Panonychus citri* and *Tetranychus urticae* CYP protein sequences. The tree was constructed using MUSCLE (Edgar, 2004) and Treefinder (Jobb et al., 2004). The tree was midpoint rooted and numbers at the branch point of each node represent the bootstrap value resulting from 1000 pseudoreplicates (LR-ELW). The scale bar represents 0.5 substitutions per site. Tetranychid CYPs clustered into the four main CYP clades: mitochondrial CYPs, CYP 2, CYP 3 and CYP 4. Colour and shape codes are as follows: *P. ulmi*, green triangle, *P. citri,* yellow triangle and *T. urticae*, red square. An arrow and an asterisk indicate CYPs associated with high levels of spirodiclofen resistance in *T. urticae* (Demaeght et al., 2013) and *P. ulmi* (this study), respectively. Tetranychid CYP protein sequences and accession numbers can be found in TABLE S2.5.

TABLE 2.1 – Comparison of the CYP gene number in *Tetranychus urticae, Panonychus ulmi* and *Panonychus citri*.

Clan	*T. urticae*	*P. ulmi**	*P. citri**
CYP 2	39	12 (16)	14 (31)
CYP 3	12	7 (8)	9 (26)
CYP 4	25	16 (30)	19 (50)
mitochondrial CYP	5	6 (9)	7 (11)
Total	81	41 (63)	49 (118)

*Numbers without brackets represent the number of *Panonychus* CYPs that were included in phylogenetic analysis (FIGURE 2.2) while number within brackets represent the total number of non-allelic CYP ORFs found in *Panonychus* species (see SECTION 2.2).

In vertebrates, mitochondrial CYPs (clan M) are associated with steroid or vitamin D metabolism while in insects this clan comprises two groups: one group involved in the ecdysteroid metabolism pathway and another group that metabolize a variety of xenobiotic compounds (Feyereisen, 2012). We identified 9 *P. ulmi* and 11 *P. citri* ORFs in this clan and detected three clear cases of 1:1:1 orthology between *P. ulmi, P. citri* and *T. urticae*: *CYP314A1, CYP315A1* and *CYP302A1* (TABLES S2.5 and 2.1, FIGURE S2.3). Together with *T. urticae CYP307A1* of the CYP2 clan (see below), these CYPs are the only *T. urticae* CYPs that could be assigned as clear orthologs of insect and crustacean CYPs. They are known to be involved in the ecdysteroid pathway in insects and their tetranychid counterparts likely fulfill an analogous role (Grbić et al., 2011). Similar to *T. urticae*, orthologs of insect CYP2 clan members *CYP306A1* and *CYP18A1* could also not be identified for both *Panonychus* species (TABLE S2.5, FIGURE 2.2). In insects, CYP306A1s are involved in the synthesis of 20 hydroxyecdysone (20E) by hydroxylating carbon 25, while *CYP18A1* is a hydroxylase/oxidase involved in ecdysteroid degradation (Grbić et al., 2011). Their absence in *Panonychus* sp. further corroborates that this gene cluster was lost as a whole and is an ancient trait, leading to the use of ponasterone A as the moulting hormone instead of 20E.

In insects, the number of CYP2 genes is relatively well conserved and several insect CYP2 genes are known to be involved in essential physiological functions. Compared to insects the number of CYP2 genes is more diverse in *T. urticae* and the cladoceran *Daphnia* sp. (Feyereisen, 2012; Grbić et al., 2011) and at present the 392 family within the CYP2 clan is best characterized in *T. urticae*. A whole genome microarray gene expression analysis revealed that several members of the CYP2 clan were upregulated in acaricide resistant *T. urticae* strains or in a *T. urticae* strain adapted to a new challenging host plant (Demaeght et al., 2013; Dermauw et al., 2013b). Furthermore, functional expression of two *T. urticae* CYP2 clan enzymes, CYP392A16 and CYP392E10, confirmed that these could metabolise the acaricides spirodiclofen and abamectin, respectively (Demaeght et al., 2013; Riga et al., 2014). We identified 16 and 31 CYP2 ORFs in *P. ulmi* and *P. citri*, respectively. In our phylogenetic analysis, there are two clear cases of 1:1:1 orthology within the CYP2 clan (*T. urticae CYP307A1*, and *CYP392A5*). The phylogenetic analysis also suggests that the potential expansion/loss events of certain CYP2 subfamilies, like CYP392A, CYP392D and CYP392E, occurred after the split between the *Tetranychus* and *Panonychus* genus. Consequently, it was not surprising that we could not identify a clear *P. ulmi*/*P. citri* ortholog of *T. urticae CYP392E10* and *CYP392A16*, suggesting that spirodiclofen or abamectin metabolism must depend on different P450s or other factors in *Panonychus* species (see SECTION 2.2.7).

Insect CYP3 and CYP4 clans comprise the insect specific families CYP4, CYP6, CYP9 and CYP325 that are well known for their involvement in environmental response/detoxification functions against xenobiotics and phytotoxins (Feyereisen, 2012). We identified 8 *P. ulmi* and 26 *P. citri* ORFs in the CYP3 clan, while 30 *P. ulmi and* 50 *P. citri* ORFs were found in the CYP4 clan. Within the CYP3 and CYP4 clan we found 5 (*T. urticae CYP382A1*, *CYP384A1*, *CYP385A1*, *CYP385B1*, *CYP385C1*) and 3 (*T. urticae CYP407A1*, *CYP391A1*, and *CYP4CL1*) clear cases of 1:1:1 orthology between *T. urticae, P. ulmi* and *P. citri*, respectively. Within the CYP4 clan, the CYP389A subfamily consists of a single gene in *T. urticae* while 4 paralogs are present in each, *P. ulmi* and *P. citri*. In contrast, the CYP389C subfamily appears to be larger in *T. urticae* compared to the two *Panonychus* species. Interestingly, Ran et al. (2012) showed that *P. citri* paralogs of *T. urticae CYP389A1* were among the most up- and down regulated CYP genes in an amitraz resistant strain. Ding et al. (2013), on the other hand, showed that a *P. citri* ortholog of *T. urticae CYP4CF2* was highly induced upon exposure to pyridaben while the expression of a *P. citri* ortholog of *T. urticae CYP4CL1* increased after induction by abamectin, azocyclotin, pyridaben, and spirodiclofen. In contrast to the CYP2 clan, none of these tetranychid CYP3 and CYP4 clan members have been functionally characterized.

2.3.3.2. Carboxylcholinesterases

Carboxylcholinesterases (CCEs) catalyse the hydrolysis of carboxylesters and have a whole gamut of physiological functions including degradation of neurotransmitters, metabolizing hormones and pheromones, regulation of behaviour and detoxification of xenobiotics (Claudianos et al., 2006; Oakeshott, 2005). CCEs are phase I detoxification enzymes and their role in xenobiotic metabolism and the development of resistance in arthropods has been thoroughly studied (recently reviewed in Feyereisen et al., 2015). A classification based on the CCE phylogeny, as proposed by (Oakeshott, 2005) groups insect CCEs into 13 clades spread over 3 classes: the dietary/detoxification enzymes (clades A–C), the generally secreted enzymes (clades D–G) and the neuro/developmental CCEs (clades I–M, mainly non-catalytic esterases) (Claudianos et al., 2006; Oakeshott, 2005). While total numbers of CCEs in insects and *T. urticae* are similar, the neuro/developmental class is expanded in *T. urticae* with two new subclades J' and J". In addition, in contrast to insects no *T. urticae* CCEs have been identified within the currently recognized classification of insect dietary CCEs (Grbić et al., 2011).

We found a total of 61 and 91 CCE non-allelic ORFs in the *P. ulmi and P. citri* transcriptome, of which 41 and 40 ORFs of *P. ulmi* and *P. citri*, respectively, were included into a phylogenetic analysis. Based on this analysis or the best BLASTx hit with *T. urticae* CCEs, *P. ulmi and P. citri* CCEs could be assigned to one of the 13 CCE clades (FIGURE 2.3, TABLES 2.2 and S2.1). Similar to *T. urticae* CCEs, the majority of *Panonychus* CCEs belong to the neuro/developmental clade, with 18 *P. ulmi* and 25 *P. citri* CCEs in clade J" and 17 *P. ulmi* and 23 *P. citri* CCEs in clade J' (TABLES 2.2 and S2.1, FIGURE 2.3). In 16 cases, 1:1:1 orthologous groups could be identified between *T. urticae* and *Panonychus* species: 1 in clade J (AChE), 1 in clade K (gliotactin), 1 in clade

L (neuroligins), 1 in clade J', 2 in clade F' (Acari/Crustacean Juvenile hormone CCEs), 3 in undetermined clades (NC) and 7 in clade J". Remarkably, within the subclade J', a putative expansion of CCEs in *T. urticae* (17 CCEs) was found, suggesting these *T. urticae* J' CCEs arose after the *Tetranychus*/*Panonychus* divergence within the Tetranychidae. Finally, in clade J' two clear *P. ulmi* orthologues of *P. citri* PcCCE16 and PcCCE39 could be identified. The latter have their best BLASTn (E-value of 0.0) hit with *P. citri* PCE1 and PCE2, respectively, which have been shown to be induced by acaricide exposure (Zhang et al., 2013).

2.3.3.3. Glutathione-S-transferases

Glutathione-S-transferases (GSTs) belong to a multifunctional enzyme family, known to play an important role in phase II of xenobiotic detoxification. In addition to pesticide detoxification by conjugation, insect GSTs have also been reported to play a role in attenuation of oxidative stress caused by pesticide exposure and sporadically by metabolism of pesticides directly (Ortelli et al., 2003). Insects cytosolic GSTs can be divided into seven classes [delta (δ), epsilon (ε), omega (ω), sigma (σ), theta (θ) and zeta (ζ)] with delta and epsilon GSTs well known for their role in detoxification of organophosphates and organochlorines (Enayati et al., 2005; Feyereisen et al., 2015). In contrast to insects, Acari also have mu-class GSTs, which were until recently thought to be verterbrate specific (Grbić et al., 2011; Reddy et al., 2011).

We found a total of 19 *P. ulmi* and 37 *P. citri* GST non-allelic ORFs of which 13 *P. ulmi* and 23 *P. citri* GSTs were included in a phylogenetic analysis (TABLES 2.3 and S2.6, FIGURE 2.4). Based on this analysis or based on the best BLASTp hit with *T. urticae*

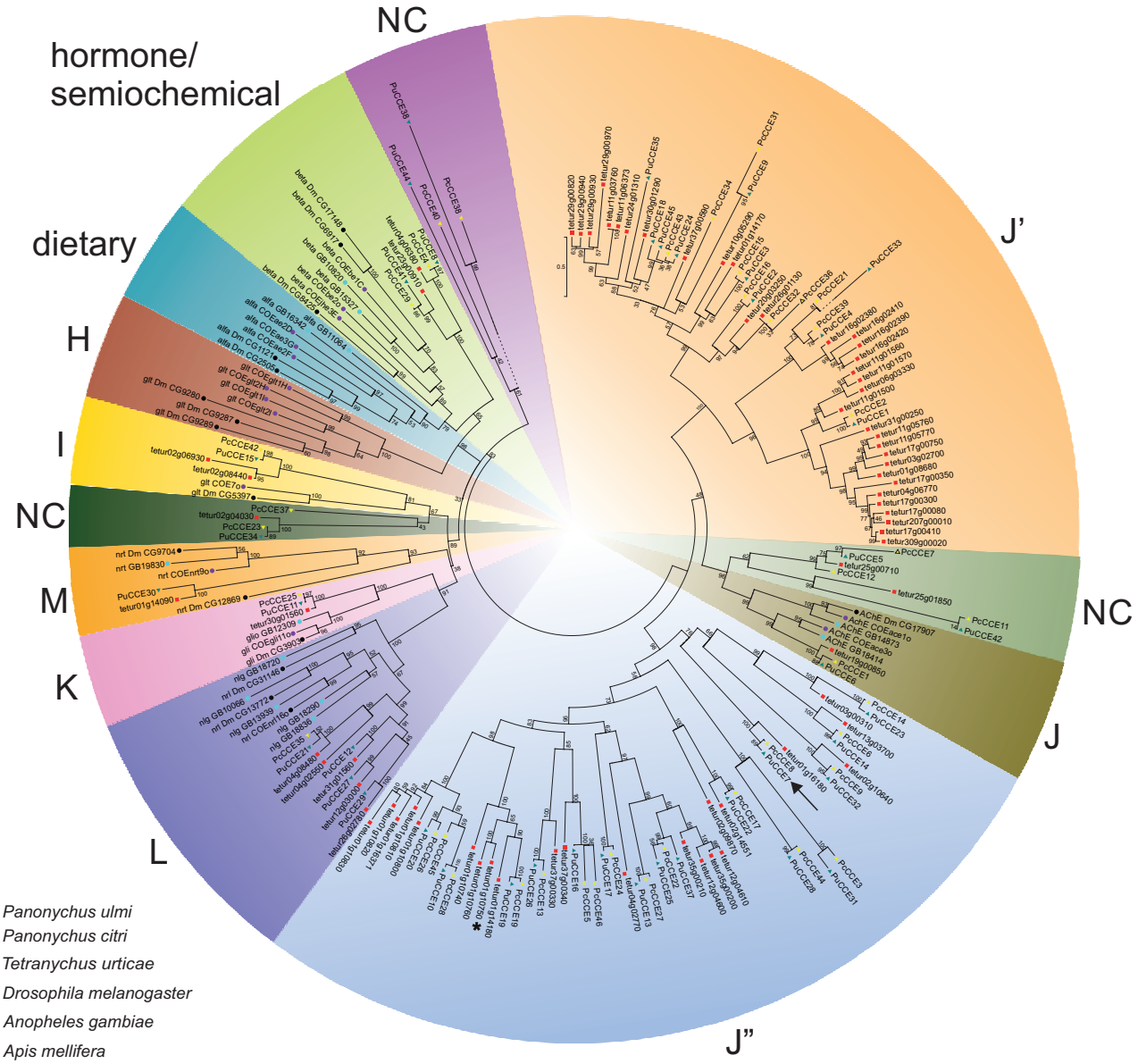

FIGURE 2.3 – Phylogenetic analysis of *Panonychus ulmi* putative CCEs. A set of *P. ulmi*, *Panonychus citri*, *Tetranychus urticae, Drosophila melanogaster, Anopheles gambiae* and *Apis mellifera* CCE protein sequences were aligned using MUSCLE, subsequently trimmed according to Claudianos et al. (2006) and subjected to a maximum-likelihood analysis using Treefinder (Jobb et al., 2004). The tree was midpoint rooted and numbers at the branch point of each node represent the bootstrap value resulting from 1000 pseudoreplicates (LR-ELW). The scale bar represents 0.5 substitutions per site. Tetranychid CCEs clustered into clades (Claudianos et al., 2006; Grbić et al., 2011): F' (Crustacean/Acari JhE), I, J (AChEs), J, J', K (gliotactins), L (neuroligins), M (neurotactins) and three undetermined clades (NC). Colour and shape codes are as follows: *P. ulmi,* green triangle, *P. citri,* yellow triangle, *T. urticae,* red square, *D. melanogaster,* black dot, *An. gambiae*, purple dot and *Ap. mellifera*, blue dot. An arrow and an asterisk indicate CCEs associated with high levels of spirodiclofen resistance in *T. urticae* (Demaeght et al., 2013) and *P. ulmi* (this study), respectively. CCE protein sequences and accession numbers can be found in TABLE S2.1.

TABLE 2.2 – Comparison of the CCE gene number in *Tetranychus urticae, Panonychus ulmi, Panonychus citri* and *Drosophila melanogaster*.

Class/Clade	*T. urticae*	*P. ulmi**	*P. citri**	*D. melanogaster*
Dietary class (Clade A, B, C)				13
Hormone/semiochemical class				
D				3
E				2
F				3
G				
F'	2	2	2 (4)	
Neuro/developmental class				
H				5
I	2	1	1	1
J	1	1	1	1
K	1	1	1	1
L	5	4 (13)	1 (20)	4
M	1	1	0 (4)	2
J'	34	10 (17)	10 (23)	
J"	22	16 (18)	17 (25)	
Undetermined clades (NC)	3	5 (7)	7 (12)	
Total	71	41 (61)	40 (91)	35

*Numbers without brackets represent the number of *Panonychus* CCEs that were included in phylogenetic analysis (FIGURE 2.3) while number within brackets represent the total number of non-allelic CCE ORFs found in *Panonychus* species (see SECTION 2.2.4).

GSTs, *P. ulmi* and *P. citri* GSTs could be assigned to 4 GST-classes: delta, mu, omega and zeta. The number of *P. ulmi* GSTs is lower than those observed in *P. citri, T. urticae* and *Ixodes scapularis* (TABLE 2.3). Although BLASTp searches against the non-redundant database performed by Niu et al. (2012) identified representatives of sigma and theta GST we were unable to find neither of those two classes in the transcriptome of *P. ulmi*. The lack of theta and sigma GSTs seems to be a common feature of Acari species analysed to date (Reddy et al., 2011; Xu et al., 2014). Similar to the phylogenetic analysis of tetranychid CYP genes (see SECTION 2.3.3.1) only limited orthologous relationships between tetranychid GSTs could be established, as illustrated by the presence of *T. urticae* specific clusters of delta and mu GSTs. In both the omega and zeta class we identified a 1:1:1 orthology between *Panonychus* contigs and *T. urticae* GSTs (*TuGSTo01*: PcGST6: PuGST8 and *TuGSTz01*: PcGST14: PuGST7). Within the mu GSTs we found two 1:1:1 orthologs (*TuGSTm11/TuGSTm05*: PuGST12: PcGST9 and *TuGSTm02*: PcGST24: PuGST11) while no 1:1:1 orthologs could be identified in the delta GSTs (FIGURE 2.4).

Using a whole genome gene-expression microarray, members of the same GST classes were also shown to be highly upregulated in multi-resistant *T. urticae* strains (Dermauw et al., 2013b). Three GST genes (*TuGSTd10*, *TuGSTd14*, *TuGSTm09*) that were upregulated in a strain MAR-AB highly resistant against abamectin and other important acaricides were also functionally expressed. Out of these, TuGSTd14 showed the highest affinity toward abamectin and had a competitive type of inhibition, suggesting the acaricide may bind to the H-site of the enzyme (Pavlidi et al.). However, as mentioned earlier no clear *Panonychus* orthologs of *TuGSTd14* could be identified in our study.

2.3.3.4. ABC transporters

The ATP Binding Cassettte (ABC) protein family is a large and ubiquitous family of proteins. Most members of this family use ATP to transport substrates across lipid membranes, and hence are referred to as ABC transporters. Based on the sequence similarity of their nucleotide binding domain (NBD, the domain that binds ATP) the ABC protein family can be divided into eight subfamilies (A to H). Although extensively characterized for their involvement in drug resistance in vertebrates, the role of ABC transporters in arthropod xenobiotic resistance is less well known. Nevertheless, ABC transporters have been associated with resistance to as many as 27 insecticides/acaricides from nine different chemical classes (Dermauw and Van Leeuwen, 2014). However, the major source of evidence linking these arthropod ABC transporters to resistance has been ABC transporter expression quantification or synergism studies (Dermauw and Van Leeuwen, 2014). A total of 103 ABC genes were identified in the *T. urticae* genome, the highest number discovered in a metazoan species to date. This high number of ABC genes in *T. urticae* is due predominantly to lineage-specific expansions of the ABCC, ABCG and ABCH subfamilies (Dermauw et al., 2013a).

TABLE 2.3 – Comparison of the cytosolic GST gene number in *Tetranychus urticae, Panonychus ulmi, Panonychus citri* and *Ixodes scapularis*.

Class	*T. urticae*[1]	*P. ulmi*[2]	*P. citri*[2]	*I. scapularis*[3]
delta	16	6 (11)	10 (16)	12[1]
mu	12	4 (5)	10 (18)	14
omega	2	2	2	3
zeta	1	1	1	3
Total	31	13 (19)	23 (37)	32

[1]In Grbić et al.(2011), the number of theta and zeta GSTs was switched: it should be zero theta *T. urticae* GSTs and one zeta *T. urticae* GST
[2]Numbers without brackets represent the number of *Panonychus* GSTs that were included in phylogenetic analysis (FIGURE 2.4) while number within brackets represent the total number of non-allelic GST ORFs found in *Panonychus* species (see Methods)
[3]Numbers derived from Reddy et al. (2011)
[4]The number of *I. scapularis* epsilon (5) and delta GSTs (7) was merged as it was not clearly shown in the *I. scapularis* GST gene family study by Reddy et al. (2011) that epsilon GSTs actually do cluster with epsilon GSTs of insects.

We found 145 *P. ulmi* and 230 *P. citri* ORFs with a tBLASTn hit with *T. urticae* ABC protein sequences (TABLES S2.7 and 2.4). NBDs from the ORFs of these contigs, when present, were extracted and used in a maximum likelihood phylogenetic analysis. Similar to previous studies (Dermauw et al., 2013a) C-terminal NBDs of ABCC transporters clustered together with NBDs of ABCB transporters (FIGURE 2.5, TABLE S2.7). Based on this analysis or, in case the ORF did not encode an NBD, based on its best BLASTx hit with *T. urticae* ABC proteins, we assigned *P. ulmi* and *P. citri* ABC ORFs to one of the 8 ABC gene subfamilies (TABLES 2.4 and S2.7, FIGURE 2.5). As ABC genes are large genes (around 2 to 5 kb in size), they can be easily fragmented into multiple contigs assembled from RNA-seq reads. This could result in an overestimation of the number of putative ABC genes. Therefore, we used the number of NBDs to estimate quantitative differences among the ABC gene families in the Tetranychidae (TABLE 2.4). The number of *T. urticae* NBDs is much higher than the number of *P. citri* and *P. ulmi* NBDs, and is mainly due to a higher number of *T. urticae* NBDs in the ABCA, ABCC, ABCG and ABCH gene subfamily. In several cases *T. urticae* ABCH NBDs do not have a counterpart in *Panonychus* species while in both the ABCA, ABCC as ABCG gene family a putative expansion of *T. urticae* NBDs compared to *Panonychus* NBDs can be suspected. Interestingly, for exactly two of these *T. urticae* ABC gene subfamilies it was shown that several genes were differentially expressed in multi-pesticide resistant strains and/or in mites transferred to challenging host plants (Dermauw et al., 2013b). Finally, NBDs of ABC transporters that are considered as conserved in arthropods [ABCB half transporters (*D. melanogaster CCG7955*, *CG1824*), ABCC (*D. melanogaster CG7806*, *sur*), ABCD, ABCE, ABCF, ABCG (*D. melanogaster CG3327*, *CG11069*, *CG31121*)] were also found in both *Panonychus* species (TABLES 2.4 and S2.7, FIGURE 2.5) (Dermauw and Van Leeuwen, 2014).

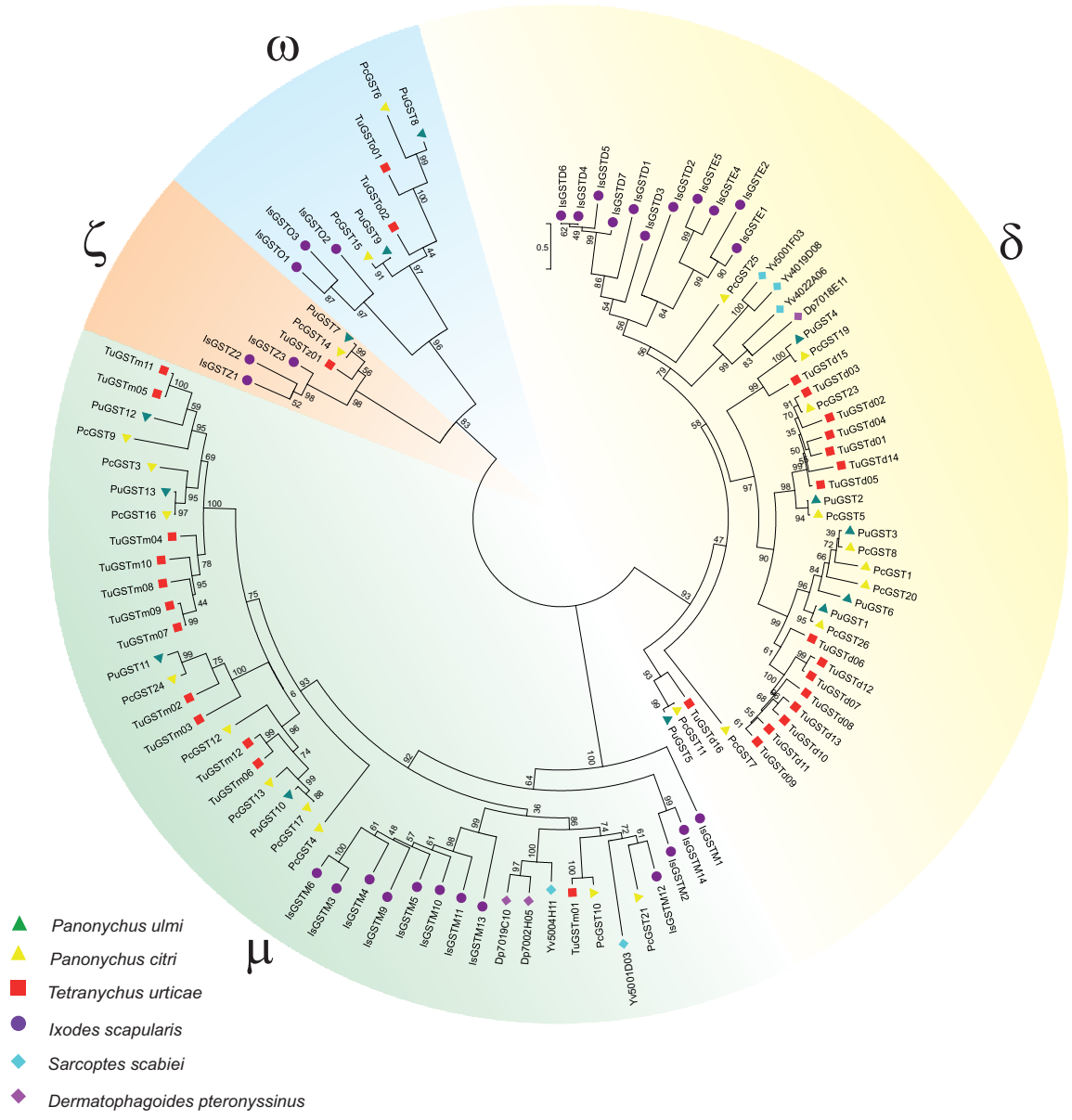

FIGURE 2.4 – Phylogenetic analysis of *Panonychus ulmi* putative GSTs. A set of *P. ulmi*, *Panonychus citri*, *Tetranychus urticae, Ixodes scapularis, Sarcoptes scabiei, Dermatophagoides pteronyssinus* GST protein sequences were aligned using MUSCLE and subjected to a maximum-likelihood analysis using Treefinder. The tree was midpoint rooted and numbers at the branch point of each node represent the bootstrap value resulting from 1000 pseudoreplicates (LR-ELW). Tetranychid GSTs clustered within classes: δ, delta class, ζ, zeta class, ω, omega class and μ, mu class. Colour and shape codes are as follows: *P. ulmi,* green triangle, *P. citri,* yellow triangle, *T. urticae,* red square, purple dot, *I. scapularis*, blue rhombus, *S. scabiei* and pink rhombus, *D. pteronyssinus*. GST protein sequences and accession numbers can be found in TABLE S2.6.

2.3.4. Horizontally transferred genes

Horizontal gene transfer (HGT) refers to the asexual transfer of genetic information between non-related species. In bacteria and fungi HGT is a strong evolutionary force contributing to adaptation and colonization of challenging environments. Due to the unique characteristics of animal biology, the prevalence and impact of HGT on animal evolution was considered to be minor. However, recent studies have uncovered a vast array of horizontally transferred genes (HTGs) in the *T. urticae* genome with plausible roles in xenobiotics detoxification or other responses to the environment. These include 80 UDP-glycosyltransferases (Ahn et al., 2014), 16 intradiol ring-cleavage dioxygenases (Dermauw et al., 2013b), 3 carotenoid desaturases, 2 carotenoid cyclase/synthases (Bryon et al., 2013; Grbić et al., 2011), 2 levanases (Grbić et al., 2011), a cyanate lyase (Wybouw et al., 2012), β-cyanoalanine synthase (Wybouw et al., 2014) and a methionine synthase (Grbić et al., 2011). Orthologs of these *T. urticae* HTGs were identified in both *Panonychus* transcriptomes (TABLES 2.5, 2.6 and S2.8). The presence of these genes in the *Panonychus* genus strongly suggests that these horizontal gene transfers occurred

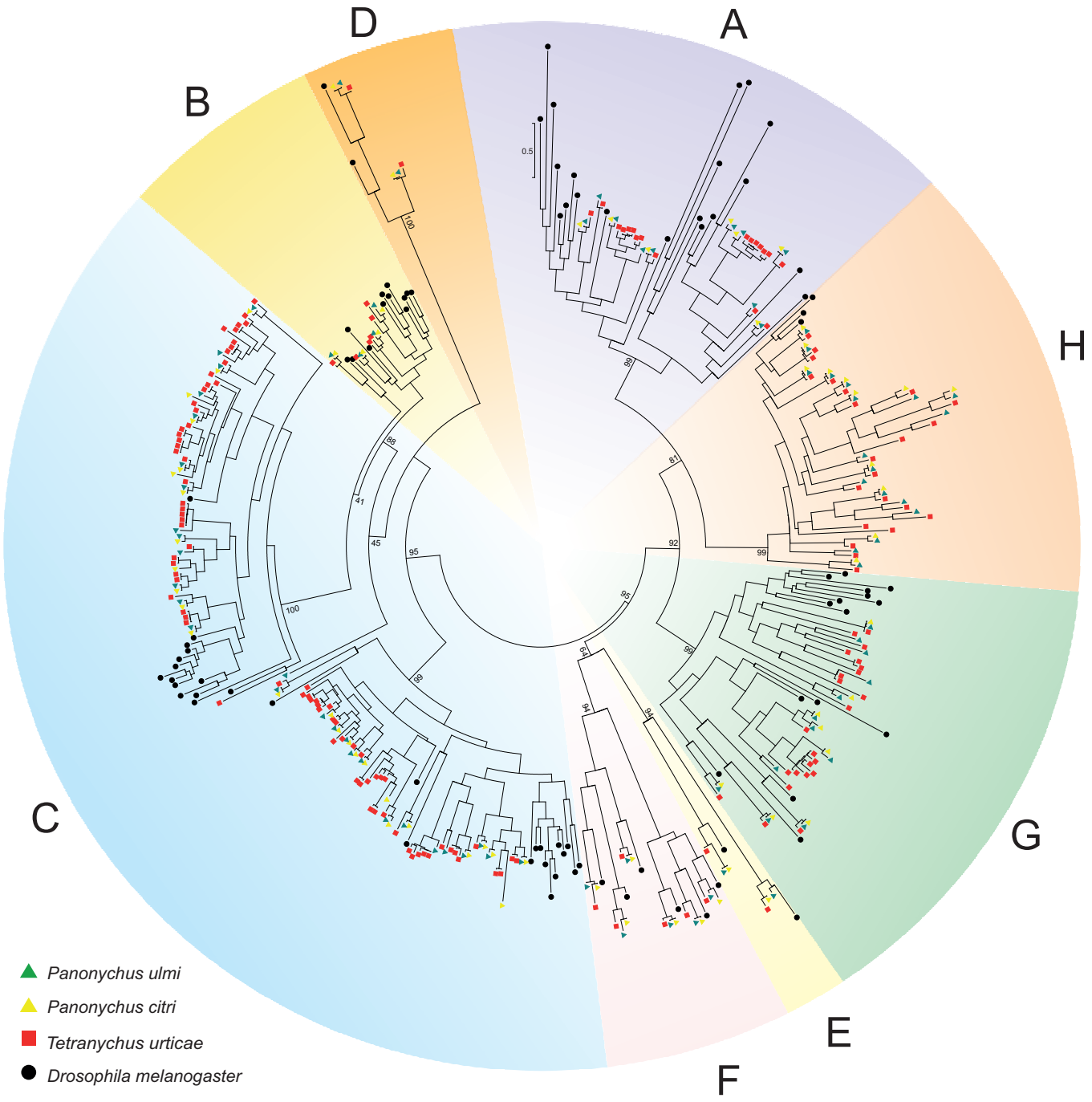

FIGURE 2.5 – Phylogenetic analysis of *Panonychus ulmi* ABC protein NBDs. Maximum likelihood midpoint rooted tree of N- and/or C-terminal NBDs extracted from *P. ulmi* and *Panonychus citri* ABC protein coding ORFs and *Tetranychus urticae* and *Drosophila melanogaster* ABC protein sequences. The scale bar represents 0.5 amino-acid substitutions per site. *P. ulmi* and *P. citri* NBDs clustered within the eight currently described ABC subfamilies (A to H). The tree was midpoint rooted and numbers at the branch point of each node represent the bootstrap value resulting from 1000 pseudoreplicates (LR-ELW). C-terminal NBDs of ABCC transporters clustered together with NBDs of ABCB transporters (Dermauw et al., 2013a). Colour and shape codes are as follows: *P. ulmi,* green triangle, *P. citri,* yellow triangle, *T. urticae,* red square and *D. melanogaster,* black dot. ABC protein sequences and accession numbers can be found in TABLE S2.7.

TABLE 2.4 – Comparison of the number of *Tetranychus urticae*, *Panonychus ulmi* and *Panonychus citri* ABC protein NBDs.

Class	*T. urticae*	*P. ulmi*	*P. citri*
A	18	10	7
B	6	4	4
C	78	28	26
D	2	2	2
E	2	2	2
F	6	6	6
G	23	12	8
H	22	18	12
Total	157	82	67

before the split of the *Tetranychus* and *Panonychus* genus. As the majority of the microbial xenologues of these unique spider mite genes code for enzymes able to detoxify and break down plant secondary metabolites, it has been argued that HGT facilitated spider mite adaptations to a phytophagous lifestyle (Wybouw et al., 2014). In the sections below we will discuss some of these tetranychid HGT families more into detail.

2.3.4.1. UDP-glycosyltransferases

UDP-glycosyltransferases (UGTs) are common in the majority of living organisms including viruses, bacteria, plants and animals. They catalyze the conjugation of small lipophilic molecules with uridine diphosphate (UDP) sugars, increasing their water solubility. As such, UGTs play an important role in the synthesis, storage and transport of secondary metabolites. In vertebrates, UGTs are also well studied because of their role in phase II drug metabolism (Ahn et al., 2014; Rowland et al., 2013). Although a role for UGTs in arthropod xenobiotic resistance was suggested more than twenty years ago (Brattsten, 1992), only recently has functional evidence for their role in xenobiotic resistance been presented (Ahn et al., 2011; Daimon et al., 2010; Lee et al., 2006; Sasai et al., 2009). It has been suggested that the UGT gene family might have been lost early in the Chelicerata lineage and subsequently re-gained in the tetranychid mites by means of the horizontal gene transfer from bacteria. This discovery provides important clues to UGTs functions in relation to detoxification and therefore host adaptation in the phytophagous mites.

We identified a total of 33 and 52 *P. ulmi* and *P. citri* UGT non-allelic ORFs, respectively. A subset of 24 *P. ulmi* and 32 *P. citri* ORFs were included in a phylogenetic analysis (FIGURE 2.6). Based on this analysis or based on the *T. urticae* best BLASTx hit, *P. ulmi* and *P. citri* UGTs could be assigned to one of the UGT subfamilies (FIGURE 2.6, TABLES 2.5 and S2.8). Within Tetranychidae the UGT201 subfamily is the largest UGT subfamily with 10, 23 and 36 UGT ORFs/genes in *P. ulmi*, *P. citri* and *T. urticae*, respectively (TABLES 2.5 and S2.8). The UGT201A, UGT201B, UGT202A and UGT204A subfamily are clearly more numerous in *T. urticae* compared to both *Panonychus* species while the opposite could be observed for the UGT201G subfamily, suggesting the UGTs in these subfamilies probably arose in *T. urticae* or were lost in *P. ulmi* after diversification within the Tetranychidae. Finally, within different UGT subfamilies clear 1:1:1 orthologous relationships could be observed between *Panonychus* UGTs and *T. urticae UGT202B1*, *UGT203G1*, *UGT203F1*, *UGT204C1*, *UGT205A3*, *UGT206A1* and *UGT207A1* (FIGURE 2.6).

TABLE 2.5 – Comparison of the UGT gene number in *Tetranychus urticae, Panonychus ulmi* and *Panonychus citri*.

Family	*T. urticae*	*P. ulmi*[1]	*P. citri*[1]
201	36	9 (10)	12 (23)
202	17	3 (5)	6 (8)
203	11	4 (5)	5 (9)
204	8	3	3
205	6	4 (7)	4 (7)
206	1	1 (2)	1
207	1	1	1
Total	80	25 (33)	32 (52)

[1]Numbers without brackets represent the number of *Panonychus* UGTs that were included in phylogenetic analysis (FIGURE 2.6) while number within brackets represent the total number of non-allelic UGT ORFs found in *Panonychus* species (see SECTION 2.2.4)

2.3.4.2. Intradiol ring-cleavage dioxygenases (IDRCDs)

Among Metazoa, tetranychid mites are the only species known to harbor genes encoding IDRCDs. In bacteria and fungi these enzymes catalyze the oxygenolytic fission of catecholic substances, allowing them to degrade aromatic rings, an essential step in the carbon cycle (Dermauw et al., 2013b; Grbić et al., 2011). However, their role in tetranychid mites has not yet been characterized. About half of the number of genes in this family were differentially expressed in mites upon host plant change and in multi-resistant *T. urticae* strains, and their expression patterns were highly correlated, suggestive of a role for this gene family in xenobiotic resistance (Dermauw et al., 2013b). Twelve *P. ulmi* and 23 *P. citri* contigs showed a tBLASTn hit with *T. urticae* IDRCD proteins. A phylogenetic analysis revealed several orthologous relationships between tetranychid IDRCDs (*tetur01g00490*, *tetur04g00150*, *tetur04g08620*, *tetur07g02040*, *tetur10g00490*, *tetur19g02300*, *tetur20g01160*), suggesting these IDRCD genes arose before the split between the *Tetranychus* and *Panonychus* genus (FIGURE 2.7). Furthermore, an expansion of IDRCDs can be observed in both *T. urticae* (17 IDRCD genes) as in *P. ulmi and P. citri* (12 and 23 genes, respectively). Remarkably, exactly those IDRCDs that are absent in both *Panonychus* species are highly upregulated in mites upon host plant change and in multi-resistant *T. urticae* strains (Dermauw et al., 2013b).

2.3.4.3. Other horizontal gene transfers

Besides UGTs and IDRCDs we also identified *Panonychus* orthologs of *T. urticae* carotenoid desaturases, carotenoid cyclases, levanases, cyanate lyase, β-cyanoalanine synthase and methionine synthase (TABLES 2.6 and S2.8). Wybouw et al. (2014) showed that a *T. urticae* strain overexpresses β-cyanoalanine synthase when adapted to cyanogenic plants. Functional expression also revealed that this enzyme is able to detoxify cyanide which is the main defensive phytochemical of these plants. In contrast to *T. urticae, Panonychus* sp. almost exclusively feed on Rosaceae (Migeon and Dorkeld, 2015), a cyanogenic plant family producing cyanide in various tissues as plant defense (Vetter, 2000). As such, β-cyanoalanine synthase might have been crucial in conferring resistance in *Panonychus* sp. to this continuous exposure to dietary cyanide.

2.3.5. Target-sites of insecticides/acaricides

Since the PSR-TK strain originates from the field and has evolved resistance to at least hexythiazox, clofentezine and

spirodiclofen (Kramer and Nauen, 2011), the *P. ulmi* transcriptome was mined for contigs encoding known target-sites of acaricides, in order to search for SNPs associated with target-site resistance. We successfully annotated acetylcholinesterase (AChE), voltage gated sodium channel (VGSC), GABA- and glutamate-gated chloride channels (Rdl and GluCl), chitin synthase 1 (CHS1), acetyl-CoA carboxylase (ACCase) and cytochrome B (cytB) (Demaeght et al., 2014; Dermauw et al., 2012; Feyereisen et al., 2015; Khajehali et al., 2010; Kwon et al., 2010a,b,c; Lummen et al., 2014; Tsagkarakou et al., 2009; Van Leeuwen et al., 2008; Van Nieuwenhuyse et al., 2009) [see TABLE 2.7 for an overview and the Insecticide Resistance Action Committee (IRAC) mode of action classes that target these proteins; Sparks and Nauen, 2015]. To obtain *P. ulmi* target-site sequences as full-length as possible, we also mined an alternative *P. ulmi* assembly, which was constructed using the Velvet/Oases package (FILE S2.3), and combined contigs from the latter assembly with the one described in this study (CLC Genomics Workbench).

Compared to insects *T. urticae* has a higher number of Rdl and GluCl genes, with the majority of insects having only one Rdl and GluCl gene while *T. urticae* has 3 and 6, respectively (Dermauw et al., 2012). Similar to *T. urticae* we identified 5 *P. ulmi* GluCl and 3 Rdl genes (FILE S2.1), suggesting that the Rdl and GluCl gene family diversified before the radiation of the Tetranychidae. Next, we compared all known target-site sequences of the spirodiclofen susceptible (HS) and resistant (PSR-TK) *P. ulmi* strains to identify non-synonymous single nucleotide polymorphisms (SNPs) that are unique for the resistant strain. For AChE, we identified the F331W substitution (*Torpedo californica* numbering) that was fixed in the PSR-TK strain but

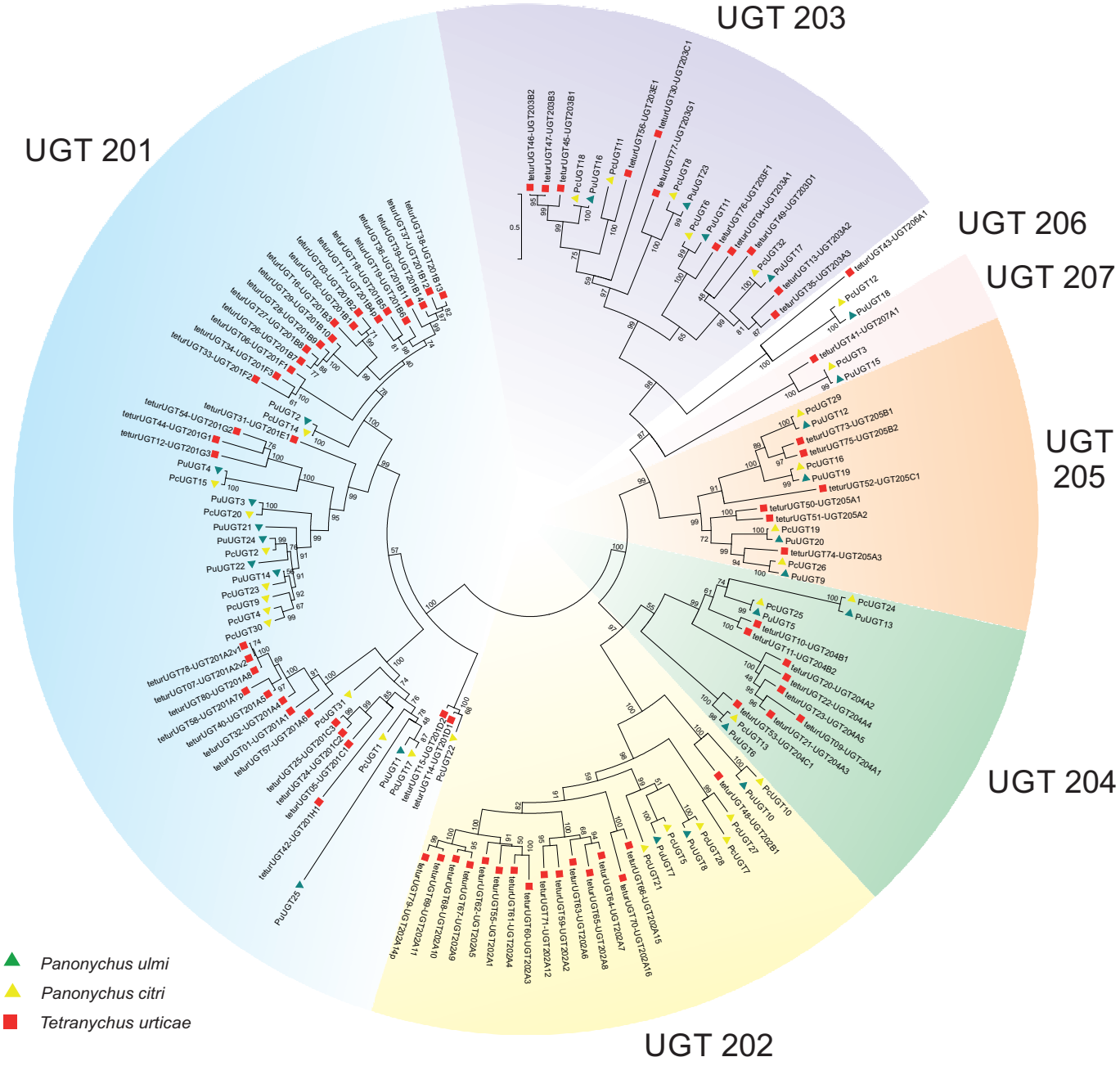

FIGURE 2.6 – Phylogenetic analysis of *Panonychus ulmi* putative UGTs. Maximum likelihood midpoint rooted tree of *P. ulmi*, *Panonychus citri* and *Tetranychus urticae* UGT protein sequences. The scale bar represents 0.5 amino-acid substitutions per site. Numbers at the branch point of each node represent the bootstrap value resulting from 1000 pseudoreplicates (LR-ELW). Color and shape codes are as follows: *P. ulmi,* green triangle, *P. citri,* yellow triangle and *T. urticae,* red square. UGT protein sequences can be found in TABLE S2.8.

TABLE 2.6 – Putative horizontally transferred genes identified in the *Tetranychus urticae* genome and *Panonychus* spp. transcriptomes.

	Carotenoid desaturase	Carotenoid cyclase/synthase	Cyanate lyase	Cysteine synthase A	IDRCD	Levanase	Methionine synthase
T. urticae	3	2	1	1	17	2	1
*P. ulmi**	2 (3)	2	1	1 (2)	8 (12)	2	1
*P. citri**	3 (6)	2 (4)	1 (2)	1 (2)	16 (23)	2 (5)	1 (5)

*Numbers within brackets show the total number of ORFs for each family of HTGs (excluding UGTs, see TABLE 2.5). Numbers without brackets depict HTG ORFs coding for proteins with an amino acid (AA) sequence length ≥ than 150 AA (carotenoid desaturases, levanases and methionine synthase), ≥ 200 AA (carotenoid synthases) and ≥ 100 AA (cyanate lyase and IDRCDs).

segregating in the HS strain. In the past, it has been shown that a substitution at this position causes resistance to organophosphate (OP) and carbamate (CB) insecticides/acaricides (Feyereisen et al., 2015) and the presence of this substitution in both *P. ulmi* strains emphasizes the scope of selection exerted by OPs and CBs during the second half of the 20th century.

Comparing ACCase sequences of both strains revealed several non-synonymous fixed SNPs in the PSR-TK strain that were not present in the susceptible HS-strain. At present, only one ACCase substitution has been associated with resistance against cyclic keto-enols: an E645K substitution in the whitefly *Trialeurodes vaporariorum* (Karatolos et al., 2012). However, the residue is not conserved in mites and at the corresponding position in the *P. ulmi* ACCase protein, the sequence is identical in both strains (a glutamine; at position 566, *T. urticae* numbering *tetur21g02170*). Recently, it has been shown that the activated

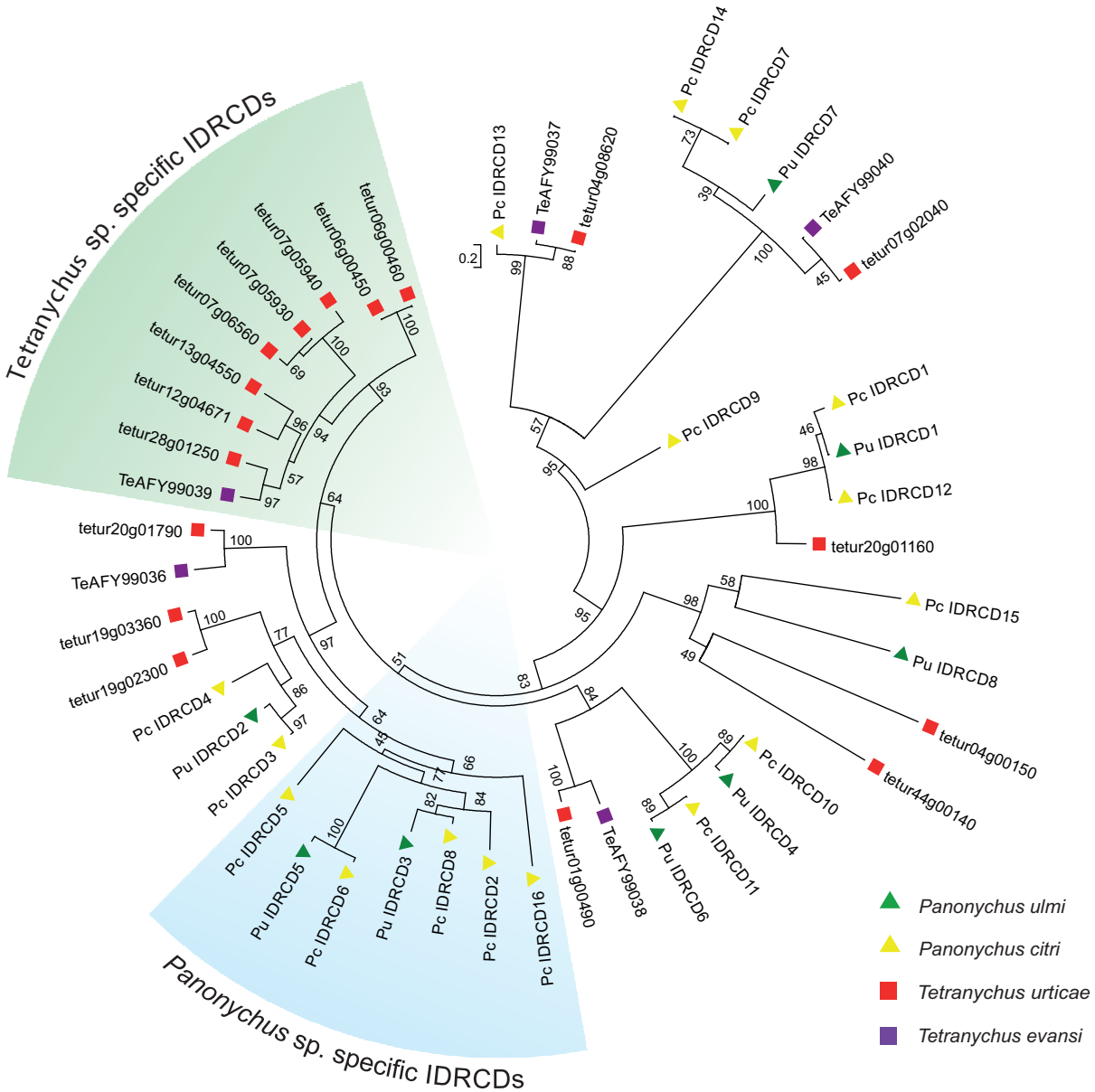

FIGURE 2.7 – Phylogenetic analysis of *Panonychus ulmi* putative IDRCDs. Maximum likelihood midpoint rooted tree of *P. ulmi*, *Panonychus citri*, *Tetranychus urticae* and *Tetranychus evansi* IDRCD protein sequences. The scale bar represents 0.2 amino-acid substitutions per site. Numbers at the branch point of each node represent the bootstrap value resulting from 1000 pseudoreplicates (LR-ELW). *Panonychus* sp. and *Tetranychus* sp. specific clades are shaded in blue and green, respectively. IDRCD protein sequences and accession numbers can be found in TABLE S2.8.

TABLE 2.7 – Known target-sites of acaricides, their *Tetranychus urticae* gene ID and presence in *Panonychus ulmi* and the IRAC acaricide classes that target these proteins.

Target-site	*T. urticae*[1] gene ID	length (nt)	*P. ulmi*[2] length (nt)	IRAC group	Reference
VGSC	*tetur34g00970*	6600	6696	3 (Sodium channel modulators)	Kwon et al., 2010a; Tsagkarakou et al., 2009
AChE	*tetur19g00850*	2064	2193	1 (AChE inh.)	Khajehali et al., 2010; Kwon et al., 2010b
ACCase	*tetur21g02170*	6957	6594	23 (Accase inh.)	Lummen et al., 2014
cytB	*FJ196444*	1063	1041	20 (Mt Complex III inh.)	Van Leeuwen et al., 2008; Van Nieuwenhuyse et al., 2009
CHS1	*tetur03g08510*	4608	4170	10 (Mite growth inh.)	Demaeght et al., 2014; Van Leeuwen et al., 2012
GluCl1	*tetur02g04080*	1338	1335	6 (Cl channel activators)	Dermauw et al., 2012; Kwon et al., 2010c
GluCl2	*tetur08g04990*	1368	987		
GluCl3	*tetur10g03090*	1629	1623		
GluCl4	*tetur22g02450*	1325	1344		
GluCl5	*tetur36g00090*	1392	1317		
GluCl6	*tetur41g00120*	1392	-		
Rdl1	*tetur12g03620*	2346	2049		
Rdl2	*tetur36g00580*	1572	1608		
Rdl3	*tetur36g00590*	2187	2019		

[1]*T. urticae* gene IDs can be accessed at the ORCAE database (http://bioinformatics.psb.ugent.be/orcae/overview/Tetur) while FJ96444 can be accessed at the NCBI database

[2]*P. ulmi* target-site sequences can be found in FILE S2.1

enol derivative of the insecticide spirotetramate interacts with the carboxyltransferase domain of ACCase (Lummen et al., 2014), suggesting this region as a prime site to expect resistance mutations. Within this domain region, we also found several residues that were different between the ACCase protein sequence of the PSR-TK and the HS-strain (FILE S2.1, FIGURE S2.4). However, it is unclear whether these substitutions play a role in resistance. Similarly to ACCase, other target-site sequences (VGSC, cytB, GluCl, Rdl and CHS1) contained non-synonymous SNPs in the PSR-TK strain that were not present in the HS strain, but none were located at positions previously reported to be involved in acaricide resistance (Feyereisen et al., 2015).

2.3.6. Differential expression analysis between an acaricide resistant (PSR-TK) and susceptible strain (HS) of *P. ulmi* using RNAseq data

RNA was extracted from 200 1-3 day old adult female *P. ulmi* mites from the PSR-TK and HS strains with four-fold biological replication (see SECTION 2.2). Using the replicated RNAseq data and the DESeq2 software, we performed a differential expression analysis between the acaricide resistant (PSR-TK) and a susceptible (HS) strain of *P. ulmi*. Only those contigs that (1) were not considered as contamination by the NCBI contamination screen (2) have strand-specific reads and (3) have a BLASTx hit were included in our differential expression analysis (see SECTION 2.2 for more details). For all *P. ulmi* contigs the number of mapped reads per contig can be found in TABLE S2.9. For further analyses described below, we excluded *P. ulmi* contigs without a BLASTx hit as a biological role of the proteins coded by these contigs cannot be assigned. We observed that 123 of 8722 *P. ulmi* contigs were significantly upregulated (Benjamini-Hochberg adjusted p-value ≤ 0.05 and $|FC| > 2$) in the PSR-TK strain compared to the HS strain, while 122 were significantly downregulated (FIGURE 2.8, TABLES 2.8, 2.9 and S2.10). For a subset of contigs (genes), the differential expression analysis based on RNAseq was validated by qPCR on cDNAs of the same populations (FIGURE 2.9).

Several up- and downregulated contigs showed high homology with sequences of arthropod bisegmented double stranded RNA viruses (biRNA viruses) (TABLES 2.8, 2.9 and S2.10). There are several reports documenting viruses infecting *Panonychus* species (Bird, 1967; Putman, 1970; Reed and Desjardins, 1982; Smith et al., 1959). As such, differential expression of biRNA viruses between the two resistant strains might reflect the different virus composition and infection status.

Among the differentially expressed contigs, 29 coded for major detoxification enzyme families or potential new players in detoxification (see above and Dermauw et al., 2013b). Among the upregulated contigs, we identified 4 CCEs, 3 CYPs, 3 GSTs, 3 ABCs, 4 UGTs, 2 lipocalins and 2 Major Facilitator Superfamily genes while 1 CYP, 2 CCEs, 1 GST, 2 ABCs and 2 IDRCDs were downregulated (FIGURE 2.8, TABLE S2.10). Such a broad response in detoxifying enzyme families is typically also encountered in the spider mite *T. urticae*, where numerous gene-expression studies of susceptible and resistant strains have shown an overlap in the gene-families involved (Demaeght et al., 2013; Dermauw et al., 2013b; Khalighi et al., 2015), but also specificity in term of the members of these families.

Two differentially expressed contigs also coded for the same small (~200 bp) fragment of the gene coding for ACCase, the target-site of cyclic keto-enols (Lummen et al., 2014) (see above), but with a six nucleotide difference between the two contig sequences. One of these contigs was highly upregulated (contig_21107) in the PSR-TK strain according to our analysis while the other was highly downregulated (contig_08025). A close inspection of mapped reads revealed that sequences polymorphism could explain this result (see TABLE S2.9). Therefore, we mapped all reads from both *P. ulmi* strains against the manually assembled *P. ulmi* ACCase gene (see above) and found no significant difference in expression (data not shown). Two contigs encoding CCEs (contig_19626/PuCCE13 and contig_00577/PuCCE7) and a CYP (contig_01016/PuCYP_18) were among the most highly upregulated contigs while another CCE contig (contig_00445/PuCCE2) was one of the most downregulated (FIGURE 2.9, TABLES 2.8, 2.9 and S2.10). RT-qPCR data confirmed the upregulation of contig_00577/ PuCCE7 and con-

TABLE 2.8 – Top 20 of upregulated contigs in the acaricide resistant *Panonychus ulmi* PSR-TK strain relative to the susceptible HS strain. See TABLE S2.10 for a full list of differentially expressed contigs.

Contig ID[1]	log_2FC	Blast2GO description	*T. urticae* BLASTx hit (E-value)[2]	*T. urticae* gene name
Contig_00184	6.06	RNA polymerase	-	-
Contig_01016	5.13	Cytochrome family	*tetur26g01470* (0)	Cytochrome P450 monooxygenase (CYP385C1)
Contig_19626	5.06	Acetylcholinesterase	*tetur35g00210* ($3E^{-171}$)	Carboxylcholinesterases (TuCCE66)
Contig_08154	3.61	Farnesoic acid o-methyl transferase-like protein	*tetur30g00770* ($3E^{-64}$)	Farnesoic acid O-methyltransferase-like protein
Contig_00577	3.59	Acetylcholinesterase	*tetur01g16180* (0)	Carboxylcholinesterases (TuCCE13)
Contig_14289	3.52	-	*tetur30g01870* ($7E^{-17}$)	Hypothetical protein
Contig_09906	3.51	Collagen alpha-4 chain	*tetur38g00240* ($1E^{-23}$)	Collagen alpha-2
Contig_21107	3.47	Acetyl- partial	*tetur21g02170* ($2E^{-22}$)	ACCase
Contig_01487	3.51	Cathepsin partial	*tetur25g00650* ($2E^{-95}$)	Cathepsin L
Contig_14168	3.47	Adenylyl cyclase associated protein	*tetur14g00690* ($2E^{-30}$)	Adenylate cyclase-associated CAP
Contig_00198	3.36	Polyprotein	-	-
Contig_08809	3.34	RNA polymerase 1	*tetur02g08750* ($3E^{-09}$)	RNA-directed RNA polymerase 1
Contig_04691	3.28	Polyprotein	-	-
Contig_06838	3.27	Glucosylceramidase isoform 2	*tetur02g12930* (0)	Glycoside hydrolase
Contig_26919	3.27	Intermediate in toll signal transduction	*tetur03g05570* ($2E^{-27}$)	Evolutionarily conserved signaling intermediate in Toll pathway
Contig_26878	3.17	Chimeric r1 r2 retrotransposon	*tetur04g02090* ($2E^{-26}$)	Similar to gag-like protein
Contig_12550	3.10	Collagen alpha-1	*tetur01g04240* ($3E^{-18}$)	Collagen alpha-1
Contig_12959	3.07	n/a	*tetur03g05210* ($2E^{-17}$)	U6 snRNA phosphodiesterase isoform X1 putative
Contig_16990	3.03	Leucine-rich repeat	*tetur14g01680* (0)	Similar to leucine-rich transmembrane protein
Contig_25316	3.00	RNA polymerase 2-like	*tetur02g08750* ($2E^{-15}$)	RNA-directed RNA polymerase 1

[1]Contigs in bold have their best BLASTx hit with biRNA viruses while underlined contigs contain a gene of which the upregulation is the result of spirodiclofen selection.
[2]E-value threshold was set at $1E^{-5}$.

tig_01016/PuCYP18 and the downregulation of contig_00445/PuCCE2, while upregulation of contig_19626/PuCCE13 was only partially confirmed (FIGURE 2.9). qPCR expression analysis also confirmed that the up- and downregulation of contig_00577/PuCCE7 and contig_00445/PuCCE2 in PSR-TK might be the result of spirodiclofen selection, with both contigs being slightly downregulated in Ge 16/09 (parental strain of PSR-TK) and highly up- and downregulated in PSR-TK relative to HS, respectively (FIGURE 2.10). In addition, the same pattern was found for a CYP (contig_01016/PuP450_18) which is highly upregulated in PSR-TK-while and slightly downregulated in GE 16/09 compared to the HS strain (FIGURE 2.10).

Previously, it was shown that the P450 inhibitor PBO and the esterase inhibitor DEF could enhance the toxicity of spirodi-

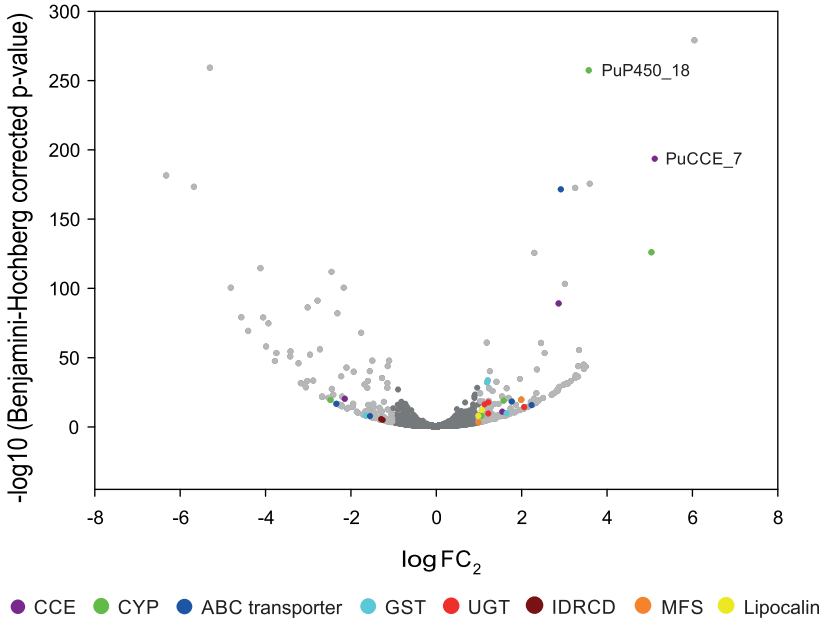

FIGURE 2.8 – Volcano plot of differentially expressed contigs in the acaricide resistant PSR-TK strain relative to the susceptible HS strain. The negative log_{10} of Benjamini-Hochberg (BH) adjusted *p*-values was plotted against the log_2FC in gene expression. *Panonychus ulmi* contigs up- and downregulated, by twofold or more (123 and 122 genes, respectively) are depicted as light grey circular dots. Circular dots of various colours depict contigs with a best BLASTx hit with *Tetranychus urticae* proteins putatively involved in xenobiotic detoxification (Dermauw et al., 2013b; see also TABLE S2.10). Contigs encoding detoxification genes putatively involved in spirodiclofen resistance are indicated in the plot by ID PuP450_18 (contig_01016, log_2(FC) = 5.1, BH adjusted p-value= $1,1E^{-193}$) and ID PuCCE_7 (contig_00577, log_2(FC)= 5.1, BH adjusted p-value =$1,4E^{-257}$).

TABLE 2.9 – Top 20 of downregulated contigs in the acaricide resistant *Panonychus ulmi* PSR-TK strain relative to the susceptible HS strain. See TABLE S2.10 for a full list of differentially expressed contigs.

Contig ID[1]	log_2FC	Blast2GO description	*T. urticae* BLASTx-hit (E-value)*	*T. urticae* gene name
Contig_10595	-6.31	Nucleolin 2-like	*tetur04g03300* ($9E^{-17}$)	4Fe-4S ferredoxin, iron-sulpur binding domain
Contig_03877	-6.05	Krab-a domain-containing protein	*tetur03g07110* ($7E^{-05}$)	Zinc finger protein
Contig_04800	-5.66	Chemosensory gustatory receptor family	*tetur07g05030* ($5E^{-25}$)	Gustatory receptor family
Contig_06558	-5.29	DNA polymerase	*tetur21g01960* ($6E^{-09}$)	Pacifastin
Contig_02213	-4.80	Histone-lysine n-methyltransferase setmar-like	*tetur04g09537* ($8E^{-06}$)	Transposable element Tc3-related transposase
Contig_04690	-4.55	Polyprotein	-	-
Contig_09905	-4.39	Collagen alpha-4 chain	*tetur38g00240* ($9E^{-24}$)	Collagen alpha-2
Contig_03088	-4.10	Farnesoic acid o methyltransferase-like protein	*tetur30g00780* ($1E^{-14}$)	Farnesoic acid O-methyltransferase-like protein
Contig_02422	-4.04	Reverse transcriptase maturase	*tetur21g01960* ($3E^{-11}$)	Proteinase inhibitor I19, pacifastin
Contig_00445	-3.99	Esterase type b	*tetur20g03250* (0)	Carboxylcholinesterases (TuCCE50)
Contig_02182	-3.97	Retrovirus-related pol polyprotein from transposon partial	*tetur09g03700* ($1E^{-18}$)	Hypothetical cell surface protein
Contig_08551	-3.92	Adenylyl cyclase-associated protein	*tetur14g00690* ($1E^{-29}$)	Adenylate cyclase-associated CAP
Contig_08025	-3.76	Acetyl – partial	*tetur21g02170* ($1E^{-29}$)	ACCase
Contig_10729	-3.73	Hypothetical protein TcasGA2_TC016169	*tetur04g02090* ($3E^{-17}$)	Similar to gag-like protein
Contig_09937	-3.41	Expressed sequence ai451617	*tetur03g07110* ($3E^{-12}$)	Zinc finger protein
Contig_04137	-3.40	Alpha-galactosidase a	*tetur02g04310* ($1E^{-42}$)	Glycoside hydrolase, catalytic core
Contig_08881	-3.21	Reverse transcriptase	*tetur63g00020* ($6E^{-18}$)	Hypothetical protein
Contig_00197	-3.16	Structural polyprotein	-	-
Contig_11839	-3.07	Paired box and transposase domain	*tetur04g09537* ($2E^{-13}$)	Transposable element Tc3-related transposase
Contig_00506	-3.04	Retrovirus-related pol	*tetur40g00380* ($4E^{-14}$)	Hypothetical protein

[1]Contigs in bold have their best BLASTx hit with *Drosophila melanogaster* biRNA viruses while the underlined contig contains a CCE gene of which the downregulation is the result of spirodiclofen selection

[2]E-value threshold was set at $1E^{-5}$

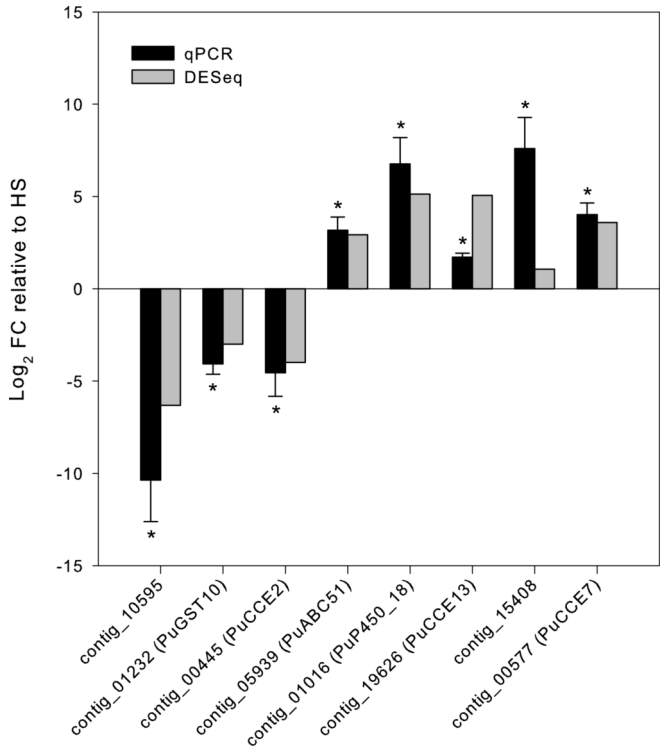

FIGURE 2.9 – Validation of differentially expressed *Panonychus ulmi* contigs by qPCR. Five upregulated and three downregulated contigs determined by differential gene expression analysis of RNA-seq data (see FIGURE 2.8) were selected for qPCR analysis. The data from qPCR were presented as mean of three replicates. Error bars represent the standard error of the calculated mean. Asterisks indicate significantly different expression values compared to the reference condition (HS).

clofen in a highly spirodiclofen resistant *T. urticae* strain (Van Pottelberge et al., 2009) and in spirodiclofen resistant *P. citri* strain (Yu et al., 2011). Similarly, Kramer and Nauen (2011) showed that PBO enhanced the toxicity of spirodiclofen in PSR-TK *P. ulmi* strain, suggesting the involvement of CYPs in spirodiclofen resistance in both tetranychid species. The upregulation

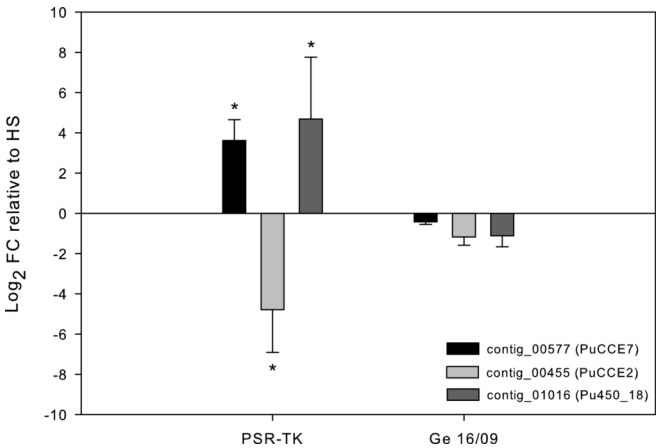

FIGURE 2.10 – Effect of spirodiclofen selection on expression of genes putatively involved in spirodiclofen resistance. qPCR quantification of expression levels of contig_01016 (PuP450_18), contig_00577 (PuCCE_7) and contig_00445(PuCCE_2) in *Panonychus ulmi* strains HS (spirodiclofen susceptible strain), PSR-TK (spirodiclofen resistance ratio > 7000 relative to HS) and Ge 16/09, the parental strain of PSR-TK (spirodiclofen resistance ratio of 59 relative to HS) (Kramer and Nauen, 2011). The data from qPCR were presented as mean of three replicates. Error bars represent the standard error of the calculated mean. Asterisks indicate significant different expression values compared to the reference condition (HS).

of a CYP (*CYP392E10*, *tetur27g01030*) and a CCE (*TuCCE-04*, *tetur01g10750*) was also strongly associated with spirodiclofen resistance in two unrelated *T. urticae* strains (SR-VP and SR-TK). Furthermore, functional expression confirmed that CYP392E10 metabolizes spirodiclofen (Demaeght et al., 2013). Remarkably, the upregulated *P. ulmi* CCE and CYP in the PSR-TK strain cluster in different clades in our phylogenetic analyses of tetranychid CCEs and CYPs than those that are upregulated in the spirodiclofen-resistant *T. urticae* strains (FIGURES 2.2 and 2.3). Thus, within the Tetranychidae, two different species are able to develop a similar resistance mechanism to spirodiclofen, but the enzymes recruited for this purpose do not need to have followed the same evolutionary path. This independent development of resistance in related species is further reinforced by the fact that only few of the differentially expressed contigs in the PSR-TK *P. ulmi* strain have a BLASTx hit with amino acid translations of differentially expressed genes in two spirodiclofen resistant *T. urticae* strains (SR-VP and SR-TK) (Demaeght et al., 2013) (see TABLE S2.11). Overall, the analysis has pointed to a number of genes and enzymes that might be causal for the resistant phenotype, and future work including functional expression should confirm that the enzymes can metabolize or sequester spirodiclofen leading to resistance, as was recently undertaken for the model species *T. urticae* (Demaeght et al., 2013).

2.4. CONCLUSIONS

Using paired-end strand specific reads from RNA of spirodiclofen susceptible and resistant *P. ulmi* strains we generated a *P. ulmi* transcriptome dataset containing 27,777 contigs. As such, our transcriptome data represents a significant increase in the genomic resources that are available for this species. Phylogenetic analyses revealed that in the majority of cases detoxification gene (CCEs, CYPs, UGTs, ABCs) classes/clades/subfamilies are more numerate in *T. urticae* compared to *P. ulmi*, suggestive of a link between detoxification gene proliferation and the polyphagous nature of *T. urticae*. Specific radiations of subfamilies in *P. ulmi* were also observed. Annotation of all major target-sites in *P. ulmi* revealed the presence of mutations in AchE that are likely to confer carbamate and organophosphate resistance. Finally, we used the replicated RNAseq data to assess differences in gene expression between a spirodiclofen resistant and susceptible *P. ulmi* strain and found that a CYP and CCEs are likely to be associated with spirodiclofen resistance in *P. ulmi*. These results are in line with a previous report on molecular mechanisms of spirodiclofen resistance in *T. urticae*. However, the upregulated CYP and CCE in the resistant strains from both species seem not to have evolved from the same common ancestor, indicating both species developed spirodiclofen resistance in a similar but nevertheless independent evolutionary manner. To conclude, the new genomic resources and data that we present in this study for *P. ulmi* will substantially facilitate molecular studies of underlying mechanisms involved in acaricide resistance

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FIGURE S2.1 – Taxonomic distribution of top BLAST hits of the *P. ulmi* transcriptome. Analysis of the taxonomic distribution of the 11,673 top BLAST hits obtained by BLASTx and BLASTn against the non-redundantprotein and nucleotide database (NCBI), respectively, and by BLASTx against the *T. urticae* proteome (http://bioinformatics.psb.ugent.be/orcae/overview/Tetur). Wheel diagram depicts the percentage distribution within different taxonomic groups.

FIGURE S2.2 – Enzyme Classification (EC) analysis of the *P. ulmi* transcriptome. The wheel diagram depicts percentage distribution of EC numbers in general EC terms.

FIGURE S2.3 – Phylogenetic analysis of *P. ulmi* mitochondrial CYPs. Maximum likelihood phylogenetic tree of *P. ulmi, P. citri, T. urticae* and *D. melanogaster* mitochondrial CYP protein sequences. The tree was rooted with *T. urticae* CYP307A1, *P. ulmi* PcP450_42 and *P. citri* Pc0cP450_21 (CYP2 clan members). The scale bar represents 0.5 amino-acid substitutions per site. Numbers at the branch point of each node represent the bootstrap value resulting from 1,000 pseudoreplicates (LR-ELW). Colour and shape codes are as follows: *P. ulmi*, green triangle, *P. citri*, yellow triangle, *T. urticae*, red square and *D. melanogaster*, black dot.

FIGURE S2.4 – ClustalW alignment of ACCase protein sequences of the PSR-TK and HS *P. ulmi* strain and *T. urticae*. The carboxyltransferase domain is shaded in grey while the residue corresponding to the E645K substitution associated with spiromesifen resistance in *T. vaporarorium* is shaded in yellow.

TABLE S2.1 – Annotation details and accession numbers of the carboxylcholinesterases.

TABLE S2.2 – qPCR primers used in this study. Sequences are given for the forward (F) and reverse primer (R).

TABLE S2.3 – Alignment summary for the CLC and Velvet/Oases assemblies.

TABLE S2.4 – BLAST2GO analysis of *P. ulmi* transcriptome.

TABLE S2.5 – Annotation details and accession numbers of Cytochrome P450s.

TABLE S2.6 – Annotation details and accession numbers of Glutathione S- transferases.

TABLE S2.7 – Annotation details and accession numbers of ATP-binding cassette family transporters.

TABLE S2.8 – Annotation details and accession numbers of HTGs.

TABLE S2.9 – The number of mapped RNA reads per contig for both *P. ulmi* strains (HS and PSR-TK). Mapped reads were counted using htseq-count, included in the HTSeq python package (Anders et al., 2013).

TABLE S2.10 – Differentially expressed contigs in the *P. ulmi* PSR-TK strain relative to the HS strain (Benjamini-Hochberg adjusted *p*-value ≤ 0.05 and $|FC| > 2$).

TABLE S2.11 – Differentially expressed contigs in the *P. ulmi* PSR-TK strain with a BLASTx (E-value treshold e-15) hit against amino acid translations of differentially expressed genes in SR-VP and SR-TK, two spirodiclofen resistant *T. urticae* strains (Demaeght et al., 2013).

FILE S2.1 – Target-site nucleotide sequences of the *P. ulmi* PSR-TK and HS-strain.

FILE S2.2 – Transcriptome assembly (CLC Genomics Workbench) of *P. ulmi* described in this study.

FILE S2.3 – Alternative transcriptome assembly (Velvet/Oases) of *P. ulmi* used for obtaining target-site sequences as full length as possible and as an extra resource for mining of HTGs.

FILE S2.4 – Complete transcriptome assembly of *P. citri* reassembled from raw reads data (Liu et al., 2011).

FILE S2.5 – Complete transcriptome assembly of *P. citri* reassembled from raw reads data (Niu et al., 2012).

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A mutation in the PSST homologue of complex I (NADH:ubiquinone oxidoreductase) from *Tetranychus urticae* is associated with resistance to METI acaricides

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Abstract. The acaricidal compounds pyridaben, tebufenpyrad and fenpyroximate are frequently used in the control of phytophagous mites such as *Tetranychus urticae*, and are referred to as Mitochondrial Electron Transport Inhibitors, acting at the quinone binding pocket of complex I (METI-I acaricides). Because of their very frequent use, resistance evolved fast more than 20 years ago, and is currently wide-spread. Increased activity of P450 monooxygenases has been often associated with resistance, but target-site based resistance mechanisms were never reported. Here, we report on the discovery of a mutation (H92R) in the PSST homologue of complex I in METI-I resistant *T. urticae* strains. The position of the mutation was studied using the high-resolution crystal structure of *Thermus thermophilus*, and was located in a stretch of amino acids previously photo-affinity labelled by fenpyroximate. Selection experiments with a strain segregating for the mutant allele, together with marker-assisted back-crossing of the mutation in a susceptible background, confirmed the involvement of the mutation in METI-I resistance. Additionally, an independent genetic mapping approach and QTL analysis identified the genomic locus of pyridaben resistance, which included the PSST gene. Last, we used CRISPR-Cas9 genome editing tools to introduce the mutation in the *Drosophila* PSST homologue.

3.1. INTRODUCTION

Inhibition of electron transport at the mitochondrial respiratory chain has been a successful mode of action especially in the case of acaricides (Lummen, 2007; Van Leeuwen and Dermauw, 2016). In the mitochondrial inner membrane, four large transmembrane complexes (complex I-IV) mediate electron transport via several redox reactions from NADPH and $FADH_2$ to oxygen as a final electron acceptor. The proton gradient created during this process is the driving force for ATP synthesis by the F_0F_1 ATPase (complex V) (Karp, 2008). A number of commercial acaricides target these processes at different sites: classical Mitochondrial Electron Transport Inhibitors (METIs) such as quinazolines, pyrimidinamines, pyrazoles and pyridazinones act at complex I (here referred to as METI site I, IRAC group 21; FIGURE 3.1) (Hollingworth and Ahammadsahib, 1995; Hollingworth et al., 1994). Acequinocyl and the carbazate bifenazate have been shown to act as inhibitors of cytochrome b at the Q_0-site in complex III (here referred to as METI site III, IRAC group 20) (Van Leeuwen et al., 2008; Van Nieuwenhuyse et al., 2009). Most recently, a number of compounds such as the beta-ketonitriles have been uncovered and commercialized as specific inhibitors of complex II (here referred to as METI site II, IRAC group 25) (Hayashi et al., 2013; Nakahira, 2011). In addition to electron transport inhibitors, a number of acaricides directly interfere with ATP synthesis at complex IV (IRAC group 12), such as organotin compounds (cyhexatin, fenbutatin oxide), propargite and diafenthiuron (Carbonaro et al., 1986; Kadir and Knowles, 1991; Miyasono et al., 1992; Ruder and Kayser, 1994).

METIs acting at site I (METI-Is) were first launched as excellent miticides in the early 90s'(Hollingworth and Ahammadsahib, 1995; Hollingworth et al., 1994; Obata et al., 2006). They immediately gained global popularity as they have broad spectrum activity against various mite species and are to some extent selective towards insect predators. Members of the METI-I acaricides (tebufenpyrad, pyridaben, fenazaquin, fenpyroximate, pyrimidifen and tolfenpyrad, FIGURE 3.1) belong to different chemical families, but all contain heterocyclic rings with two nitrogen atoms, associated with long hydrophobic tails and a tertiary butyl group or alkyl moiety.

METI-Is inhibit the function of the proton translocating NADH:ubiquinone oxidoreductase which is the largest and most complex multi-subunit structure of the respiratory chain, with a total mass of approximately 1MDa (Wirth et al., 2016). The enzyme is crucial for the oxidative energy conversion in eukaryotic cells and its malfunction is the most common reason underlying mitochondrial disorders in humans (Martin et al., 2005; Rodenburg, 2016; Wong, 2013). Nonetheless, the catalytic mechanism of Complex I remains only partially understood. The study of the mode of action of different inhibitors is believed to be critical in obtaining structural and functional evidence on the detailed mechanisms of ubiquinone reduction and proton translocation at Complex I (Brandt, 2006; Hirst, 2010; Tocilescu et al., 2010a). Although specific binding sites for ubiquinone and different inhibitors may not be identical (Fendel et al., 2008; Tocilescu et al., 2007), many structurally diverse compounds have been described that inhibit complex I and are considered to interfere with ubiquinone reduction (Esposti, 1998; Lummen, 1998). According to the initial notion based on the studies relying on Michaelis-Menten type kinetic analyses (reviewed in Esposti, 1998) there are three types of Complex I inhibitors and at least two quinone reaction sites that can be independently blocked by the inhibitors of a different type. This idea, however, was revised in the study by Okun et al. (1999), where competition experiments consistently demonstrated that all tested hydrophobic inhibitors of complex I share a common binding domain with partially overlapping sites. Thanks to the availability of structural data on complex I (Fiedorczuk et al., 2016; Vinothkumar et al., 2014; Zickermann et al., 2015), supported by earlier biochemical studies (Schuler et al., 1999; Shiraishi et al., 2012), evidence is accumulating that the ubiquinone and inhibitor binding site consists of the nuclear encoded PSST and the 49 kDa subunits (FIGURE 3.2a,b), forming a pocket located in the proximal half of the peripheral arm which docks onto the membrane arm. The mitochondrial encoded subunit ND1 forms the access path towards the ubiquinone reduction site at the interface of the 49-kDa and PSST subunits (Earley et al., 1987; Murai et al., 2011; Murai et al., 2009; Schuler and Casida, 2001a). In addition, PSST is the most likely carrier of iron-sulfur cluster N2, which has been proposed

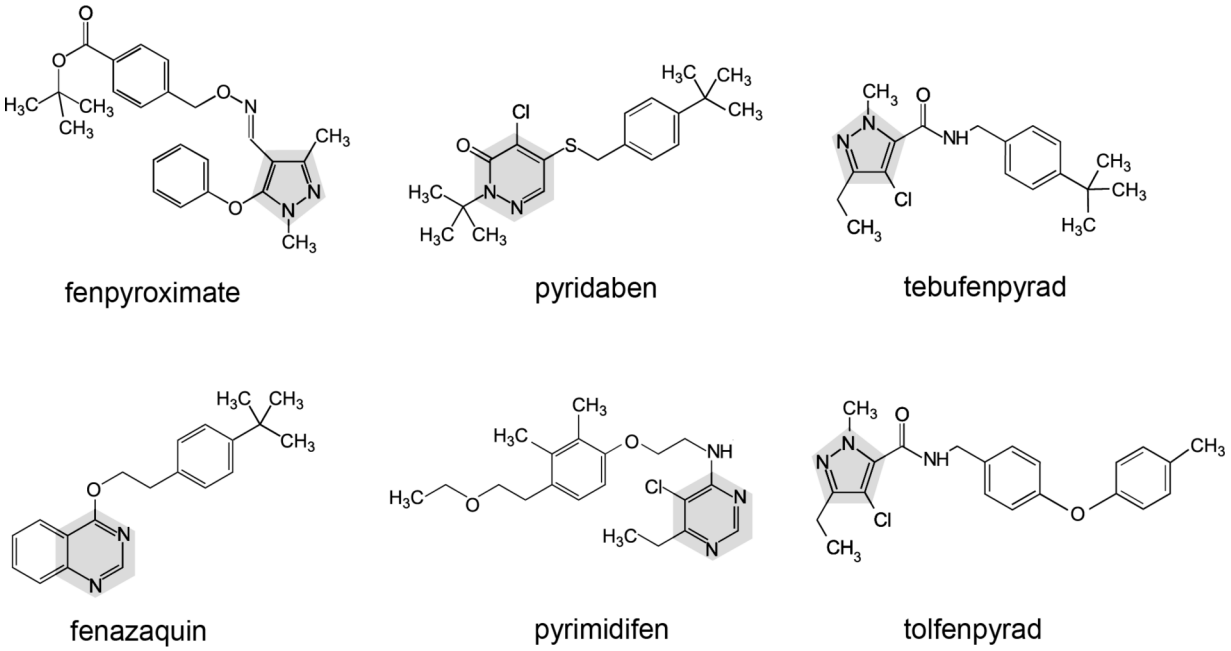

FIGURE 3.1 – Chemical structures of METI-I acaricides. Nitrogen heterocycles are shaded grey.

to be a direct electron donor for the ubiquinone reduction (Duarte et al., 2002; Friedrich, 1998; Magnitsky et al., 2002).

Arthropods have developed two main mechanisms that can lead to pesticide resistance: mechanisms that decrease exposure due to quantitative or qualitative changes in major detoxification enzymes and transporters (pharmacokinetic mechanisms) and mechanisms that decrease sensitivity due to changes in target-site sensitivity caused by point mutations (pharmacodynamic mechanisms) (Feyereisen et al., 2015; Li et al., 2007; Van Leeuwen and Dermauw, 2016). Resistance to acaricidal METI-Is has been reported from around the world, in particular for the polyphagous pest *Tetranychus urticae*, a herbivore with an extreme host range including many agriculturally important crops (Jeppson et al., 1975; Migeon and Dorkeld, 2006-2015). However, there are currently no reports that document target-site related mutations that might be associated with METI-I resistance. Moreover, mutations that alter inhibitor interactions with the binding site in complex I have not been documented for any metazoan species, and are mainly restricted to yeast as a model.

METI-I resistance in spider mites has most often been linked with increased P450 activity (Kim et al., 2004, 2006; Stumpf and Nauen, 2001; Van Pottelberge et al., 2009), and this was put forward as the reason for the commonly observed cross-resistance patterns between different METI-I compounds (Kim et al., 2004; Stumpf and Nauen, 2001; Van Pottelberge et al., 2009). As was also suggested by metabolomics studies, P450 monooxygenase driven hydroxylation of the tertiary group may be a mutual mechanism of oxidative detoxification for METI-I

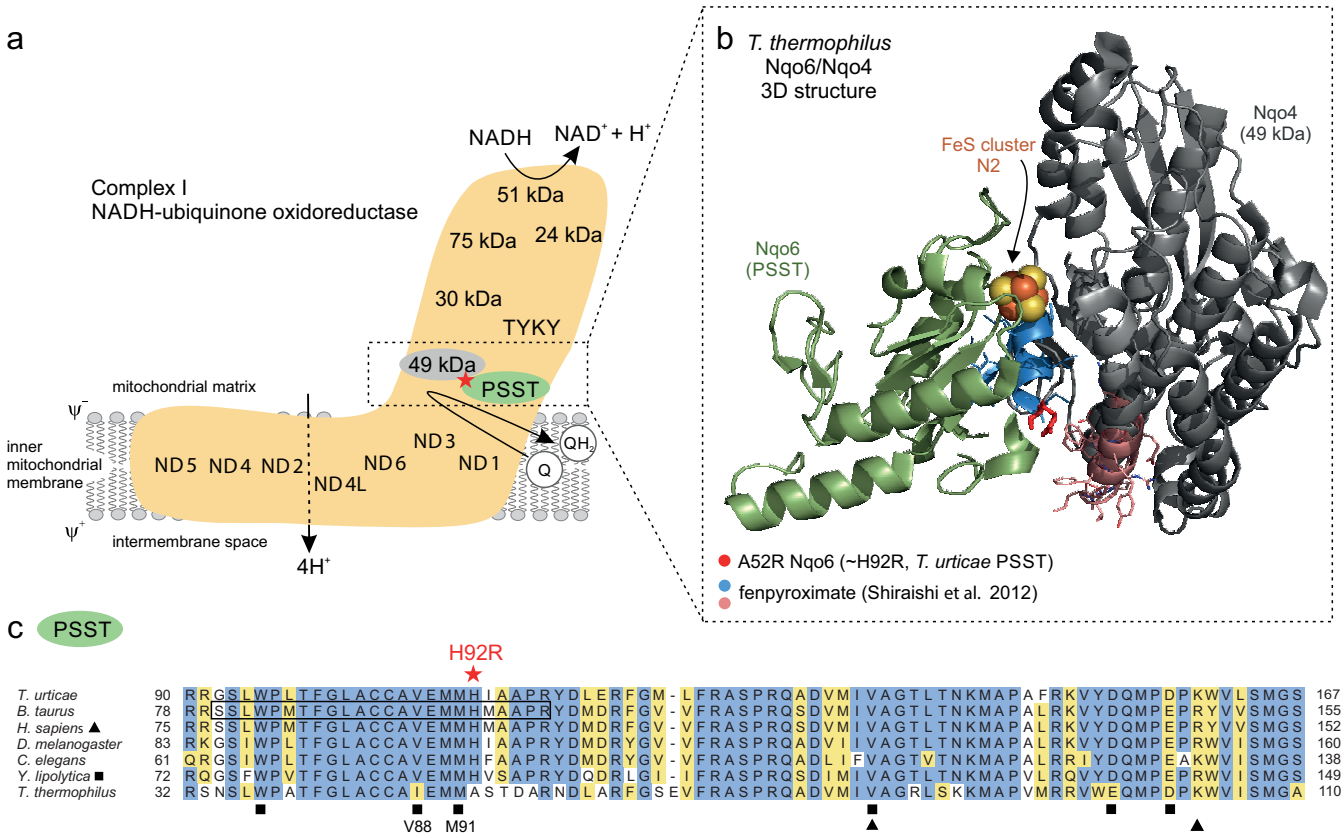

FIGURE 3.2 – Identification of the H92R substitution in the PSST homologue of complex I (NADH:ubiquinone oxidoreductase) from *Tetranychus urticae*. (a) Structure of complex I. Mitochondrial (ND1, ND2, ND3, ND4, ND4L, ND5 and ND6) and nuclear (24 kDa, 30 kDa, 49kDa, 51 kDa, 75 kDa, TYKY, PSST) encoded subunits. The 49kDa and PSST subunit, believed to interact with Complex I inhibitors (Schuler and Casida, 2001b; Shiraishi et al., 2012; Zickermann et al., 2015) are shaded with a grey and green oval, respectively. A red star indicates the location of the H92R substitution discussed in panel b and c (b) 3D structure of the interface between the Nqo4 and Nqo6 subunit of *Thermus thermophilus* [PDB: 4HEA, (Baradaran et al., 2013)]. The 3D structure was visualized using pymol version 1.7.4.0 structure viewer (Schrodinger, LLC) and is depicted as a 'cartoon'. Nqo6 and Nqo4 are the *T. thermophilus* homologues of the *Bos taurus* PSST and 49kDa subunit, respectively. The protein sequence of Nqo6 was altered to contain an A52R substitution (with R52 being depicted as red 'sticks'), corresponding to the H92R (*Yarrowia lipolytica* numbering) substitution in *T. urticae* PSST. Residues of *T. thermophilus* Nqo6 and Nqo4 aligning with those residues of *B. taurus* PSST and 49 kDA of which recently it was shown that they were photo-affinity labeled by a photoreactive derivative of fenpyroximate (Shiraishi et al., 2012; see also panel c), were depicted as 'lines' and shaded blue and pink, respectively. The iron-sulphur cluster N2 is shown as a yellow-orange space-filled model. (c) Alignment of the *T. urticae* PSST subunit with those of other Metazoa, *Y. lipolytica* (Fungi) and *T. thermophilus* (Bacteria). All protein sequences in the alignment can be accessed via the UniProt database [*T. urticae* (T1K9K2), *B. taurus* (P42026), *Homo sapiens* (Q7LD69), *Drosophila melanogaster* (Q9VXK7), *Caenorhabditis elegans* (Q94360), *Y. lipolytica* (Q6C2Q1) and *T. thermophilus* (Q56218)]. Protein sequences were aligned using MUSCLE (Edgar, 2004) and visualized in BioEdit (Hall, 1999). An 80% threshold was used for identity (blue background) and similarity shading (yellow background). A red star on top of the alignment indicates the position of the H92R (*Y. lipolytica* numbering; H110R, *T. urticae* numbering) substitution in the *T. urticae* PSST subunit identified in this study. Black triangles and squares below the alignment specify mutation positions that were investigated in the PSST homologue of *H. sapiens* [V122M (Triepels et al., 1999), R145H (Lebon et al., 2007)] and *Y. lipolytica* [W77E (Angerer et al., 2012), V88F,I (Angerer et al., 2012; Tocilescu et al., 2007), V88L,M (Tocilescu et al., 2007), M91E,K (Angerer et al., 2012; Fendel et al., 2008), M91C (Fendel et al., 2008), V119M (Ahlers et al., 2000a), D136N (Ahlers et al., 2000b; Garofano et al., 2003), E140Q (Ahlers et al., 2000b; Garofano et al., 2003)]. Residues V88 and M91 of *Y. lipolytica* are labeled (see SECTION 3.4). *B. taurus* PSST residues that were photoaffinity labeled by a photoreactive derivative of fenpyroximate are framed (Shiraishi et al., 2012).

acaricides (Hollingworth and Ahammadsahib, 1995; Motoba et al., 2000; Stumpf and Nauen, 2001). Recently it was actually shown that a functionally expressed P450, CYP392A11 of *T. urticae*, was able to directly hydroxylate fenpyroximate to a non-toxic metabolite (Riga et al., 2015).

Presence of a single common P450 based mechanism of (cross)-resistance would presumably be manifested in a similar pattern of inheritance in different strains and for different METI compounds in a specific strain. This is, however, not the case since for example, in a *T. urticae* strain cross-resistant to pyridaben, tebufenpyrad and fenpyroximate, resistance is conferred by a single gene for pyridaben and fenpyroximate, while tebufenpyrad resistance is polygenic (Van Pottelberge et al., 2009). In addition, the P450 synergist PBO strongly synergized pyridaben and tebufenpyrad resistance in this strain, while no synergism was found for fenpyroximate resistance (Van Pottelberge et al., 2009). Together with other studies on the effect of synergists on METI-I toxicity and the different genetic patterns in spider mite species (Goka, 1998; Stumpf and Nauen, 2001), this indicates that other mechanisms in addition to P450 based detoxification are probably in place. However, the complexity and limited availability of sequence information on complex I subunits has not allowed, until recently, to investigate whether these include target-site based mechanisms.

In this study, we exploited the available whole genome sequence of *T. urticae* to identify, annotate and sequence the relevant complex I subunits that are thought to form the binding site of METI-Is in both susceptible and characterized METI-I resistant strains. A single point mutation in the *T. urticae* homologue of the *Bos taurus* PSST subunit (hereafter referred to as *T. urticae* PSST) was identified and its potential role in METI-I resistance was further studied by modeling, selection experiments and genetic mapping. Using marker-assisted introgression, we introduced the mutation into a *T. urticae* line with susceptible genetic background and studied its effect on the resistant phenotype. Last, in a reverse genetic approach using CRISPR-Cas9 technology combined with homologous recombination-directed gene modification, we introduced the mite mutation in the *Drosophila melanogaster* PSST homologue and studied the resulting phenotype.

3.2. MATERIALS AND METHODS

3.2.1. Acaricides

The acaricides used in this study were commercial formulations (Fyto Vanhulle, Belgium) of fenpyroximate (Naja; 50 g a.i. l^{-1} SC), pyridaben (Sanmite; 150 g a.i. l^{-1} SC) and tebufenpyrad (Pyranica; 200 g a.i. l^{-1} SC).

3.2.2. *T. urticae* strains

The METI-I resistant MR-VP strain was the same as used by Van Pottelberge et al. (2009) and was originally collected from bean plants at the national botanical garden (Brussels, Belgium) in September 2005 and since then kept in the laboratory at a constant selection pressure of 1000 mg l^{-1} tebufenpyrad. The METI-I resistant strain Akita, was the same as the one described by Stumpf and Nauen (2001) and maintained in the laboratory under constant selection pressure of 500 mg l^{-1} fenpyroximate. The pyridaben resistant NPR strain was previously described in Sugimoto and Osakabe (2014). The susceptible strain NS was described in Asahara et al. (2008). For quantitative trait loci (QTL) analysis, strain NPR was additionally selected with 10,000 mg l^{-1} of pyridaben, while NS was reversely selected for pyridl^{-1} pyridaben. The Hennep strain was collected at Bourgoyen-Ossemeersen (Gent, Belgium) in June 2011 from the brittlestem hemp-nettle *Galeopsis tetrahit* L. All *T. urticae* strains were maintained on 3-week-old potted kidney bean plants (*Phaseolus vulgaris* L., cv. Prelude or Speedy) in a climatically controlled room or incubator at 25 ± 1°C, 60% relative humidity, and 16:8 light:dark photoperiod

3.2.3. Selection for METI-I resistance

The *T. urticae* strain Hennep, segregating for H92R was selected with either 1000 mg l^{-1} fenpyroximate, 1000 mg l^{-1} pyridaben or 300 mg l^{-1} tebufenpyrad. Selection was initiated by placing approximately 1000 adult female mites on detached bean leaves sprayed with one of the METI-I acaricides using a hand pressurized sprayer (Birchmeier, Switzerland) until runoff. The survivors were kept at a constant selection pressure for six generations until no mortality was observed. In total, three biological replicates were performed for each METI-I acaricide selection experiment (resulting in nine METI-I selected *T. urticae* lines). Genomic DNA of each METI-I acaricide selected line and the original unselected strain (Hennep) was isolated from 200 adult females according to the standard phenol-chloroform protocol (Van Leeuwen et al., 2008). The frequency of the H92R mutation in the population was investigated by PCR and proportional sequencing (Van Leeuwen et al., 2008) (TABLES S3.1 and S3.2).

3.2.4. Full dose response bioassays

Adulticidal bioassays were conducted using a standard method described in Van Leeuwen et al. (2004). In short, 20–30 young adult female mites were transferred to the upper side of 9 cm^2 square-cut kidney bean leaf discs on wet cotton wool, which had been sprayed with 0.8 ml of spray fluid at 1 bar pressure in a Cornelis spray tower (1.5 ± 0.06 mg aqueous acaricide deposit cm^{-2}) (Vanlaecke and Degheele, 1993). The plates were then placed in a climatically controlled room at 26 ± 0.5°C, 60% RH and 16:8 light:dark photoperiod. Four replicates of at least five concentrations of each acaricide and a control (deionized water) were tested. Mortality was assessed after three days. Mites were scored as being alive if they could walk normally after being prodded with a camel's hair brush. All mortalities obtained for a water control were lower than 10%. LC_{50}-values, slopes, resistance ratios and 95% confidence limits were calculated by probit analysis (POLO, LeOra Software, Berkeley, USA) (Robertson and Preisler, 1992).

3.2.5. Sequencing of *T. urticae* PSST and 49 kDa subunits

Tetranychus urticae complex I subunits, most likely involved in the ubiquinone/inhibitor binding: PSST and 49 kDa (Schuler and Casida, 2001b; Schuler et al., 1999; Shiraishi et al., 2012; Zickermann et al., 2015) were identified in the *T. urticae* proteome by a BLASTp search using *B. taurus* PSST and 49 kDa as query (UniProt accessions P42026 and P17694, respectively). Total RNA was extracted from 200 young adult females and homogenized according to the protocol of the RNeasy mini kit (Qiagen). Residual DNA was removed by a DNAse treatment (TURBO DNA-free™ Kit, Ambion). One µg of RNA was used for 1st strand cDNA synthesis with the Maxima First Strand

cDNA synthesis kit for RT-PCR (Fermentas Life Sciences). *Tetranychus urticae* PSST and 49 kDa subunit encoding genes were PCR–amplified as detailed in TABLE S3.1. PCR product sequencing was performed at the Macrogen facility (Amsterdam, The Netherlands). All primer sequences used in this experiment can be found in TABLE S3.2.

3.2.6. Detection of the H92R mutation in geographically diverse *T. urticae* strains

A selection of *T. urticae* strains from diverse geographical regions (TABLE S3.3; Asahara et al., 2008; Grbic et al., 2011; Khajehali et al., 2011; Stumpf and Nauen, 2001; Sugimoto and Osakabe, 2014; Tsagkarakou et al., 2002; Van Leeuwen et al., 2006, 2005; Van Pottelberge et al., 2009) was screened for the presence of the H92R mutation. RNA was extracted from 200 adult female mites according to the protocol of the RNeasy mini kit (Qiagen). cDNA was prepared as described above, using Maxima First Strand cDNA synthesis kit for RT-PCR (Fermentas Life Sciences). PCR amplification of the PSST subunit gene fragment carrying the mutation, was performed with primers PSST_in_Gene (For PCR conditions and primers sequences refer to TABLES S3.1 and S3.2, respectively). Sequencing of PCR products was performed at Macrogen (Amsterdam, The Netherlands). The mutation frequency was estimated by comparing the height of the wild type (cytosine) and the mutated (guanine) peaks in electropherograms, as previously described (Van Leeuwen et al. 2008)

3.2.7. Modelling of Complex I structure

The crystal structure of the respiratory complex I of *T. thermophilus* (PDB: 4HEA; Baradaran et al., 2013) was studied using PyMOL 1.7.4.0 structure viewer (Schrodinger, LLC). The *T. thermophilus* crystal structure of complex I was determined at 3.3 A resolution (Baradaran et al., 2013) and was used in this study as it is at present the only complete solved structure of respiratory complex I (Sazanov, 2015). To investigate the role of the H92R substitution in altered inhibitor binding, the corresponding amino acid (Alanine at position 52) of Nqo6 of *T. thermophilus* was altered to an arginine.

3.2.8. Back-crossing experiment

A haploid male of the METI-I resistant strain MR-VP (with the R92 genotype for PSST) was crossed with a virgin female of the METI-I susceptible strain NS (with the H92/H92 genotype for PSST). The resulting heterozygous virgin females were back-crossed to NS males and the resulting heterozygotes (with H92/R92 genotype) were screened by PCR (TABLES S3.1 and S3.2), after they had laid sufficient amount of eggs. This process was repeated for six generations. In the last generation, a cross was carried out between the back-crossed heterozygous virgin females and their first born sons representing a H92 or R92 genotype, respectively, which resulted in congenic homozygotic lines with either the H92/H92 genotype (C-lines) or the 92R/92R genotype (R-lines). The experiment was performed in two biological replicates.

3.2.9. Mapping of pyridaben resistance to a *T. urticae* genomic scaffold

Prior to the QTL analysis, we determined scaffolds involving pyridaben resistance by cross experiments between NPR and NS strains using 5 microsatellite loci: TkMS15, TuCT13, TuCT09, TuCT67, and Tu27 (Accession numbers: *AB107765*, *AB263085*, *AB263084*, *AB263090*, and *AJ419831*; Navajas et al., 1998; Nishimura et al., 2003; Uesugi and Osakabe, 2007) of parents and F2 males.

An NPR female was mated with a NS male on a kidney bean leaf disc (2 cm in diameter) placed on water-soaked cotton in a Petri dish (9 cm in diameter). Females were allowed to lay eggs for five days, after which DNA samples of the parental female and male were prepared as described in SECTION 3.2.11. Developed teliochrysalis females were reared individually on fresh leaf discs. Consequently, twenty F1 virgin females were allowed to lay F2 haploid eggs for 10 days. During this period, the females were moved to a fresh leaf disc every day. F2 haploid eggs were reared to adulthood, and 458 adult F2 males were obtained. The adult F2 males were treated by dipping into pyridaben solution at the criterion concentration of 200 mg l^{-1} for 10 s. This concentration was sufficiently high to kill susceptible males (Sugimoto and Osakabe, 2014). After 5 days, the number of survivors was counted under a binocular microscope; mites that could move normally were scored as alive while mites that were paralyzed after touching with a fine brush were scored as dead. As a result, we obtained 231 surviving males (50.4% in mortality), and 192 F2 males were applied to microsatellite genotyping.

Single mite PCR products (TABLE S3.1) were diluted with 380 µl of distilled water, and 1µl of the diluted PCR products were used for genotyping by fragment analysis using ABI PRISM 3130 Genetic Analyser with Gene Mapper (Applied Biosystems, Foster City, CA). Then, recombination rates between each microsatellite and pyridaben resistance were calculated, and whether the observed recombination rates fitted to the expected value under no linkage was tested by a Chi-square test using R software version 3.0. Linkage among microsatellites and a pyridaben resistance gene and their orders in the linkage group was estimated using the 'OneMap' package (Margarido et al., 2007) in R software version 3.2.2.

3.2.10. QTL analysis

Reciprocal crosses between the NPR and NS strain were performed. Four teliochrysalis females of one strain and a male of the other strain were introduced on a kidney bean leaf square (2×2 cm) placed on water-soaked cotton in a Petri dish (9 cm in diameter). Four Petri dishes (replicates) were used in each direction of the reciprocal crosses. After 2 days, females were individually moved to fresh leaves and allowed to lay F1 eggs for 2 days (15 ♀♀ [3.8 ♀♀ ± 0.5 SD] in NS♀ × NPR♂ and 12 ♀♀ [3.0 ♀♀ ± 0.8 SD] in NPR♀ × NS♂; other females had been drowned). One Petri dish for NS♀ × NPR♂ was omitted from the following treatments because no F1 females emerged. Virgin F1 females (6.1 ♀♀ ± 2.4 SD and 7.3 ♀♀ ± 2.5 SD in NS♀ × NPR♂ and NPR♀ × NS♂, respectively) and an F1 male derived from the same parental female were introduced together to a fresh leaf square and allowed to mate (back cross) and lay B1 eggs for 9 days. Then, developed B1 teleiochrysalis females were individually moved to fresh leaf squares (142 ♀♀ [14.2 ♀♀ ± 3.4 SD] in NS♀ × NPR♂ and 170 ♀♀ [15.5 ♀♀ ± 3.3 SD] in NPR♀ × NS♂). B1 females derived from one F1 female in NPR♀ × NS♂ were omitted in this treatment to reduce difference in the number of F1 females between the reciprocal crosses. After molting they were allowed to produce B2 haploid male eggs for 3 days. Then, developed B2 adult males were applied to test

susceptibility to pyridaben by spraying with the solution at criterion concentration of 200 mg l^{-1} (adhesion amount: 1.911 ± 0.155 mg cm^{-2}). We checked mortality of the B2 males 2 days after spray. We used a mortality data set of B2 males derived from 135 B1 females (70 and 65 originally from NS♀ × NPR♂ and NPR♀ × NS♂, respectively). The mortality data for each B1 female consisted of more than 10 B2 males (15.0 ♂♂ ± 3.9 SD per B1 female). During these manipulations, DNA template samples of parents, and B1 females (311 ♀♀ including the 135 ♀♀ described above) were prepared (see SECTION 3.2.11).

We designed primer sets for 12 new microsatellite loci (tu07ms_g1–g12) distributed over scaffold 7 (Grbic et al., 2011). Performing fragment analysis, we tested whether the new and two known primer sets amplifying microsatellite loci, TuCT04 and TkMS15, worked as diagnostic markers for progeny of NPR × NS crosses. Although amplification was successful in all the cases (TABLE S3.1), crossed individuals were distinguishable in obtained fragments only with TuCT04 (GenBank: AB263083), and 4 microsatellite loci, tu07ms_g10, tu07ms_g7, tu07ms_g4, tu07ms_g3 (GenBank: LC090067, LC090066, LC090065, and LC090064, respectively) (TABLE S3.2). The single mite PCR products (see SECTION 3.2.11, PCR conditions in TABLE S3.2) were diluted with 180 µl of distilled water, and 1µl of the diluted PCR products were used for genotyping by fragment analysis using ABI PRISM 3130 Genetic Analyser with Gene Mapper (Applied Biosystems, Foster City, CA).

Linkage among microsatellite loci was estimated based on the genotypes of parents and 311 B1 females using the 'OneMap' package (Margarido et al., 2007) in R software version 3.2.2. Genotype data of tu07ms_g4 and TuCT04 were unavailable in 63 and 197 B1 females, respectively, because parental females and a male had the same alleles. Then, QTL analysis was performed using 'qtl' package (Broman et al., 2003) with 'rlecuyer' package (Haley-Knott regression; 1,000 permutations). Prior to the QTL analysis, mortalities of B2 males for each B1 female were transformed by an arcsine square root transformation.

3.2.11. Single mite DNA preparation

In order to perform genotyping on single mite specimens for the back-crossing experiment, individual *T. urticae* mites were homogenized in 20 µl STE buffer (100 mM NaCl, 10mM Tris-HCl and 1mM EDTA) with 1 mg ml^{-1} proteinase K (Sigma-Aldrich). Homogenate was incubated at 37°C for 30 min followed proteinase K inactivation for 5 min at 95°C. After cooling, homogenate was directly used in PCR reaction. For the microsatellite analysis, mites were individually homogenized in 20 µl of lysis buffer (10 mM Tris-HCl buffer [pH 8.0], 100 mM EDTA [pH 8.0], 0.5% Igepal CA-630 [Sigma-Aldrich Co., St. Louis, USA], 10 mM NaCl, and 1 mg ml^{-1} proteinase K [Takara, Kusatsu, Japan]). The homogenate was incubated at 65°C for 15 min and then at 95°C for 10 min. The lysate of females and males was diluted with 380 and 180 µl of 0.1×TE buffer, respectively, and preserved at −20°C until use in microsatellite analysis.

3.2.12. CRISPR-Cas9 genome editing in *Drosophila*

3.2.12.1. Drosophila *DNA purification and amplification of the* Drosophila *PSST subunit*

DNA was purified from *Drosophila* tissues by DNAzol® (MRC) according to manufacturer instructions. Prior to design of the dsDNA template and sgRNAs, the full length *Drosophila* PSST encoding gene (*Dmel_CG9172*) of y^1 M{nos-Cas9.P}ZH-2A w^* flies (nos.Cas9; stock #54591 at Bloomington Stock Center; Port et al., 2014) was PCR-amplified with primer pair CG9172_F/CG9172_R and sequenced with two more internal primers (CG9172INT_1, CG9172INT_2) as well as the external ones (for PCR details and primers sequences see TABLES S3.1 and S3.2, respectively) to inspect for potential point mutations that might interfere with identification of the target sequence by the Cas9 protein and/or homologous recombination.

3.2.12.2. sgRNAs design and cloning

sgRNA1 and 2 were designed with the online CRISPR design tool from the Zhang Lab (http://CRISPR.mit.edu/) and screened for potential off-target sites with CRISPOR online software (Haeussler et al., 2016). The sgRNA sequences can be found in TABLE S3.2 and FILE S3.1. Oligos were 5' phosphorylated by the manufacturer and supplemented with 5' overhangs to facilitate cloning into the pU6-BbsI-chiRNA plasmid via the BbsI restriction sites (Addgene; Gratz et al., 2013). The predicted Cas9-cutting sites were located 118 nt upstream and 145 nt downstream from the introduced H92R mutation, for sgRNA1 and sgRNA2, respectively (FIGURE S3.1). To prepare vectors for the *in vitro* transcription of sgRNAs, oligonucleotides were cloned according to a standard protocol (flyCRISPR protocols, see http://flycrispr.molbio.wisc.edu/protocols/gRNA). The pU6-BbSI-chiRNA vectors containing cloned sgRNA1 or 2 oligos, were transformed to DH5α *E. coli*. Inserts were confirmed by sequencing with T7 and T3 primers.

3.2.12.3. Design of dsDNA donor for homologous recombination mediated repair and screening for recombinants

The double stranded DNA donor with a total length of 2264 bp (FILE S3.1), was synthesized by GeneScript and provided in a form of lyophilized 4 µg pUC57 plasmid preparation. Homology arms containing sequence immediately adjacent to the cleavage sites were 1000 bp each. To prevent Cas9 mediated digestion of the donor template, three silent point mutations were introduced in the seed region corresponding to the sequence of each sgRNA. The donor template was designed to introduce a non-synonymous A308G mutation (H103R, *Drosophila* numbering) in *Dmel_CG9172* which corresponds to the A329G mutation (resulting in a H92R substitution, *Yarrowia lipolytica* numbering) in *T. urticae* PSST (see FIGURE 3.2). To enable efficient screening for recombinants in a large sampling group, the donor template was altered with synonymous point mutations, which in case of successful homologous recombination, removed Tsp45I restriction site. DNA of recombined flies was then PCR amplified with the diagnostic primers (TABLE S3.2) and further screening was facilitated by the target mutation itself, since the A308G (*Drosophila* numbering) mutation introduces a Fsp I restriction site (FIGURE S3.1, for more details, see SECTION 3.2.12.4).

3.2.12.4. Generation and selection of genome modified flies

Fly CRISPR-Cas9 protocols were performed as previously described in Douris et al. (2016). Briefly, we used transgenic flies with the genotype y^1 M{nos-Cas9.P}ZH-2A w^* (nos.Cas9; stock #54591 at Bloomington Stock Center), that carry a transgene expressing Cas9 protein during oogenesis under control of *nanos* regulatory sequences (Port et al., 2014). Embryos were injected with a plasmid mixture containing 75 ng/µl of each gRNA plas-

mid and 100 ng/µl of donor plasmid in injection buffer (2 mM Sodium phosphate pH 6.8-7.8, 100 mM KCl), according to optimal concentrations defined in Ren et al. (2014). Injected G_0 adults were back-crossed with nos.Cas9 flies and G_1 progeny was initially screened *en masse* to identify crosses that had produced G_1 flies that underwent HDR events.

Screening was performed on DNA purified from sets of ~30 individuals per vial. We used a 'mutant-specific' primer pair (primers CG9172_dia_F/ CG9172_dia_R, Table S3.2) that was designed specifically on the modified donor sequence that corresponds to the CRISPR targets, yielding a diagnostic 294 bp fragment that can be additionally cleaved with *Fsp*I to two fragments of 164 and 130 bp.

In case the presence of genome modified alleles was indicated in the pool by the amplification of the diagnostic fragment, several individual G_1 flies from the same original cross were individually screened, to positively identify G_1 flies that indeed carry genome modified alleles and can generate fly lines bearing the mutation. Lines originating from positive G_1 flies were balanced by crossing individual female G_2 flies (expected to be heterozygous for the mutant allele at a 50% ratio) to males from a strain containing an X chromosome balancer (FM7c *y Hw w B*).

3.2.12.5. Sequence verification

For sequence verification of the flies containing the genome modified allele, a 559 bp fragment generated by primer pairs CG9172_ver_F/ CG9172_ver_R, encompassing the modified genomic region, was amplified and sequenced. To verify that the whole gene was intact and carried no other mutations than the designed one, a specific pair of primers (CG9172_F/ CG9172_R) was used to amplify the *Drosophila* PSST open reading frame, and two more internal primers (CG9172INT_1, CG9172INT_2) were further used for sequencing together with external ones (Figure S3.2, Table S3.2).

3.2.12.6. Recombination of X chromosome

To investigate if the H103R mutation (*Drosophila* numbering) is indeed the cause of lethality in homozygous females and hemizygous males or whether another X-linked locus bearing a deleterious allele is perhaps a potential source of the lethal phenotype, heterozygous females carrying the genome modified X chromosome were crossed with yellow-white (yw) males to separate this X chromosome from the balancer. Subsequently heterozygous F1 females (where recombination was now possible) were crossed with yw males. After 2-3 generations, the population was screened for males with red eyes, carrying the nos.Cas9 mini-white marker and simultaneously for the presence of mutation H103R.

3.3. Results

3.3.1. Identification of a mutation in the *T. urticae* homologue of the PSST subunit of NADH: ubiquinone oxidoreductase (complex I) and assessment of mutation incidence and frequency in resistant and susceptible strains

Tetranychus urticae homologues, tetur11g03360 and tetur07g05240 of the bovine complex I subunits 49 kDa and PSST, presumed to form the quinone binding pocket of complex I, were identified in the *T. urticae* proteome using a BLASTp approach (*T. urticae* gene IDs encoding the homologues of 49 kDA and PSST subunit can be accessed at http://bioinformatics.psb.ugent.be/ orcae/ overview/Tetur). The full-length genes encoding these complex I *T. urticae* subunits were PCR amplified and first sequenced in two characterized METI-I resistant strains: Akita and MR-VP (for PCR conditions and primers used see Tables S3.1 and S3.2) (Stumpf and Nauen, 2001; Van Pottelberge et al., 2009). PCR products were aligned with the corresponding sequences of the reference genome strain (London strain; Grbic et al., 2011). The gene encoding the 49 kDa subunit of Akita and MR-VP (GenBank accession KX806602 and KX806603) was 99.6 and 100% identical to that of the London strain, respectively. The PSST gene of Akita and MR-VP (GenBank accession KX806604 and KX806605) was 99.1 and 98.7% identical to that of the London strain, respectively. While in the 49 kDa gene only synonymous mutations were observed, two different non-synonymous mutations were observed in the PSST gene of the METI-resistant *T. urticae* strains: a A403T mutation causing a methionine to leucine substitution (M135L *T. urticae* numbering, M117L *Y. lipolytica* numbering) in MR-VP only and a A329G mutation that causes a histidine to arginine substitution (H110R *T. urticae* numbering, H92R *Y. lipolytica* numbering) in both MR-VP and Akita. The H92R mutation was fixed and located in the conserved part of the *T. urticae* PSST subunit (Figure 3.2b,c). To further link the mutation with resistance, we amplified and sequenced the region harboring H92R in ten additional *T. urticae* strains from various geographical origins, including a third METI-I resistant strain NPR (Table S3.3) from Japan. While in all populations the frequency of the mutation was estimated by proportional sequencing to be between 0 and 40%, only in the METI-I resistant strains (Akita, MR-VP, NPR) the mutation was fixed (Table S3.3).

3.3.2. Localization of the PSST H92R substitution in complex I of *T. thermophilus*

We studied the *T. thermophilus* complex I structure since, it is the only complex I structure of which the full atomic structure has been resolved at present (Sazanov, 2015). Graphical representation of the coordinates of the *Thermus* structure revealed that A52R in Nqo6 (corresponding to H92R in *T. urticae* PSST) is positioned in the cavity, on the distal side of the interface between Nqo6 and Nqo4 (*Thermus* homologue of the 49 kDa subunit) (Figure 3.2b,c). The mutation is located next to a stretch of highly conserved amino acid residues (blue colored residues of Nqo6 in Figure 3.2b) that was photoaffinity-labeled by a photoreactive derivative of fenpyroximate (Shiraishi et al., 2012) (Figure 3.2b,c).

3.3.3. The effect of METI-I selection on H92R mutation frequency

We next investigated the effect of selection with METI-Is fenpyroximate, pyridaben and tebufenpyrad on the frequency of H92R in a *T. urticae* population (Hennep strain) segregating for this allele. The frequency of the mutation was estimated by PCR and proportional sequencing (Van Leeuwen et al., 2008) on DNA isolated from 200 randomly chosen adult females (see Tables S3.1 and S3.2 for PCR details). In all three replicated selected lines for each acaricide, selection increased the frequency of H92R from approximately 20% in the unselected Hennep strain to 100% in all nine METI-I selected lines from the Hennep strain. The frequency of H92R did not change in the control

Hennep strain population, between start and end of the selection experiment. At the phenotypic level, LC_{50} values from the non-selected Hennep strain rose from 86, 32 and 28 mg l^{-1} (confirming susceptibility), to higher than 1000, 1000 and 300 mg l^{-1} for fenpyroximate, pyridaben and tebufenpyrad, respectively (TABLE S3.4).

3.3.4. Marker-assisted back-crossing of H92R into a susceptible background.

The H92R mutation was uncovered in field collected strains with unknown historical exposure to acaricides, and the strains were kept as METI-I resistant reference strains under selection pressure in the laboratory for a decade (see SECTION 3.2.2 for details). Hence, several factors might have accumulated that contribute to the extreme high resistance levels documented (Van Pottelberge et al., 2009). In order to assess the relative phenotypic effect of the mutation and at least partially uncouple the H92R mutation from other resistance conferring genes, we introgressed the mutation (using the MR-VP strain) into the genomic background of strain NS (which is susceptible to METI-Is and where we could not detect the H92R allele). Two independent back-crossing experiments were performed, giving rise to (1) the lines R1 and R2 with the H92R substitution and (2) the control lines C1 and C2 where in the last cycle the wild-type allele (H92) was selected. Susceptibility of the congenic homozygotic lines C1 and C2 (H92/ H92) and lines R1 and R2 (92R/92R) was assessed in bioassays with the three METI-I acaricides (FIGURE 3.3, see TABLE S3.5 for details). For pyridaben, resistance ratios (RRs) of the R1 and R2 lines was 63.8 and 58.2 fold compared to NS. In contrast, in the C1 and C2 line pyridaben RRs were limited to 1.3 and 0.8. Accordingly, RRs calculated for tebufenpyrad were 39.4 and 30.0 in the R1 and R2 lines, while reaching only 2.1 in the C1 and C2 lines. Last, fenpyroximate RRs calculated for R1 and R2 were 21.9 and 4.7, respectively. The corresponding RRs for the C1 and C2 lines were 0.8 and 0.06, respectively. Overall, these results strongly point towards a significant effect of H92R on METI-I toxicity, but also further confirm that additional factors are needed to attain the high RR of the METI-I resistant parental strain.

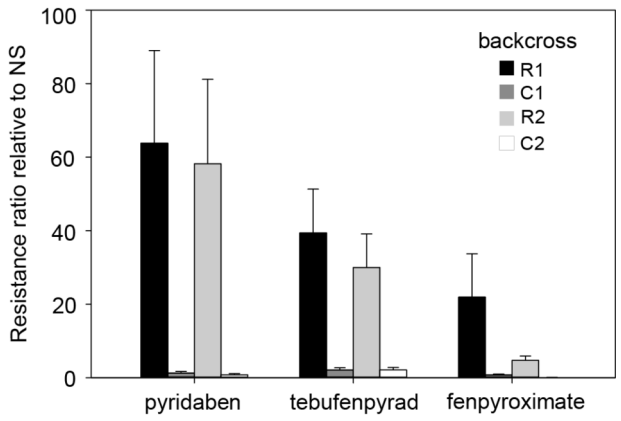

FIGURE 3.3 – Susceptibility of back-crossed *Tetranychus urticae* lines to METI-I acaricides. Resistance ratios were calculated as the LC_{50} of back-crossed lines C1, C2 (both with H92/H92 genotype), R1 and R2 (both with R92/R92 genotype) divided by the LC_{50} of the parental susceptible strain NS. Error bars represent 95% confidence limits calculated by probit analysis (POLO, LeOra Software, Berkeley, USA) (Robertson and Preisler, 1992).

TABLE 3.1 – Recombination rates between each microsatellite locus and pyridaben resistance in F2 males of NPR ♀ × NS ♂ cross after the selection with 200 mg l^{-1} pyridaben.

Loci (Scaffold No.)	No. F2 males analyzed	Recombination rate (%)
TuCT09 (3)	191	51.31
Tu27 (4)	192	47.40
TkMS15 (7)	192	5.73*
TuCT67 (9)	192	54.17
TuCT13 (35)	188	28.19*

*P<0.01 by Chi-square test against the expected values under the no linkage status.

3.3.5. Genetic mapping of the pyridaben resistance locus in *T. urticae* and QTL analysis

Prior to QTL analysis, we determined the scaffold(s) (Grbic et al., 2011) that were linked with pyridaben resistance in strain NPR (homozygous for H92R) and NS using five available microsatellite markers linked to scaffolds 3, 4, 7, 9 and 35 (for details, see SECTION 3.2.9). Significant linkage was found between the pyridaben resistance conferring gene(s), here denoted by 'PyrR' and two microsatellite markers, TkMS15 (5.73%) and TuCT13 (28.19%) (TABLE 3.1). The TkMS15 is located at the position of 1.16 Mb in scaffold 7 (3.7 Mb), while TuCT13 is on scaffold 35. Because the recombination rate between the resistance gene and TkMS15 was likely small enough to contain the resistance gene, we continued QTL analysis with markers on scaffold 7.

On scaffold 7, the order of the microsatellites was estimated as follows: tu07ms_g10–tu07ms_g7–tu07ms_g4–tu07ms_g3–TuCT04 (LOD = 5; log-likelihood = −510.5572), which did not correspond with the genomic sequence data. The software also provided an alternative order (TuCT04–tu07ms_g10–tu07ms_g7–tu07ms_g4–tu07ms_g3, log-likelihood = −514.6042) which was in line with the sequence data (FIGURE 3.4). Instability of the location for TuCT04 might be due to the small sample number for this locus, and log-likelihood values were not widely different between those orders. Therefore, we employed the latter order to perform QTL analysis.

Both simple interval mapping (sim) and composite interval mapping (cim) showed higher lod values for the location of pyridaben resistance gene (37 cM [LOD = 44.3] by sim and 34 cM [LOD = 11.1] by cim) between tu07ms_g10 (28.87 cM) and tu07ms_g7 (38.41 cM; FIGURE 3.4). As a result, QTL of the pyridaben resistance gene was determined between those microsatellite loci (34 cM; FIGURE 3.4). The location of the resistance gene corresponds roughly with that of the PSST gene in physical map (FIGURE 3.4). Additionally, LOD scores by cim were exceeding 5% in the region between markers tu07ms_g7 and tu07ms_g4, a region containing five (one pseudo and four full-length) cytochrome P450 genes.

3.3.6. Introduction of H92R in the PSST homologue of *Drosophila* by CRISPR-Cas9 followed by homologous recombination-directed gene modification (HDR)

The PSST homologue of *Drosophila*, *Dmel_CG9172*, was identified by a BLASTp approach using *B. taurus* PSST (UniProt accession P42026) as query. The CRISPR-Cas9 genome modification procedure was started by injecting *Drosophila* embryos with a mix of 2 sgRNAs and a dsDNA template for homologous recombination (HR). Screening with the CRISPOR

on-line tool (Haeussler et al., 2016) showed 100% specificity for both guide RNAs, according to Hsu et al. (2013). Both sgRNAs had no predicted off-targets with perfect matches near (12bp) the PAM regions indicating their uniqueness in the genome. The sgRNAs directed the Cas9 endonuclease to cut 118 nt upstream and 145 nt downstream from H103 (*Drosophila* numbering, corresponding to H92 in *Y. lipolytica*). The dsDNA template equipped with 1kb long arms mediating HR, was designed to introduce H103R. In addition several silent mutations were introduced for protection from being digested by Cas9 and to facilitate screening for recombinants (for details see SECTION 3.2.12.3, FIGURE S3.1). Following injection of y^1 M{nos-Cas9.P}ZH-2A w^* (referred further below as nos.Cas9) *Drosophila* embryos with the relevant gRNAs/Donor plasmid mix, 47 G_0 flies that reached adulthood were mated with nos.Cas9 flies of the opposite sex. From these crosses, 27 produced G_1 progeny (20 either died prematurely or the mating did not produce progeny). Samples of circa 30 G_1 individuals from each cross were collected and subjected to screening *en masse*, which provided indications for the presence of HDR-derived alleles within the sample at nine different lines, i.e. ~33% of the total number of lines that gave G_1 progeny. Twenty to thirty G_1 virgin females from each of three different original (G_0) lines were individually crossed to FM7c *y Hw w B* male flies and after generating G_2 progeny were individually screened to identify positive heterozygotes. Several independent lines were established from different G_1 individuals (originating from three different G_0 flies / injection events) after balancing. However, no male flies bearing the modified allele emerged from this cross (only FM7c males), while heterozygous females (bearing the modified allele opposite to the balancer chromosomes) were normally present in the progeny (at roughly 2:1 ratio compared to FM7c males). Consequently, it was not possible to generate homozygous females, and the lines were kept as stable balanced lines (heterozygous females) against an FM7c X chromosome. Further, to verify, that indeed H103R and no other sex chromosome-linked deleterious mutation was responsible for the lethal phenotype, we outcrossed the balancer and allowed recombination on the X chromosome for two-three generations. Screening of male progeny, however, showed no indication for the presence of the mutation.

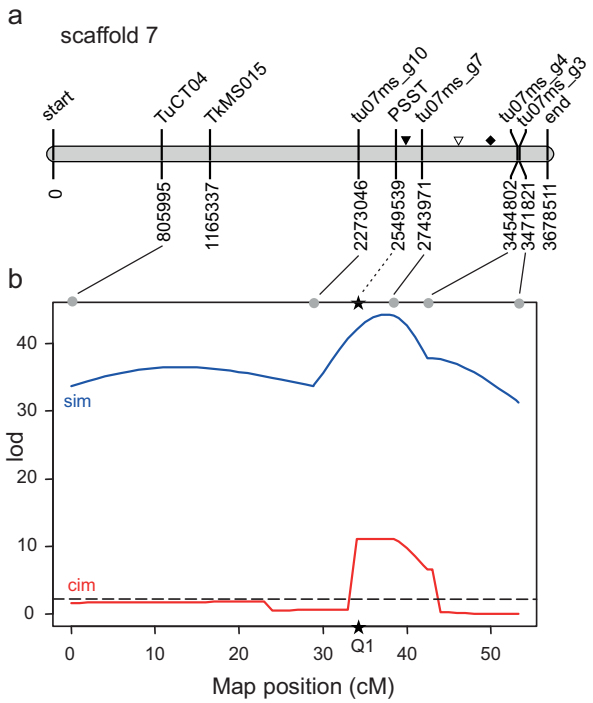

FIGURE 3.4 – Genetic mapping of the pyridaben resistance locus in *Tetranychus urticae*. (a) Position (number of bases) of *T. urticae* microsatellites and the PSST gene on the physical map of scaffold 7. The position of the P450 monooxygenase genes (*CYP385A1*, the CYP392A1-CYP392A4 cluster and CYP390B1) on scaffold 7 is indicated with a solid triangle, hollow triangle and solid rhombus, respectively. (b) Lod score plot with microsatellite data for scaffold 7. Simple interval mapping (sim) and composite interval mapping (cim) are shown as a blue and a red line, respectively. A horizontal broken line shows $\alpha = 5\%$ for cim. Q1 represents QTL position, which is roughly (dashed line) corresponding with the PSST position on the physical map of scaffold 7.

3.4. DISCUSSION

To function normally, living organisms require energy in the form of ATP. Mitochondrial respiratory complex I plays a major role in the energy production, linking respiratory electron transport with proton translocation across the mitochondrial membrane, which contributes to generation of the proton motive force required for ATP synthesis. As a consequence, Complex I deficiency is the most frequently encountered single enzyme deficiency in patients with a mitochondrial disorder (Martin et al., 2005; Rodenburg, 2016; Wong, 2013). For example, mutations R145H and V122M in the human complex I NDUFS7 (homologue *of B. taurus* PSST) have been reported to be associated with Leigh syndrome (Lebon et al., 2007; Triepels et al., 1999) (FIGURE 3.2).

Numerous hydrophobic compounds are known to inhibit the ubiquinone reductase reaction of complex I (Esposti, 1998; Lummen, 1998), of which rotenone as one of the first discovered insecticides, has been first studied in this context (Fukami, 1976). A multitude of other chemicals are used today as modern mite control agents, in particular the METI-I acaricides. Resistance to METI-Is was first reported in the '90s but is now considered to be widespread. Several studies have investigated resistance mechanisms leading to a consensus that in most cases P450 monooxygenase mediated metabolism is a main resistance conferring factor, possibly also responsible for the broad cross-resistance pattern observed between different METI-Is compounds (Hollingworth and Ahammadsahib, 1995; Motoba et al., 2000; Stumpf and Nauen, 2001; Sugimoto and Osakabe, 2014; Van Pottelberge et al., 2009; Van Pottelberge et al., 2008). Involvement of P450s was most often inferred from synergism data. Also, enzymatic experiments with model substrates, sometimes revealed up to 20-fold higher P450 activity in resistant strains (Van Pottelberge et al., 2009). However, only recently functional expression of a specific P450, CYP392A11, has shown direct metabolism of both cyenopyrafen (a beta-ketonitrile acting at complex II) and fenpyroximate (METI-I) (Riga et al., 2015). Nevertheless, the study of METI-I resistance has also suggested that multiple mechanisms are at play, given the differences in genetics of resistance between tetranychid species and within strains, combined with lack of PBO synergism (Devine et al., 2001; Goka, 1998; Van Pottelberge et al., 2009).

METI-I target-site resistance has not been documented (until association in this study with H92R), but was also never screened for in currently published studies. This is probably related to the complexity of the multimeric NADH:ubiquinone oxidoreductase, and the lack of sequence data of subunits for relevant organisms. We here exploited the available genome sequence of *T.*

urticae (Grbic et al., 2011), together with existing structural data (Fiedorczuk et al., 2016; Vinothkumar et al., 2014; Wirth et al., 2016; Zickermann et al., 2015) and insights based on photo-affinity labeling (Schuler et al., 1999; Shiraishi et al., 2012), to mine for relevant subunits in both susceptible and resistant strains.

Comparing full length sequences between strains uncovered a mutation, H92R, in the PSST subunit of three METI-I resistant *T. urticae* strains. The mutation was fixed in these phenotypically characterized resistant strains, but it was also segregating in others, indicating a potentially global relevance of alleles linked to METI-I resistance. Short-term selection with pyridaben, fenpyroximate and tebufenpyrad lead to fixation of the mutation in a population segregating for this allele, while it simultaneously drastically decreased susceptibility. This correlative support of mutation and phenotype was further investigated by marker-assisted back-crossing. Back-crossing is a well-established breeding strategy where a characteristic is introgressed from a donor parent into the genomic background of a recurrent parent (Hospital, 2005). The aim is to reduce the genetic background of a former to the minimum. So, to uncouple the mutation from other resistance conferring genes, we introgressed it in a reference susceptible background and found remaining resistance levels that mounted up to 30-60 fold for tebufenpyrad and pyridaben, compared to the NS strain. Lower and less consistent resistance levels were documented for fenpyroximate. This probably indicates that the effect of the mutation is not necessarily identical for all METIs, which might not be surprising given the structural different families of compounds. In addition, resistance levels associated with the mutation in the susceptible background, were clearly less pronounced when compared to the original MR-VP resistant strain, confirming the role of multiple factors in resistance. In addition, back-crossing reduces the proportion of donor genome by one-half (50%) at each generation, except on the chromosome carrying the characteristic. On this chromosome, the rate of decrease is slower (Hanson, 1959; Naveira and Barbadilla, 1992; Stam and Zeven, 1981) resulting in so called linkage drag. To therefore further reinforce the likelihood of H92R to be involved in resistance, we also mapped the resistance locus using traditional recombination mapping and QTL analysis using a genetically independent (from MR-VP) METI-I resistant strain from Japan, NPR. Using a set of SNP-markers and microsatellites, the resistance locus in this strain was mapped to a 470 kb (34 cM) region on scaffold 7 which includes the PSST locus. Additionally, since LOD scores by cim were exceeding 5% probability around the location of tu07ms_g4 (FIGURE 3.4), we scanned the region between PSST and tu07ms_g4 and found one pseudo and five full-length cytochrome P450 monooxygenase genes, *CYP385A1* (*tetur07g05500*), *CYP392A1* (*tetur07g06410*), *CYP392A2p* (*tetur07g06440*), *CYP392A3* (*tetur07g06460*), *CYP392A4* (*tetur07g06480*) and *CYP390B1* (*tetur07g08209*) (FIGURE 3.4a), which could also be involved in resistance. However, available genome wide expression data of the METI-I resistant strain MR-VP does not show markedly increased expression of these P450 genes, but instead increased expression of *CYP392A12, CYP392A11, CYP392D2* (Khalighi et al., 2016). The latter P450 genes are all located on scaffold three and recently CYP392A11 has been shown to metabolize fenpyroximate (Riga et al., 2015). One could argue that in strain NPR other P450 genes might be linked to pyridaben resistance than those in the MR-VP strain, warranting further research. Nevertheless, it was previously shown that pyridaben resistance is maternally inherited in NPR, but only in the eggs (Sugimoto and Osakabe, 2014). This suggests that maternal factors are important in early life stages to survive METI-I toxicity, but are diluted during development. Mitochondria in the egg are all of maternal origin, and would hence contain PSST protein synthesized by the mother, potentially linking this phenomenon to the uncovered mutation in this study. In summary, correlation analysis, selection experiments and genetic mapping all support a role for H92R in METI-I resistance.

A direct evidence of the role of H92R in METI-I resistance could in principle be obtained by characterizing inhibitor kinetics on spider mite preparations rich in NADH:ubiquinone oxidoreductase activity. Such assays have been described (Motoba et al., 1992; Nakano et al., 2015), but where in our hand not straightforward in terms of repeatability, which did not allow to characterize potential relative small differences in IC_{50}s of the METI-I compounds tested. However, assays are standardized for fly material, and especially for *D. melanogaster*, multiple protocols are described to generated good fragmented mitochondrial membrane preparations accessible for electron donors and acceptors of the assays (e.g., Ferguson et al., 2005). We therefore used a CRISPR-Cas9 technology to introduce the mutation in the PSST homologue of *D. melanogaster*. Genome modified flies could in theory also be used to assess differences in toxicity associated with the mutation, although METI-I acaricides are not very active on flies or insects in general. CRISPR-Cas9 modified *Drosophila* lines were successfully created, but unfortunately the mutation could not be brought to homozygosity in any of the independently generated lines, and hemizygous males were not found. One could wonder whether unspecific activity of the Cas9 endonuclease could result in this lethal phenotype. However, *in silico* screening for off-target regions showed that both guide RNAs were 100% specific, indicating that any off-target effect of our CRISPR-Cas9 approach is highly unlikely. In addition, outcrossing and allowing recombination of the X-chromosome failed to generate hemizygous males carrying the mutation, and so the phenotype could not be uncoupled from the mutation. This strongly suggests that it is the mutation that causes lethality.

Starting with the discovery of the first inhibitor induced mutation in the 49 kDa homologue of *Rhodobacter sphaeroides* (Darrouzet and Dupuis, 1997), several other studies used insecticidal complex I inhibitors to obtain resistant complex I mutants in model organisms (Lummen, 2007). Particularly, an extensive site-directed mutagenesis approach on *Y. lipolytica* gave rise to an abundance of examples where single point mutations either in PSST or the 49 kDa subunit resulted in decrease in quinone/inhibitor binding or diminished enzymatic activity (Ahlers et al., 2000a,b; Angerer et al., 2012; Fendel et al., 2008; Grgic et al., 2004; Kashani-Poor et al., 2001; Kerscher et al., 2004; Tocilescu et al., 2007, 2010a,b) (FIGURE 3.2). The structural model of complex I of *T. thermophilus* shows that A52R, corresponding to H92R in *T. urticae* PSST, is positioned in the cavity, on the distal side of the interface between Nqo6 (*T. thermophilus* homologue of PSST) and Nqo4 (*T. thermophilus* homologue of the 49 kDa subunit), in close proximity of the amino acid residues known to play a vital role in complex I functioning (FIGURE 3.2b,c). Valine at position 88 (V88) of the PSST homologue in *Y. lipolytica* (also known as NUKM) is located only four amino acids away from H92R (FIGURE 3.2). V88 forms, together with two residues

of 49 kDa subunit (V145, V460), a hydrophobic platform around tyrosine at position 144, being responsible for placing quinone at a perfect distance from the iron sulfur cluster N2 (Kashani-Poor et al., 2001; Tocilescu et al., 2010a; Tocilescu et al., 2007). Angerer et al. (2012) postulated that methionine 91 (M91) (Figure 3.2) situated in the direct neighborhood of H92, is one of the few hydrophobic residues, so called M-domains involved in binding hydrophobic tail of ubiquinone. Substitution of M91 with cysteine, lysine or glutamate resulted in insensitivity to inhibitors and diminished enzymatic activity (Angerer et al., 2012; Fendel et al., 2008). In a recent study, Zickermann et al. (2015) co- crystallized complex I with brominated derivatives of 2-decyl-4-quinazolinylamine (DQA); a true analogue of ubiquinone (Tocilescu et al., 2010b), revealing that the inhibitors planar aromatic ring system stacks between the tip of loop β1-β2 in the 49-kDa subunit and M91 of PSST. The above-mentioned examples support the notion that due to its close proximity to V88 and M91, substitution of H92 may, if not directly then allosterically, affect inhibitor binding. Additional evidence comes from photoaffinity studies. Shiraishi et al. (2012) performed photoaffinity labeling experiments using two fenpyroximate derivatives [125I]APF and [125I]AIF. Analysis of the fragmentation patterns of the labeled PSST and 49 kDa subunits indicated that the residues labeled by [125I]APF and [125I]AIF are located in the Ser43−Arg66 (PSST, numbering based on mature PSST protein, after removal of the transit peptide) and Asp160−Arg174 (49 kDa) regions (Figure 3.2b,c) (Shiraishi et al., 2012). Notably, H92 is located in the stretch of PSST subunit residues labelled by [125I]APF (Figure 3.2). Shiraishi's findings are in line with a previous photoaffinity study, which established that pyridaben, a structure similar to fenpyroximate, also binds to the PSST subunit (Schuler et al., 1999).

In the absence of crystallographic information, we can only speculate, why the H92R mutation is lethal in the *Drosophila* flies and not in *T. urticae*. It could be that the mutation-induces secondary/tertiary structure alterations that only occur in the fly protein. However, as mentioned above, H92 is located in a very conserved domain of the PSST subunit, where a single amino acid substitution has an immediate impact on protein activity, inhibitor and/or quinone binding. In addition, according to an on-line prediction tool for the functional effect of amino acid substitutions, PROVEAN (Choi et al., 2012), the H92R substitution is detrimental for both *Drosophila* and *T. urticae*, which is, considering the distinctive chemical futures of histidine and arginine (Betts and Russell, 2003), not unexpected. Hence, future research will be needed to further unravel this species-specific difference in lethality of the H92R mutation in PSST.

To conclude, the development of resistance to METI-I group of inhibitors in the polyphagous spider mite *T. urticae*, presents a significant challenge for agriculture. Although METI-I resistance has been traditionally attributed to P450 mediated detoxification, our findings suggest an additional, target-site based resistance mechanism. All experimental evidence obtained throughout this study, starting from the discovery of H92R in METI-I resistant strains, supplemented with genetic evidence and the location of the mutation being relevant for inhibitor binding, supports a causal link between H92R and METI-I resistance in *T. urticae*. In addition, our study contributes to a general understanding of the complex I structure and mechanism of inhibitor binding.

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FIGURE S3.1 – Details of the CRISPR design. The sgRNA sequences are shaded blue. sgRNA1 is located towards the 5' end of the sequence and sgRNA2 towards the 3' end. The corresponding protospacer adjacent motif (PAM) sequences are shaded yellow. A guanine residue required for a more efficient U6-driven in vivo transcription, added to the 5' end of the sgRNA1 oligonucleotide, is not depicted. Blue arrows show the predicted target-sites of Cas9 endonuclease. Nucleotides marked in red show silent point mutations introduced to the dsDNA donor template, in regions corresponding to sgRNA sequences, to prevent Cas9 mediated digestion of the donor template. The wild type Dmel_CG9172 (PSST) gene contains Tsp45I restriction site (shaded green), which in case of homologous directed recombination is removed by an A->G mutation. Recombination was additionally confirmed with a 'mutant-specific' primer pair (primers CG9172_dia_F/ CG9172_dia_R, TABLE S3.2) shown in orange. Introduction of the A->G mutation (H103R, *D. melanogaster* numbering; H92R, *Y. lipolytica* numbering) creates a target-site for FspI (shaded magenta) which can be further used to digest the CG9172_dia_F/ CG9172_dia_R amplification product.

FIGURE S3.2 – Nucleotide sequence of the genome modified *D. melanogaster* homologue of the *B. taurus* PSST gene. The coding sequence is indicated with a black font, while untranslated regions (UTRs) and introns are indicated with an orange and green font, respectively. Amino acid translations (blue font) are shown on top of the nucleotide sequence. Homozygous and heterozygous (R=G/A, S=G/C, M=A/C, Y=T/C) synonymous single nucleotide polymorphisms (compared to the CG9172 sequence available in FlyBase) are shaded in green and yellow, respectively. The position of the CRISPR-Cas9 introduced A308G mutation, resulting in the H103R (*Drosophila* numbering; H92R, *Y. lipolytica* numbering) substitution in *D. melanogaster* PSST, is shaded red.

TABLE S3.1 – PCR components and conditions.

TABLE S3.2 – Primers used in this study.

TABLE S3.3 – Strains used in this study and estimated H92R frequency.

TABLE S3.4 – Toxicity of METI-I acaricides to a *T. urticae* field strain (Hennep) before and after selection with pyridaben, tebufenpyrad and fenpyroximate and corresponding frequencies of H92R.

TABLE S3.5 – Toxicity of tebufenpyrad, fenpyroximate and pyridaben to adult *T. urticae* females of back-crossed lines C1 and C2 (H92/H92 genotype), back-crossed lines R1 and R2 (R92/R92 genotype) and their susceptible parent strain (NS).

FILE S3.1 – Sequences of sgRNAs and dsDNA template used in CRISPR-Cas9 approach.

4

The relative contribution of target-site mutations in complex acaricide resistant phenotypes as assessed by marker assisted backcrossing in *Tetranychus urticae*

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Abstract. The mechanisms underlying insecticide and acaricide resistance in insects and mites are often complex, including additive effects of target-site insensitivity, increased metabolism and transport. The extent to which target-site resistance mutations contribute to the resistance phenotype is, however, not well studied. Here, we used marker-assisted backcrossing to create 30 congenic lines carrying nine mutations (alone, or in combination in a few cases) associated with resistance to avermectins, pyrethroids, mite growth inhibitors and mitochondrial complex III inhibitors (QoI) in a polyphagous arthropod pest, the spider mite *Tetranychus urticae*. Toxicity tests revealed that mutations in the voltage-gated sodium channel, chitin synthase 1 and cytochrome b confer high levels of resistance and, when fixed in a population, these mutations alone can result in field failure of acaricide treatment. In contrast, although we confirmed the implication of mutations in glutamate-gated chloride channels in abamectin and milbemectin insensitivity, these mutations do not lead to the high resistance levels that are often reported in abamectin resistant strains of *T. urticae*. Overall, this study functionally validates reported target-site resistance mutations in *T. urticae*, by uncoupling them from additional mechanisms, allowing to finally investigate the strength of the conferred phenotype *in vivo*.

4.1. INTRODUCTION

Insecticide resistance is a major threat for the chemical control of insects and mites in public health and agriculture. At present, the Insecticide Resistance Action Committee (IRAC) distinguishes between at least fifty-five different chemical classes and more than twenty-five distinct mode of action (MoA) groups (Sparks and Nauen, 2015). MoA diversity is of key importance for effective Insecticide Resistance Management (IRM). However, the costs involved in the discovery, development and marketing of chemicals with new properties, increased immensely and slow down the development of compounds with new MoA. In addition, concerns about the environment and human health, integrated in new regulations, demand molecules with better selectivity (Sparks, 2013). To preserve the utility and diversity of available and newly developed insecticides/acaricides, it is of utmost importance to understand the resistance mechanisms against these compounds (Sparks and Nauen, 2015) and develop diagnostic tools that support monitoring activities and resistance management.

A number of mechanisms have been shown to underlie insecticide resistance, most often quantitative or qualitative changes in major detoxification enzymes and transporters (pharmacokinetic mechanisms) and/or target-site mutations (pharmacodynamic mechanisms) (Feyereisen et al., 2015; Li et al., 2007). When resistance is caused by a combination of factors (polygenic resistance), the overall resistance levels may be the sum of contribution of each individual factor (Bohannan et al., 1999; Raymond et al., 1989) but synergistic or antagonistic interactions between resistance loci also occur (Moore and Williams, 2005; Williams et al., 2005; Zhang et al., 2016). The relative contribution of each individual resistance locus to complex insecticide/acaricide resistance phenotypes has only been sporadically investigated (Hardstone and Scott, 2010). In particular, the relative importance and strength of target-site mutations is often hard to assess by merely associating a phenotype with mutation frequency in field populations, where prolonged selection may have led to the accumulation of additional resistance mechanisms. Furthermore, the majority of studies that look into epistatic interactions and/or resistance levels confirmed by a single genetic factor, are sometimes difficult to interpret if resistance alleles are not investigated in a common genetic background (Liu and Pridgeon, 2002; McEnroe and Naegele, 1968; Peyronnet et al., 1994; Shi et al., 2004; Zhang et al., 2016). Therefore, analysis of a resistance trait requires the studied strains to be identical, except for its causal gene (Georghiou, 1969; Mckenzie et al., 1982). Functional validation of resistance mutations has been reported after recombinant expression. Inhibitor-protein interactions are then quantified via enzymatic reactions or ligand binding assays such as voltage-clamp electrophysiology. Although they provide strong evidence of the effect of a mutation on the affinity for the compound to the target-site, they are less suitable to assess the relative phenotypic consequences *in vivo* (Cully et al., 1994; Ludmerer et al., 2002). A more precise way to determine the effect of a mutation *in vivo* is to introduce it in a defined susceptible genetic background, by utilizing genome editing techniques, such as CRISPR-Cas9 (Douris et al., 2016; Zimmer et al., 2016), in species where this approach is applicable. In species where genome editing tools are not yet available, a more feasible alternative is to repeatedly backcross resistant individuals with susceptible ones (Georghiou, 1969; McCart et al., 2005; Roush and Mckenzie, 1987). Marker-assisted backcrossing provides a straight-forward and relatively precise method to untangle a mutation of interest from other mechanisms that might have been co-selected. The impact of a modifier or interactions between modifiers can be then analyzed by comparing the genetically identical strains that differ only in a small region on the chromosome, which harbors the resistant locus of interest (Bajda et al., 2017; Brito et al., 2013).

The two-spotted spider mite, *Tetranychus urticae* (Chelicerata: Acari: Acariformes) is an important agricultural pest, that thrives on more than a 1,000 plant species (Jeppson et al., 1975; Migeon and Dorkeld, 2006-2015). Its short life cycle, high fecundity and haplo-diploid system facilitates a rapid evolution of acaricide resistance. Today, *T. urticae* has developed resistance to more than 90 different chemical compounds, including major groups of currently used acaricides (Sparks and Nauen, 2015; Van Leeuwen et al., 2009, 2013). In *T. urticae* and other related spider mites, very high resistance ratios (RRs) have been reported for a number of compounds (RR>10,000) (Kramer and Nauen, 2011; Van Leeuwen et al., 2009) with numerous cases of cross-resistance to newly introduced acaricides, for example, Khalighi et al. (2014). Several target-site mutations have been uncovered and were associated with acaricide resistance in populations of *T. urticae*, recently summarized in Van Leeuwen and Dermauw (2016). These include mutations leading to amino acid substitutions in acetylcholinesterase (*AChE*) (G119S, A201S, T280A, G328A and F331W) that are associated with resistance to organophosphates and carbamate (Khajehali et al., 2010). The L1024V and A1215D + F1538I substitutions in the voltage-gated sodium channel (VGSC) have been linked to resistance to Type I (absence of α-cyano group) and Type II (presence of α-cyano group) pyrethroids (Kwon et al., 2010a; Tsagkarakou et al., 2009). Six orthologous glutamate–gated chloride channel (GluCl) genes have been reported in spider mites and substitutions in G314D and G326E in GluCl1 and GluCl3, respectively, were associated with resistance to abamectin (Dermauw et al., 2012; Kwon et al., 2010b). The G126S, I136T, S141F, D161G, P262T substitutions (in different combinations) identified in the cytochrome b (cytb) cause strong bifenazate resistance (Mitochondrial Qo inhibitors: QoI) (Van Leeuwen et al., 2008). A substitution I1017F in the chitin synthase 1 gene (*CHS1*) has been linked with high levels of resistance to mite growth inhibitors, etoxazole, clofentezine and hexythiazox (Demaeght et al., 2014; Van Leeuwen et al., 2012). Most recently, an H92R substitution in the PSST subunit of the Mitochondrial Respiratory Complex I, has been associated with resistance to pyridaben, tebufenpyrad and fenpyroximate (Mitochondrial Electron Transport Inhibitors, site I, METI-I) (Bajda et al., 2017). As resistance in spider mites often has a polygenic basis, the relative contribution of target-site resistance to the overall resistance levels is currently unknown. One notable exception for *T. urticae* is the H92R mutation in the PSST subunit, which was introduced into a susceptible background by repeated backcrossing and shown to confer moderate levels of METI resistance (Bajda et al., 2017).

In this study, we investigated the relative contribution of nine known target-site mutations conferring resistance to abamectin, pyrethroids, bifenazate and mite growth inhibitors. We adopted the method of Bajda et al. (2017) and succeeded in generating 30 congenic resistant and susceptible lines of *T. urticae*. When a combination of mutations in homologous genes was reported,

the phenotypic levels of resistance were examined for both the single mutations, as well as their combination.

4.2. MATERIALS AND METHODS

4.2.1. Acaricides

Acaricides used in this study were commercial formulations of bifenazate (Floramite, 240 g l^{-1} SC) and acequinocyl (Cantack 164 g l^{-1} SC), etoxazole (Borneo, 120 g l^{-1} SC), hexythiazox (Nissorun, 250 g l^{-1} SC) and clofentezine (Apollo, 500 g l^{-1} SC), abamectin (Vertimec 18 g l^{-1} EC), milbemectin (Milbeknock 10 g l^{-1} EC), bifenthrin (Talstar 100 g l^{-1} EC), fluvalinate (Mavrik 240 g l^{-1} EW) and analytical grade fenpropathrin (Sigma Aldrich).

4.2.2. Spider mite strains

The susceptible Wasatch strain is an inbred line, originally collected from tomato in a greenhouse near Salt Lake City, Utah, USA. The pyrethroid susceptible strain KOP8 is an inbred line derived from the Houten strain (Chatzivasileiadis and Sabelis, 1997). Wasatch does not contain any of the so far described mutations. KOP8 harbors the A1215D substitution, potentially associated with pyrethroid resistance. The GH strain carries the L1024V genotype (*Musca domestica* numbering) of the VGSC gene and was collected from greenhouse grown maize in Utah USA. The TuSB9 strain carrying the A1215D and F1538I mutations (*Musca domestica* numbering) in VGSC was previously described (Tsagkarakou et al., 2009). The MAR-AB strain carrying G314D and G326E substitutions (*Tetranychus urticae* numbering) in GluCl1 and GluCl3, respectively, was previously described in Dermauw et al. (2012). Strains with mutations associated with bifenazate resistance, HOL3 (cytb, P262T – *Tetranychus urticae* numbering) and BR-VL (cytb, G126S and S141F – *Tetranychus urticae* numbering) were described in Van Leeuwen et al. (2008) and Van Leeuwen et al. (2006), respectively. The EtoxR strain carrying the I1017F mutation (*Tetranychus urticae* numbering) in the chitin synthase (CHS1) gene was previously described (Van Leeuwen et al., 2012). An overview of strains is presented in TABLE 4.1. All *T. urticae* strains were maintained on 3-week old potted kidney bean plants (*Phaseolus vulgaris L.*) in a climatically controlled room or incubator at 25 ± 1°C, 60% relative humidity, and 16:8 light: dark photoperiod.

4.2.3. Backcrossing experiments

To assess the relative resistance levels associated with mutations, we used a marker assisted backcrossing approach to produce near-isogenic sister lines (FIGURE 4.1 and TABLE 4.1). The crossing procedure was previously outlined in Bajda et al. (2017). In short, a haploid male of the resistant strain was crossed with a virgin female of the susceptible strain. The resulting heterozygous virgin females were backcrossed to susceptible males and heterozygote genotypes were identified by a TaqMan molecular assay or PCR and sequencing as it is described in SECTION 4.2.5. This process was repeated for six to nine generations. In the last generation, a cross was carried out between the backcrossed heterozygous virgin females and their first born sons representing either a susceptible (absence of mutation) or the resistant (presence of mutation) genotype. This finally resulted in congenic homozygous lines for the mutation and the wild type allele. The final crosses were performed as follows (see TABLE 4.1): For the mutations in GluCls, G314D in GluCl1 and G326E in GluCl3, MAR-AB males were crossed with Wasatch virgin females in order to separate the mutations in different lines, as they are inherited independently (Dermauw et al., 2012), after which they were introgressed separately: ♀314D/314G × ♂314D or ♂314G to generate GluCl1_R1-R3 and GluCl1_C, ♀326E /326G × ♂326E or ♂326G to produce homozygous congenic GluCl3_R1-R3 and GluCl3_C, respectively. Mutations were later joined in a single line by dedicated crosses as follows: ♀GluCl1_R1 × ♂GluCl3_R1, ♀GluCl1_R2 × ♂GluCl3_R2, ♀GluCl1_R3 × ♂GluCl3_R3 and ♀GluCl1_C × ♂GluCl3_C to produce GluCl1+3_R1,R2,R3 and C, respectively. For the mutations in VGSC; the ♀1024V/1024L × ♂1024V or ♂1024L were crossed to obtain homozygous congenic lines VGSC_R1-R3 and VGSC_C1, respectively, ♀1215D+1538I/1215D+1538F × ♂1215D+1538I or ♂1215D+1538F to obtain homozygous congenic VGSC_R4-R6 and VGSC_C2, respectively. For the mutation in CHS1; ♀1017F/1017I × ♂1017F or ♂1017I were crossed to generate homozygous congenic CHS1_R1-R3 and CHS1_C, respectively.

For the mitochondrial mutations in cytb (HOL3 and BR-VL) that are inherited completely maternally, simple crosses between resistant females and susceptible males were performed for 7 generations, as to create a line with the nuclear genome of the susceptible parent (Wasatch), but bearing the mitochondrial haplotype of the resistant line (FIGURE 4.1). Three crosses were set up to produce lines carrying P262T mutation in PEWY motif

TABLE 4.1 – Summary of crosses performed to create congenic *Tetranychus urticae* lines. VGSC mutations were numbered according to *Musca domestica* numbering, whereas GluCl1, GluCl3, cytochrome b and chitin synthase substitutions according to *T. urticae* numbering. *IRAC mode of action group number is shown between brackets. Superscript numbers indicate which mite stage was used in the toxicity assay ([1]larval toxicity assay, [2]egg toxicity assay, [3]adult toxicity assay). Refer to SECTION 4.2.6 for more details.

Strain	Resistant to*	Target-site mutation	Crossed to	Backcrossed lines	
MAR-AB	Abamectin (6)[3]	GluCl1 (G314D)	Wasatch	GluCl1_C, GluCl1_R1, R2, R3	GluCl1+3_C, GluCl1+3_R1, R2, R3
MAR-AB	Abamectin (6)[3]	GluCl3 (G326E)	Wasatch	GluCl3_C, GluCl3_R1, R2, R3	
GH	Pyrethroids (3A)[3]	VGSC (L1024V)	Wasatch	VGSC _C1, VGSC _R1, R2, R3	
TuSB9	Pyrethroids (3A)[3]	VGSC (F1538I + A1215D)	KOP8	VGSC_C2, VGSC_R4, R5	
HOL3	Bifenazate (20A)[3]	Cytochrome b (P262T)	Wasatch	cytb_R1, R2, R3	
BR-VL	Bifenazate (20A)[3], acequinocyl (20B)[3]	Cytochrome b (G126S + S141F)	Wasatch	cytb_R4, R5	
EtoxR	Mite growth inhibitors (10)[1,2]	Chitin synthase (I1017F)	Wasatch	CHS1_C, CHS1_R1, R2, R3	

of cytb (cytb_R1-R3- and consequently three lines with G126S + S141F mutations in cd1 region of cytb: (cytb_R4-R6).

4.2.4. Single mite DNA extraction

In order to perform single mite genotyping for I1017F (EtoxR), P262T (HOL3) and G126S + S141F (BR-VL) individual *T. urticae* mites were homogenized in 20 µl STE buffer (100 mM NaCl, 10mM Tris- HCl and 1mM EDTA) with 1 mg ml^{-1} proteinase K (Sigma-Aldrich). Homogenate was incubated at 37°C for 30 min followed proteinase K inactivation for 5 min at 95°C.

For G314D, G326E (MAR-AB) and F1538I, A1215D (TuSB9), L1024V (GH) single mite DNA was extracted following the CTAB method (Navajas et al., 1999). In short, individual mites were homogenized in 200 µl of extraction buffer (2% CTAB, 1.4M NaCl, 0.2% β-mercaptoethanol, 20 mM EDTA, 100 mM Tris – HCl, pH:8.0) and incubated at 65°C for 15 min. Equal volume of chloroform: isoamylalcohol (24:1) was used in order to remove proteins. The DNA was precipitated by isopropanol and washed with 75% ethanol. The pellet was air-dried and re-suspended in 20 µl DEPC treated water.

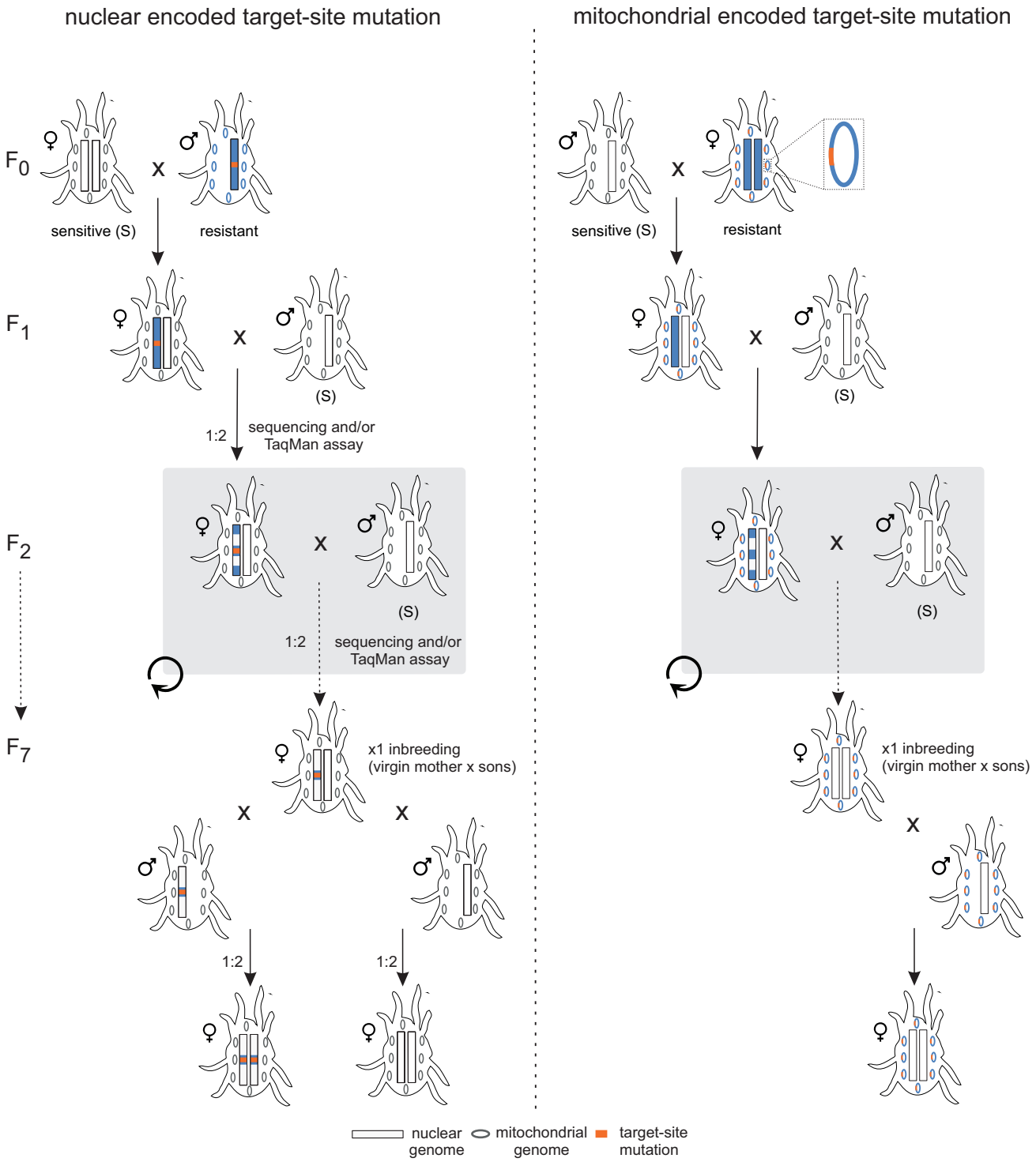

FIGURE 4.1 – Schematic diagram of marker-assisted backcrossing of nuclear and mitochondrial encoded resistance mutations. The susceptible genotype is depicted by white-colored chromosomes (rectangles) and mitochondria (ovals), while those of the resistant genotype are depicted in blue. An orange color indicates whether the resistance mutation is either nuclear or mitochondrial encoded.

4.2.5. Genotyping

Single mite genotyping was performed with standard PCR and sequencing (mutations I1017F, P262T, G126S + S141F and L1024V) and/or TaqMan method (Ilias et al., 2017) (mutations F1538I, G314D and G326E). PCRs were conducted in 50 µl final volume with 10 µl 5× Phusion HF Buffer, 0.2 mM of each dNTP, 0.5 µM each primer, 1 µl template, 0.5 µl polymerase with cycling conditions; 30 s at 98°C followed by 35 cycles 5 s at 98°C, 10 s at 55°C, 15 s at 72°C and 5 min of final extension. Reactions were performed in Bio-Rad T100™ Thermal Cycler. PCR products were purified with E.Z.N.A.® Cycle Pure Kit DNA purification kit (OMEGA bio-tek) and sequenced at Macrogen sequencing facility (Amsterdam). Sequencing data were analyzed using BioEdit 7.0.1 software (Hall, 1999). Primers used for the PCR reactions and sequencing are listed in TABLE S4.1.

TaqMan assay was performed as previously described (Ilias et al., 2017). In short, all assays were carried out in 15 µl total volume containing 2 µl of genomic DNA, 7.5 µl TaqMan Universal PCR Master Mix, 0.8 µM of each primer and 0.2 µM of each probe. Samples were run on CFX Connect, Real-Time PCR Detection System (Biorad) using the temperature cycling conditions of: 10 min at 95°C followed by 40 cycles of 92°C for 15 s and 60°C for 1 min. The increase in VIC and FAM reporter dyes, representing individuals with the resistant and susceptible alleles, respectively, was monitored in real time using the CFX Manager software. Positive and negative template controls were included in each run to aid genotype scoring. Primers and probes used for the TaqMan assay are listed in TABLE S4.1.

4.2.6. Toxicity bioassays

To assess the toxic effects of etoxazole and hexythiazox, larval bioassays (1) were performed as previously described by Van Pottelberge et al. (2009a). For the ovicide clofentezine, bioassays were performed on eggs (2) instead of larvae. Approximately fifty females were allowed to lay eggs for 5 h on the upper side of 9 cm^2 square-cut kidney bean leaf discs on wet cotton wool. For adulticidal bioassays (3) (Van Leeuwen et al., 2004), 20–30 young adult female mites were transferred to arenas, prepared as described above. Plates were sprayed with 1 ml of spray fluid at 1 bar pressure with a Potter Spray Tower (Burkard Scientific, UK) to obtain a homogenous spray film (2 mg deposit / cm^3). Experiments were then placed in a climatically controlled room at 25±0.5°C, 60% RH and 16/8 h (light/dark) photoperiod. Three to four replicates of at least five serial dilutions of each acaricide and a control (deionized water or 1:100 dilution of the mixture of N,N-dimethylformamide and emulsifier W, depending on the acaricide used) were tested. Fenpropathrin was of technical grade and formulated in 3:1 v/v mixture of N,N-dimethylformamide and emusulfier W and subsequently diluted in deionized water as previously described(Van Leeuwen et al., 2007). Mortality was assessed after 24 h for bifenazate and acequinocyl and 48 h for all other acaricides. Mites treated with growth inhibitors, were considered as unaffected, if at the time of scoring displayed the same developmental stage as water treated control. Adult mites were scored as being alive if they could walk twice the distance of their body size after being prodded with a camel's hair brush (Sato et al., 2005). All mortalities obtained for control treatment were lower than 10%. LC_{50} values, slopes, RRs and 95% confidence limits were calculated by probit analysis (POLO, LeOra Software, Berkeley, USA) (Robertson, 1992). In case 5,000 mg l^{-1} did not cause 50% mortality, no further attempts were made to determine LC_{50}s and RR was calculated by dividing 5,000 mg l^{-1} by the LC_{50} of susceptible strain. The effect of the treatment on the susceptible parent and the experimental line was considered significantly different if the hypothesis of equality of slopes and intercepts was rejected ($p = 0.05$) (Robertson et al., 2007). If a regression line – illustrating dose response – could not be derived (LC_{50} of the experimental line was found to be higher than 5,000 mg l^{-1}), the effect of treatment was considered different when the LC_{90} of the susceptible control was lower than 5,000 mg l^{-1}.

4.3. RESULTS

4.3.1. Establishment of congenic lines

The initial crosses between parental resistant and susceptible strains are outlined in TABLE 4.1. Briefly, the susceptible strain Wasatch, which does not carry any of the mutations studied here, was used for the most of the backcrossing experiments (TABLE 4.1). To study the mutations in GluCl1 (G314D) and GluCl3 (G326E) associated with abamectin resistance, virgin females of Wasatch were crossed with males of the abamectin resistant strain MAR-AB carrying both GluCl mutations. Similarly, for the L1024V mutation associated with pyrethroid resistance, Wasatch virgin females were crossed with males of the pyrethroid resistant strain GH that carries L1024V. The effect of A1215D + F1538I mutations in pyrethroid resistance was examined through crossing males of TuSB9 with females of the parental susceptible strain KOP8 (carrying the A1215D only). To study the effect of mutations in the mitochondrial encoded cytb (P262T and G126S + S141F) that confer resistance to bifenazate, virgin females of bifenazate resistant strains HOL3 (P262T) and BR-VL (G126S + S141F), were crossed to males of Wasatch. Last, to introduce the mutation I1017F associated with resistance to mite growth inhibitors, virgin females of Wasatch were crossed with EtoxR males (TABLE 4.1).

For the nuclear encoded mutations, the final cross between heterozygous backcrossed females and their sons resulted in congenic homozygous lines with either the mutation fixed or absent (FIGURE 4.1, TABLE 4.1, see SECTION 4.2.3 for outline experimental setup). Since mutations in GluCl1 and GluCl3 are not genetically linked (Dermauw et al., 2012), the impact of each mutation could be assessed separately. Once homozygous backcrossed lines carrying a mutation either in GluCl1 (GluCl1_R1-R3) or in GluCl3 (GluCl3_R1-R3) and their respective congenic control lines (GluCl1_C and GluCl3_C) were generated, the mutations were joined again by dedicated crosses, giving rise to GluCl1+3_R1-R3. The susceptible control GluCl1+3_C was obtained with the cross GluCl1_C × GluCl3_C. One replicate with genotype A1215D + F1538I (pyrethroid resistance mutations) and one with genotype G126S + S141F (bifenazate resistance mutations) were lost during backcrossing and only two biological replicates VGSC_R4, R5 and cytb_R4 and R5 could be analyzed for each genotype.

4.3.2. Toxicity assays

4.3.2.1. Parental strains

Abamectin and milbemectin were tested against the parental susceptible strain, Wasatch and the resistant strain, MAR-AB

(G314D + G326E), with the latter one exhibiting high resistance levels to abamectin (1354.9 fold) and moderate resistance to milbemectin (71.7 fold) in comparison to Wasatch (TABLE S4.2).

The parental susceptible strains, KOP8, which carries only the A1215D VGSC substitution, and Wasatch showed high susceptibility to bifenthrin, fluvalinate and fenpropathrin whereas the GH (L1024V) and TuSB9 (A1215D + F1538I) resistant strains were highly resistant to the aforementioned pyrethroids (TABLE 4.2).

The Wasatch strain and the parental resistant strains HOL3 (P262T) and BR-VL (G126S + S141F) were tested against bifenazate. The resistant strains exceeded 2,000 fold of resistance to bifenazate. Additionally, the parental susceptible strain and BR-VL were treated with acequinocyl, with the latter one showing moderate levels of resistance (RR of 28.9 fold) (FIGURE 4.2, TABLE S4.3).

Etoxazole, clofentezine and hexythiazox were tested against the EtoxR strain (I1017F) and the susceptible strain Wasatch. The EtoxR strain showed extremely high levels of insensitivity to all three compounds used, with RR values exceeding 40,000 for etoxazole, 4,000 for hexythiazox and 2,000 for clofentezine (TABLE S4.4, FIGURE 4.3).

4.3.2.2. Backcrossed strains

Abamectin and Milbemectin

The introgressed strains carrying resistance mutation in only one of the GluCls (either GluCl1 or GluCl3) showed minor resistance to abamectin and milbemectin with RR values up to 3.3 folds for GluCl1 and up to 1.6 for GluCl3, respectively (FIGURE 4.4, TABLE S4.2). However, when mutations were joined by dedicated crosses, individuals carrying both mutations (GluCl1+3_R1-3 congenic lines) showed higher resistance levels to both compounds. The RR values obtained for abamectin and milbemectin were up to 19.8 and 13.7 fold, respectively (FIGURE 4.4, TABLE S4.2).

Pyrethroids

The backcrossed strains VGSC_R1-3 and VGSC_R4,5 exhibited high levels of resistance to all pyrethroids used in this study (bifenthrin, fluvalinate and fenpropathrin), with RR values being greater than 200 fold in some cases. In contrast, the backcrossed susceptible lines VGSC_C1 and VGSC_C2 were susceptible to all three compounds (TABLE 4.2).

TABLE 4.2 – Toxicity of pyrethroids (bifenthrin, fluvalinate and fenpropathrin) to adult females of backcrossed lines VGSC_C1, VGSC_R1-R3 (L1024V genotype), VGSC_C2, VGSC_R4,5 (F1538I+ A1215D genotype) and their parental strains (Wasatch, GH, KOP8, TuSB9). [a] Number of mites used in toxicity tests. [b] RR compared to Wasatch in case of GH, VGSC_C1 and VGSC_R1-3 or KOP8 in case of TuSB9, VGSC_C2 and VGSC_R4,5 lines. a: Treatment effect was significantly different when compared to Wasatch or KOP8.

Compound	Strain	Genotype	N[a]	LC_{50} mg l^{-1} (95% CI)	Slope ± SE	χ^2 (df)	RR (95% CI)[b]
Bifenthrin	Wasatch	L1024	404	3.8 (2.1-4.7)	3.9 ± 0.8	17 (13)	-
	GH	L1024V	443	1031.0 (721.7-1406.8)a	1.5 ± 0.1	14 (13)	271.8 (185.3-398.8)
	KOP8	A1215D+F1538	354	4.1 (3.0-4.8)	3.2 ± 0.6	8 (16)	-
	TuSB9	A1215D+F1538I	517	1,715.8 (696.5-2474.8)a	2.3 ± 0.4	24 (16)	423.5 (272.4-658.4)
	VGSC_C1	L1024	382	5.09 (3.4-6.2)a	4.9 ± 0.8	26 (13)	1.3 (1.0-1.8)
	VGSC_C2	A1215D+F1538	436	4.6 (3.3-5.5)	4.8 ± 0.8	29 (16)	1.1 (0.9-1.5)
	VGSC_R1	L1024V	670	353.3 (277.1-410.3)a	3.7 ± 0.6	20 (19)	93.2 (69.1-125.7)
	VGSC_R2	L1024V	560	328.2 (260.7-390.5)a	3.0 ± 0.5	13 (18)	86.5 (63.1-118.8)
	VGSC_R3	L1024V	427	405.4 (329.8-466.5)a	3.8 ± 0.7	13 (13)	106.9 (79.4-143.9)
	VGSC_R4	A1215D+F1538I	554	508.9 (261.6-670.8)a	2.6 ± 0.6	16 (12)	125.6 (87.5-180.3)
	VGSC_R5	A1215D+F1538I	435	538.8 (380.6-670.2)a	3.6 ± 0.5	21 (12)	134.0 (100.4-176.1)
Fluvalinate	Wasatch	L1024	479	102.2 (82.7-118.5)	3.9 ± 0.6	18 (17)	-
	GH	L1024V	118	>5,000a	-		>45
	KOP8	A1215D+F1538	294	92.4 (67.3-117.5)	4.7 ± 1.1	15 (11)	-
	TuSB9	A1215D+F1538I	186	>5,000a	-	-	>50
	VGSC_C1	L1024	436	83.0 (63.2-98.5)	3.7 ± 0.6	16 (15)	0.8 (0.6-1.0)
	VGSC_C2	A1215D+F1538	508	87.0 (69.3-102.4)	3.7 ± 0.5	19 (15)	0.9 (0.8-1.2)
	VGSC_R1	L1024V	188	>5,000a	-	-	>45
	VGSC_R2	L1024V	180	>5,000a	-	-	>45
	VGSC_R3	L1024V	213	>5,000a	-	-	>45
	VGSC_R4	A1215D+F1538I	194	>5,000a	-	-	>50
	VGSC_R5	A1215D+F1538I	161	>5,000a	-	-	>50
Fenpropathrin	Wasatch	L1024	360	21.3 (15.8-26.9)	3.1 ± 0.5	23 (19)	-
	GH	L1024V	97	>5,000a	-	-	>230
	KOP8	A1215D+F1538	297	13.7 (11.0-16.9)	2.8 ± 0.5	8 (15)	-
	TuSB9	A1215D+F1538I	182	>5,000a	-	-	>360
	VGSC_C1	L1024	476	35.2 (26.2-44.2)a	2.1 ± 0.3	5 (16)	1.7 (1.2-2.3)
	VGSC_C2	A1215D+F1538	396	21.5 (15.9-26.8)a	3.5 ± 0.5	15 (19)	1.6 (1.1-2.2)
	VGSC_R1	L1024V	153	>5,000a	-	-	>230
	VGSC_R2	L1024V	155	>5,000a	-	-	>230
	VGSC_R3	L1024V	180	>5,000a	-	-	>230
	VGSC_R4	A1215D+F1538I	171	>5,000a	-	-	>360
	VGSC_R5	A1215D+F1538I	156	>5,000a	-	-	>360

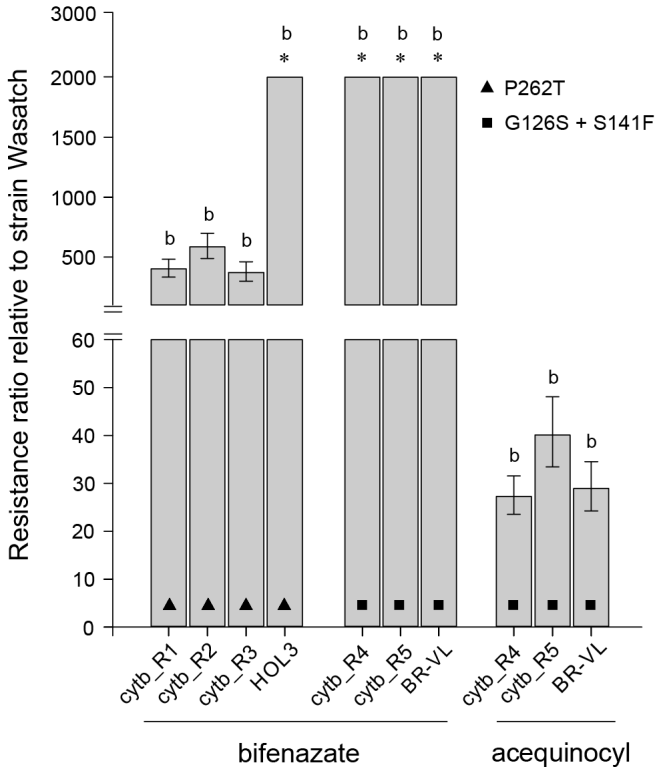

FIGURE 4.2 – Susceptibility of backcrossed *Tetranychus urticae* lines cytb_R1-R3 (P262T) and cytb_R4, R5 (G126S + S141F) and the resistant parental strain HOL3 and BR-VL to QoI acaricides bifenazate and acequinocyl. The RRs were calculated as the LC_{50} values of the backcrossed lines divided by the LC_{50} of the parental susceptible strain Wasatch. Stars indicate strains for which the LC_{50} value exceeded 5,000 mg l^{-1}. Error bars represent 95% confidence limits calculated by probit analysis. Letters above bars indicate lines where acaricide treatment had statistically the same (a) or different (b) effect comparing to Wasatch (PoloPlus LeOra Software) (Robertson, 1992).

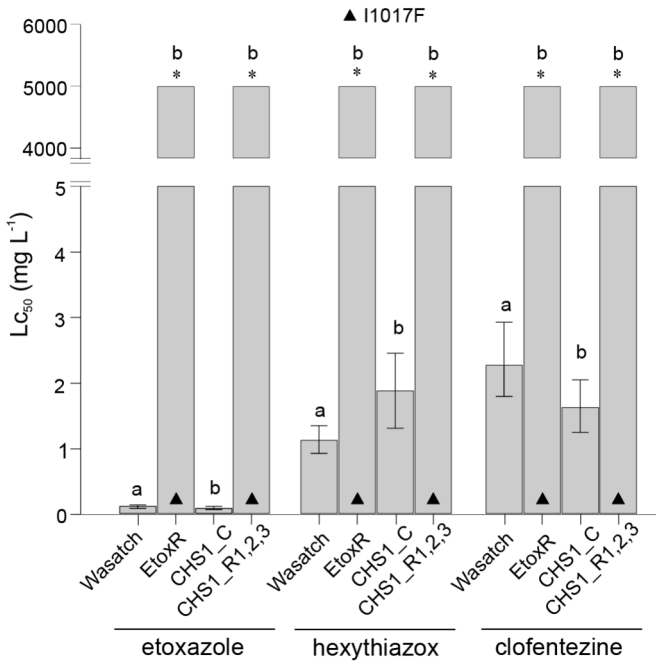

FIGURE 4.3 – Susceptibility of backcrossed *Tetranychus urticae* lines CHS1_R1-R3 (I1017F) and CHS1_C and their susceptible and resistant parental strains, to mite growth inhibitors etoxazole (most left), hexythiazox (middle) and clofentezine (most right). Bars represent the acaricide concentration at which 50% of the individuals are affected. Error bars represent the 95% confidence limit calculated by probit analysis. As LC_{50} values exceeded 5,000 mg l^{-1} for all CHS1_R1, R2 and R3 for each mite growth inhibitor tested, only one bar depicts LC_{50}s. Stars indicate lines for which, LC_{50} value exceeded 5,000 mg l^{-1}. Letters above bars indicate lines where acaricide treatment had statistically the same (a) or different (b) effect comparing to Wasatch (PoloPlus LeOra Software) (Robertson, 1992).

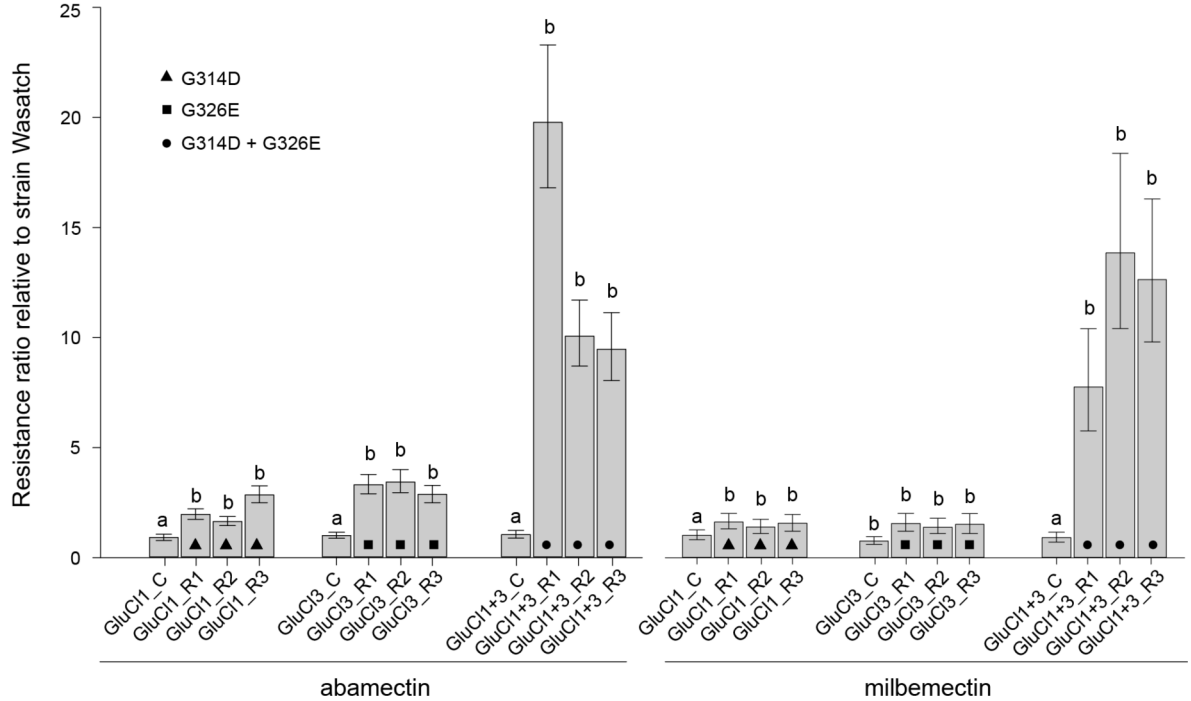

FIGURE 4.4 – Susceptibility levels of backcrossed *Tetranychus urticae* lines GluCl1_R1-R3 (G314D), GluCl1_C, GluCl3_R1-R3 (G326E), GluCl3_C, GluCl1+3_R1-R3 (G314D+G326E), GluCl1+3_C to abamectin and milbemectin. The RRs were calculated as the LC_{50} values of the backcrossed lines divided by the LC_{50} of the parental susceptible strain Wasatch. Error bars represent the 95% confidence limit calculated by probit analysis. Letters above bars indicate lines where acaricide treatment had statistically the same (a) or different (b) effect comparing to Wasatch (PoloPlus LeOra Software) (Robertson, 1992).

Mitochondrial QoI

The backcrossed strains cytb_R1, R2 and R3 carrying the P262T mutation in cytb sequence showed high resistance levels to bifenazate (FIGURE 4.2, TABLE S4.3). Interestingly, the combination of cytb substitutions; G126S and S141F provided higher level of resistance to bifenazate compared to P262T, since RRs for both cytb_R4 and R5 were higher than 2,000 fold. The importance of G126S and S141F in the observed levels of cross-resistance to acequinocyl in BR-VL was confirmed (FIGURE 4.2, TABLE S4.3).

Mite growth inhibitors

The backcrossed strains homozygous for I1017F mutation displayed extreme levels of resistance to all three mite growth inhibitors tested (FIGURE 4.3, TABLE S4.4). RRs estimated for etoxazole, hexythiazox and clofentezine exceeded 40,000, 4,000 and 2,000 fold, respectively (FIGURE 4.3, TABLE S4.4). In contrast, the backcrossed control line was highly susceptible to the aforementioned compounds (FIGURE 4.3, TABLE S4.4).

4.4. DISCUSSION

Field collected *T. urticae* strains often exhibit very high levels of resistance to multiple acaricides used for their control. Due to the identification of acaricide target-site sequences (Grbic et al., 2011; Van Leeuwen et al., 2013) and implementation of recently developed genetic mapping tools (Bajda et al., 2017; Van Leeuwen et al., 2012; Van Leeuwen and Dermauw, 2016), a number of mutations has been uncovered in the target-site of frequently used acaricides. However, to what extent these mutations determine the resistant phenotype is mostly unknown. Resistant field strains investigated so far, typically display a broad altered transcriptional response with the putative involvement of many detoxifying enzymes and transporters that might affect acaricide toxicity (Demaeght et al., 2013; Dermauw et al., 2013; Khalighi et al., 2016). Crossing experiments have revealed that a complex genetic make-up typically underlies resistance, implying the additive effect of multiple mechanisms (Dermauw et al., 2012; Van Pottelberge et al., 2009b,c). Moreover, the extent by which resistant alleles confer resistance can also vary according to the genetic background in which they are expressed (McKenzie et al., 1982; Schrag et al., 1997).

Several studies have used congenic backcrossed lines to assess insecticide related fitness cost/advantage and pleiotropic effects (Arnaud et al., 2002; ffrench-Constant and Bass, 2017; Helle, 1962; Wang and Wu, 2014; Xiao et al., 2017; Yuan et al., 2017). By substituting phenotypic selection with molecular marker-assisted backcrossing, the potential accumulation of alleles with additive effect can be uncoupled (Roush and Mckenzie, 1987). Such a setup has been previously used to assess the effects of *Aedes aegypti* kdr mutations on pyrethroid resistance and its fitness cost (Brito et al., 2013) and recently, to investigate resistance levels to METI-I acaricides caused by a mutation in the PSST subunit of complex I in *T. urticae* (Bajda et al., 2017).

Here, we analyzed the relative phenotypic contribution of target-site resistance mutations, previously uncovered in highly resistant *T. urticae* field populations. We adopted a marker-assisted backcrossing procedure described in Bajda et al. (2017) to untangle the target-site resistance loci from potential complex additive genetic mechanisms. Although in this study we cannot exclude a possible effect of closely linked loci (Hospital, 2001), previous research involving resistance gene mapping by means of bulk segregant analysis, revealed a high recombination rate in *T. urticae* (Demaeght et al., 2014; Van Leeuwen et al., 2012) which makes us believe that the procedure performed here, resulted in near-isogenic lines.

Both abamectin and milbemectin resistance has been reported frequently in spider mite populations worldwide (Nicastro et al., 2010; Sato et al., 2005; Yorulmaz and Ay, 2009) exhibiting >1000 fold resistance in some cases(Dermauw et al., 2012). These molecules target both GluCls and GABA gated chloride channels (GABACl), although GluCls are considered the main target (Clark et al., 1994; Wolstenholme, 2010). In contrast to insects with a single copy, the genome of *T. urticae* harbors six orthologous GluCl genes (Dermauw et al., 2012). Two non-synonymous mutations have been associated with resistance to abamectin, the G314D in GluCl1 and G326E in GluCl3 (Dermauw et al., 2012; Kwon et al., 2010b). When G314D and G326E were introgressed separately, only low levels of resistance remained. However, when both mutations were joined by dedicated crosses, resistance levels increased to 10-20 fold (henceforth, for the schematic visualization of a relative contribution of the mutations in resistance levels, please consult FIGURE 4.5). These resistance levels are comparable with a previous study, where an abamectin resistant strain homozygous for both GluCl mutations was investigated. Resistance levels in that strain reached only 20-fold (Kwon et al., 2010b, 2015), suggesting that target-site mutations were the only factor contributing to resistance. A possible explanation for the relatively low resistance levels conferred by the combination of two GluCl mutations may lie in the number of genes involved in channel assembly. Glutamate-gated chloride channels typically consist of five subunits, which in *T. urticae* can be encoded by 5 different GluCl genes. Hence, if the channel consists of a combination of subunits carrying the resistance associated substitution (GluCl1 and/or GluCl3) and a GluCl2 subunit (GluCl2 does not carry a resistance associated substitution, while GluCl4 and GluCl5 naturally carry substitutions that interfere with abamectin binding; see Dermauw et al., 2012), abamectin binding might still be possible. In addition, we cannot exclude the possibility of heteromeric channel assembly, consisting of GluCls and GABACl (Cully et

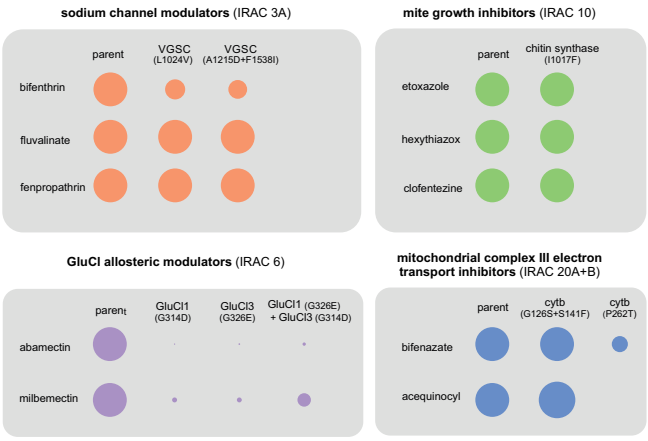

FIGURE 4.5 – Schematic representation of the relative contribution of target-site resistance mutations in overall resistance levels to acaricides belonging to different mode of action. The size of the circle shape reflects the observed levels of resistance (RR vs susceptible parent strain). Only comparisons between the backcrossed lines versus its resistant parent ('parent') are drawn to scale (TABLE 4.1).

al., 1994; Ludmerer et al., 2002). In such case, the existence of mutations in GluCl1 and GluCl3 alone would also not be capable to fully prevent channel blocking. Consequently, our results also reconfirm the importance of additional mechanisms in abamectin resistance (Clark et al., 1994; Pavlidi et al., 2015; Riga et al., 2014). Studies with synergists and biochemical tests have previously implied the involvement of detoxification enzymes in resistance in many field collected strains worldwide (Campos et al., 1996; Pavlidi et al., 2015; Stumpf and Nauen, 2002). For instance, a P450 (CYP392A16) was reported to be overexpressed in abamectin resistant strains and detoxifies abamectin rapidly (Riga et al., 2014). Therefore very high abamectin resistance levels in the MAR-AB strain (TABLE S4.2) may be attributed to a joint action of P450 detoxification and decreased sensitivity of the target-site, potentially even acting synergistically.

Milbemectin belongs to the same insecticidal class as abamectin and acts on the same target-site. Whether cross-resistance might occur between both compounds is therefore of crucial importance, and still a matter of debate. Here, we show that the combination of both GluCl mutations confers resistance levels of about 10-fold, indicating potential cross-resistance risks between milbemectin and abamectin, as has been previously suggested (Nicastro et al., 2010; Sato et al., 2005).

Pyrethroid resistance has been documented globally in *T. urticae* with resistance levels exceeding 10,000 folds in some cases (Herron et al., 2001; Van Leeuwen et al., 2005). Unlike most other arthropods, spider mites have mutations in unique positions on VGSC (Ding et al., 2015; Kwon et al., 2010a; Tsagkarakou et al., 2009), instead of the known kdr (L1014F) and super-kdr (M918T) mutations (*Musca domestica* numbering). The super-kdr mutation has been identified only once in a *Tetranychus evansi* strain (Nyoni et al., 2011). Three point mutations have been located in the sodium channel of spider mites, L1024V and F1538I in combination with A1215D (Kwon et al., 2010a; Tsagkarakou et al., 2009). Backcrossing experiments indicated the major effect of both L1024V and A1215D + F1538I mutations in pyrethroid resistance (FIGURE 4.5). Interestingly, the KOP8 strain has the A1215D mutation uncoupled from F1538I and is susceptible to all pyrethroids, indicating that the mutation alone has no effect on pyrethroid toxicity. So far, the mutation F1538I has been studied most thoroughly and its effect in resistance to pyrethroids has been confirmed by electrophysiological studies (Tan et al., 2005). Here, we showed that both L1024V and A1215D + F1538I mutations confer high resistance levels to all pyrethroid compounds, irrespectively of their type, i.e. presence of α-cyano group and/ or extended halogenated acidic moiety, suggesting that the sodium channel mutations can cause field failure of the pyrethroids.

QoI with acaricidal properties have been introduced for the control of mite infestations relatively recently (Dekeyser, 1996; Grosscurt and Avella, 2005). Nonetheless, high levels of resistance to bifenazate have already been reported in the field (Van Leeuwen et al., 2008; Van Nieuwenhuyse et al., 2009). Backcrossing revealed that the combination of cd1 helix mutations G126S and S141F in cytb confers high levels of bifenazate resistance. In contrast, the backcrossed lines carrying the P262T substitution, showed LC_{50} values of 960, 1,400.6 and 886.1 mg l^{-1}, respectively, while the parental resistant HOL3 was resistant to bifenazate concentrations up to 5,000 mg l^{-1} (FIGURE 4.5). The backcrossed lines with cd1 helix mutations, showed similar levels of resistance to acequinocyl compared to the parental strain, confirming that acequinocyl cross-resistance is completely maternally inherited, and thus linked with the mutation (Van Nieuwenhuyse et al., 2009). One of the possible explanations for the discrepancy between bifenazate resistance of the parental strain HOL3 and those of the backcrossed strains is the presence of additional resistance mechanisms. Indeed, although a strong correlation between the P262T frequency and bifenazate resistance has been reported, to what extent resistance is inherited maternally has only been described for acequinocyl (Van Nieuwenhuyse et al., 2009). This is in contrast to cd1 helix mutations, that have been shown to confer resistance levels that are completely maternally inherited (Van Leeuwen et al., 2006), suggesting that no additional mechanisms are involved or needed to attain very high resistance levels. Another explanation could be that the P262T substitution only confers high resistance levels in combination with specific nuclear encoded protein variants that co-evolved with mitochondrial encoded cytb mutations, and that uncoupling results in a loss of phenotype.

Resistance to clofentezine and hexythiazox has been frequently reported, and more recently, resistance against etoxazole has been also spreading (Herron et al., 1993; Kobayashi et al., 2001; Uesugi et al., 2002). Insensitivity to etoxazole is thought to be monogenic and recessive (Kobayashi et al., 2001; Uesugi et al., 2002), which is in line with target-site resistance as the main mechanism. Screening for I1017F revealed that the mutation has been segregating in populations from different regions of the world for a long period, although etoxazole has been only recently used to control spider mites, especially in Europe (Van Leeuwen et al., 2012). This lead to the hypothesis that the mutation was selected by other molecules such as hexythiazox or clofentezine, which was later confirmed in a follow-up genetic mapping study (Asahara et al., 2008; Demaeght et al., 2014). Here, we provide clear evidence that the I1017F substitution confers very high levels of resistance to etoxazole, hexythiazox and clofentezine (FIGURE 4.5). Our results confirm target-site based cross-resistance, despite the fact that the three mite growth inhibitors belong to chemically diverse classes (Aveyard et al., 1986; Ishida et al., 1994; Yamada et al., 1987). In a recent study, Douris et al. (2016) found a mutation (I1042M) in CHS1 gene of *Plutella xylostella* resistant to benzoylureas (BPUs), at the position corresponding to I1017F in *T. urticae*. Using the CRISPR-Cas9 approach coupled with homology directed repair (HDR), both the lepidopteran and spider mite mutations (I1056M/F) were introduced in the *Drosophila CHS1* gene (kkv). Flies carrying either of two mutations were found highly resistant to etoxazole, but also to a number of BPUs and the hemipteran chitin biosynthesis inhibitor buprofezin. The study, together with the results reported here, provide convincing evidence that chitin synthesis inhibitors BPUs, buprofezin and mite growth inhibitors, etoxazole, hexythiazox and clofentezin all directly interact with CHS1 and share a similar molecular MoA.

4.5. CONCLUSIONS

Resistance mechanisms in insects and mites can be complex and the relative strength of target-site mutations in resistance phenotypes is often not well known. Here, we have used a marker-assisted backcrossing approach to look at the phenotypic effect of the main and currently relevant target-site mutations reported to confer resistance to abamectin, pyrethroids, mite growth in-

hibitors and QoI. Mutations in VGSC, CHS and cytb confer high levels of resistance and their presence in populations alone is enough to cause field failure after acaricide treatment. In contrast, although we confirmed the functional importance of GluCl mutations and the cumulative effect of mutations in multiple channels, mutations in only two channels genes does not lead to the high resistance levels that have been reported for abamectin resistance. Overall, our results functionally validate the importance of mutations that have been inferred from correlation analysis and genetic mapping.

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TABLE S4.1 – Primers and probes used in this study

TABLE S4.2 – Toxicity of abamectin and milbemectin to adult females of backcrossed lines GluCl1_C (G314/G314), GluCl1_R1-3 (G314D/G314D), GluCl3_C (G326/G326), GluCl3_R1-3 (G326E/G326E), GluCl1/3_C (G314/G314; G326/G326), GluCl1/3_R1-R3 (G314D/G314D; G326E/G326E) and their parental strain (Wasatch, MAR-AB). [a]Number of the mites used in toxicity tests. [b]Resistance ratio compared to Wasatch. a: Treatment effect was significantly different when compared to Wasatch

TABLE S4.3 – Toxicity of bifenazate to adult females of back-crossed lines Cytb_R4-5 (G126S+ S141F/G126S+S141F) and Cytb_R1-R3 (P262T/P262T) and toxicity of acequinocyl to Cytb_R4-5, supplemented with toxicity data for both acaricides versus parental resistant strain BR-VL and bifenazate versus parental resistant strain HOL3. [a]Number of the mites used in toxicity tests. [b]Resistance ratio compared to Wasatch. a: Treatment effect was significantly different when compared to Wasatch

TABLE S4.4 – Toxicity of etoxazole, hexythiazox and clofentezine to larvae of *Tetranychus urticae* of back- crossed lines CHS1_C (I1017/I1017 genotype), CHS1_R1-R3 (I1017F/I1017F genotype), and their parental strains (Wasatch and EtoxR). [a]Number of larvae used in toxicity tests with etoxazole and hexythiazox or number of eggs used in toxicity tests with clofentezine. [b]Resistance ratio compared to Wasatch. a: Treatment effect was significantly different when compared to Wasatch

5

Key point mutations that underlie target-site resistance in the two-spotted spider mite, *Tetranychus urticae*, reveal only limited fitness costs

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Abstract. The frequency of insecticide/acaricide target-site resistance is increasing in arthropod pest populations and is typically underpinned by single point mutations that affect the binding strength between the insecticide/acaricide and its target-site. Theory predicts that although resistance mutations clearly have advantageous effects when selection pressure is present, they might convey negative pleiotropic effects on other aspects of fitness. Resistance due to *de novo* mutations is thus likely to disappear in the absence of insecticide/acaricide treatment if such fitness costs are in place, a process that would counteract the spread of resistance in agricultural crops. Unfortunately, most studies that associate target-site mutations to pleiotropic effects on arthropod fitness are hampered by the confounding different genetic backgrounds of the strains under study. Here, we used near-isogenic lines of the spider mite pest *Tetranychus urticae* that carry well-characterized acaricide target-site resistance mutations to quantify potential fitness costs. Specifically, we analyzed P262T in the mitochondrial cytochrome b, the combined G314D and G326E substitutions in the glutamate-gated chloride channels, L1024V in the voltage gated sodium channel, and I1017F in chitin synthase 1. Five fertility lifetable parameters and six single-generation life history traits were quantified and compared across a total of 15 mite lines. In addition, we monitored the temporal resistance level dynamics of populations with different starting frequency levels of the chitin synthase resistant allele to further support our findings. Only three target-site resistance mutations, I1017F and the co-occurring G314D and G326E mutations, were shown to significantly and consistently alter certain fitness parameters in *T. urticae*. The other two mutations, P262T and L1024V did not result in any consistent change in fitness parameter analyzed in our study. Our findings are discussed in the context of the global spread of *T. urticae* pesticide resistance within an integrated pest management context.

5.1. INTRODUCTION

Control of arthropod pests in agriculture remains heavily dependent on the application of insecticides and acaricides, a practice that has resulted in the current widespread occurrence of resistant populations of more than 500 arthropod species (Sparks and Nauen, 2015). Resistance of an arthropod population to a pesticide is typically defined as the heritable decrease in the susceptibility to the sprayed pesticide that leads to inadequate field control (http://www.irac-online.org/about/resistance/). Adaptation to xenobiotics, including insecticides and acaricides, is mainly attributed to an elevated activity of detoxification enzymes (toxicokinetic change) and/or a reduced binding strength of the xenobiotic to its target-site (toxicodynamic change). Within the latter mechanism, non-synonymous point mutations in the sequence coding for the pesticide target-site often result in a reduced binding strength of the compound (Feyereisen et al., 2015). Theory predicts that *de novo* point mutations in essential target-genes can have pleiotropic effects on the individual, meaning it could affect other phenotypic traits in addition to pesticide resistance (Crow, 1957; ffrench-Constant and Bass, 2017; Fisher, 1999). Indeed, these point mutations may, for instance, impose a structural constraint and put the conserved protein function and therefore the arthropod's fitness at a disadvantage. Pesticide resistance could thus result in fitness costs in populations living in a pesticide-free environment. As a consequence, alleviating pesticide use could result in a lower frequency of target-site resistance-alleles and, in turn, a lower resistance level of the population (Crow, 1957; Georghiou and Taylor, 1977). Target-site resistance alleles can, however, be maintained in the population through several mechanisms. The resistance fitness cost may be negligible or compensated via additional mutation. These additional mutations, or fitness modifiers, restore fitness to its original level and have been well studied in antibiotic-resistant bacteria (Björkman et al., 2000; Comas et al., 2012; Gagneux et al., 2006; Levin et al., 2000). A notable example of a fitness modifier within arthropods has been found in the Australian blow fly, *Lucilia cuprina,* where the scalloped wings gene *ScI* is a likely candidate for the fitness and wing asymmetry modifier in the diazinon-resistant flies (Davies et al., 1996). Alternatively, in theory, the resistance locus can be linked to a locus that confers resistance to another kind of insecticide to which the population continues to be exposed. The non-selected resistance locus can thus persist by mere linkage disequilibrium.

Experimental verification whether the mutations that underlie insecticide/acaricide resistance indeed carry fitness costs typically relies on two methodologies (Roush and Daly, 1990). The first method investigates various single generation life history parameters. However, here the cost of a causal resistance mutation can easily be missed in experimental designs that only look at a specific fitness component. Indeed, population growth depends on a multitude of interdependent life history traits and their cumulative effect on the population dynamics can only be estimated via complex parameters such as fertility life table parameters (FLT) (Roush and McKenzie, 1987). The second approach, although not always performed in cages is often referred to as 'population cage' experiment because of its analogy to the traditional cage studies investigating *Drosophila melanogaster* genetics, analyzes fitness differences by placing resistant and susceptible genotypes in direct competition (Moore, 1952). These inter-genotype competition experiments are run in absence of pesticide exposure and allow to track the frequency of resistance alleles (or resistance itself) over multiple generations.

Most previous studies that assess pesticide resistance-related fitness costs, suffer from multiple design weaknesses [see reviews by ffrench-Constant and Bass (2017) and Kliot and Ghanim (2012)], such as evaluating genetically unrelated populations in the experimental set-up. The different genetic background and adaptive variations in life history traits across such populations hamper any reliable claim of a causal effect of the point mutation of interest to the observed differences in population growth dynamics (Raymond et al., 2011; The Anopheles gambiae 1000 Genomes Consortium, 2017; Varzandeh et al., 1954). An elegant solution to overcome this experimental limitation is to backcross the target-site mutation of interest into a susceptible genomic background over multiple generations, hereby generating near-isogenic lines. This procedure maximizes the chance that the observed difference in population growth is caused by the target-site mutation under investigation (Bajda et al., 2017; Brito et al., 2013; Riga et al., 2017). Unfortunately, the biological characteristics of many insect and mite pests render the generation of near-isogenic lines extremely difficult and time consuming.

The two-spotted spider mite, *Tetranychus urticae* (Chelicerata: Acari: Tetranychidae), is one of the most notorious agricultural arthropod pests worldwide. *Tetranychus urticae* infests a wide range of different plant species (>1,000), of which many are economically important crops (Jeppson et al., 1975; Migeon and Dorkeld, 2006-2017). Control of *T. urticae* populations is mainly accomplished by acaricide application and has led to a record number of populations resistant to pesticides with varying modes of action (Van Leeuwen and Dermauw, 2016; Van Leeuwen et al., 2010). Target-site resistance has been widely reported in *T. urticae* field populations and, facilitated by available genomic resources, a number of point mutations conferring acaricide target-site resistance has been uncovered. Recently, Riga et al. (2017) investigated to what extent point mutations in a set of highly conserved acaricide target-sites underlie the high resistance levels observed in the respective *T. urticae* field populations. Eight point mutations within four conserved genes were back-crossed in a susceptible genomic background by marker-assisted back-crossing. The study revealed that the presence of target-site mutations in the highly conserved chitin synthase 1 (CHS1; I1017F), voltage gated sodium channel (VGSC; L1024V, F1538I) and mitochondrial cytochrome b (cytb; S141F + G126S, P262T) is sufficient to attain very high resistance levels (without the need of additive effects conferred by other genetic mechanisms). In contrast, the presence of the mutations in glutamate-gated chloride channels 1 and 3 (GluCl1; G314D and GluCl3; G326E) in a susceptible genotype results in lower resistance levels when compared the resistant field populations (Riga et al., 2017), although *in vitro* work established that the G326E mutation in single receptor genes abolishes the antagonistic interactions of macrocyclic lactones (Mermans et al., 2017).

In the current study, we further take advantage of this set of isogenic lines to evaluate the potential fitness costs associated with 5 target-site resistance mutations. Specifically, we focused on P262T in the mitochondrial *cyth* associated with bifenazate resistance (Van Leeuwen et al., 2008), the combined effect of G314D and G326E in the *GluCl* channels associated with avermectin resistance (Dermauw et al., 2012; Kwon et al., 2010b), L1024V in the *VGSC* associated with pyrethroid resistance

(Kwon et al., 2010a), and I1017F in *CHS1* associated with resistance to etoxazole, clofentezine, and hexythiazox (Demaeght et al., 2014; Van Leeuwen et al., 2012). Five FLT parameters: the net reproductive rate (R0), intrinsic rate of increase (rm), mean generation time (T), the finite rate of increase (LM), and the doubling time (DT) as well as six single-generation life history traits: immature stage survivorship, developmental time, sex-ratio, adult longevity, daily and total fecundity, were analyzed and compared across a total of 15 lines. In addition, we monitored the temporal resistance level dynamics of populations with different starting frequencies of the *CHS1* resistant allele to further support our findings. Our results uncover a limited effect of target-site resistance mutations on population growth in the *T. urticae* pest and help to understand and predict the occurrence and spread of *T. urticae* pesticide resistance within an integrated pest management context.

5.2. Materials and Methods

5.2.1. Spider mite strains

A total of 15 *T. urticae* near isogenic lines were used in this study. The near isogenic lines were generated in a previous study using a marker-assisted backcrossing technique (Bajda et al., 2017; Riga et al., 2017). Briefly, these were lines that carry introgressed nucleus-encoded nonsynonymous mutations in *CHS1* gene (CHS1_R1-R3), *VGSC* gene (VGSC_R2 and R3), *GluCl* genes (GluCl1+3_R1-3), and their congenic susceptible controls (CHS1_C, VGSC_C1 and GluCl1+3_C, respectively) as well as lines carrying an introgressed mitochondrial-encoded mutation in *cytb* gene (cytb_R1-R3). The congenic susceptible control for the latter mutation was the susceptible Wasatch strain. An overview of these lines is shown in Table 5.1. All *T. urticae* strains were maintained on detached 3-week-old kidney bean leaves (*Phaseolus vulgaris* L.) on wet cotton wool inside lid-covered plastic trays. Except when analyzing temporal etoxazole resistance dynamics, all mite cultures and experiments were kept under laboratory conditions at 25 ± 1°C, 60% relative humidity, and 16:8 h light: dark photoperiod.

5.2.2. Total development time, sex ratio, and immature stage survivorship (ISS)

From stock cultures of each of the backcrossed lines, four replicates of 100 adult female mites were randomly chosen and placed on a feeding arena (one 3-week-old bean leaf, lined with tissue paper to prevent escape). Mites were allowed to lay eggs for 4-5 h and were subsequently moved to a new feeding arena for another 4-5 h. The amount of eggs laid per plate was recorded for immature stage survivorship (ISS) calculations. ISS was defined as the fraction of females reaching adulthood. On the eighth day after egg laying, mite development was followed every 12 h. Eclosion was timed and adult individuals were sexed.

5.2.3. Oviposition and adult longevity

Thirty-five single-pair crosses were established per line by placing a female teliochrysalis with an adult male on a leaf disk of 3 cm^2. Disk edges were lined with tissue paper to prevent mites from escaping. Once females reached adulthood, each pair was transferred daily to a fresh leaf disk. Males that did not survive for 2 days after their female partner reached adulthood, were replaced. The ovipositional period was divided in three parts: pre-oviposition, oviposition, and post-oviposition. The pre-oviposition period was determined as the time spanning between adult female emergence and the first egg and was estimated from observations taken every 12 h. The oviposition period was defined as the time between the first and last day of egg-laying. The post-oviposition period was measured from the day when no more eggs were deposited for a given female, until her death. Oviposition and the subsequent post-oviposition periods were monitored on a daily basis until the death of each female. Data from females that died due to experimental manipulation were excluded from further analysis.

5.2.4. Temporal dynamics of etoxazole resistance

Etoxazole resistance was evaluated monthly for a period of 9 months for three *T. urticae* populations originating from a cross CHS1_R2 x CHS1_C, with different initial proportions of etoxazole-resistant and -susceptible mites: R70: 70/30, R50: 50/50, and R30: 30/70. Two hundred adult females were placed as the founding population and maintained on potted non-sprayed bean plants in Panasonic climate chamber MLR-352H-PE, at 28°C with a photoperiod of 16:8 h light:dark. To assess etoxazole resistance over time, larval bioassays were performed as previously described by Van Pottelberge et al. (2009). Briefly, 50 adult females were allowed to lay eggs for 5 h on the upper side of 9 cm^2 square-cut bean leaf discs on wet cotton wool. Larvae were sprayed with 1 ml of etoxazole (100 mg l^{-1}) at 1 bar pressure with a Potter Spray Tower (Burkard Scientific, UK) (2 mg/ cm^2 deposit). A commercial formulation of etoxazole (Borneo, 120 g l^{-1} SC) was used in this study. The discriminating etoxazole concentration is based on previously obtained mortality data (Van Leeuwen et al., 2012). Mortality was assessed after 48 h. Here, mites that displayed the same developmental stage as the water-treated control were con-

Table 5.1 – Characteristics of the *Tetranychus urticae* near-isogenic lines used in this study. The VGSC mutation was numbered according to *Musca domestica* numbering, whereas substitutions in GluCl1, GluCl3, cytb, and CHS1 followed *T. urticae* numbering. IRAC mode of action group number is shown between brackets.

Gene	Near-isogenic line/susceptible strain*	Mutation	Target-site resistance to
CHS1	CHS1_C	-	-
	CHS1_R1, R2, R3	I1017F	Mite growth inhibitors (10)
Cytb	wasatch	-	-
	cytb_R1, R2, R3	P262T	Bifenazate (20A)
VGSC	VGSC _C1	-	-
	VGSC _R2, R3	L1024V	Pyrethroids (3A)
GluCl1 and GluCl3	GluCl1+3_C,	-	-
	GluCl1+3_R1, R2, R3	G314D + G326E	Avermectins (6)

*Lines carrying mutation in *cytb*, were compared to the susceptible strain Wasatch.

sidered as unaffected. All mortalities obtained from control treatment were lower than 10%. At each time point, the resistance level of the three populations was assessed using 3-4 replicates of approximately 50 larvae on each.

5.2.5. Statistical analysis

ISS and sex ratio are categorical variables and were analyzed with logistic regression using the *glm* function with a binomial error distribution (package *stats*) (Nelder, 1974).

Female longevity, total and daily fecundity, and duration of pre-oviposition, oviposition, and post-oviposition periods were analyzed in a linear model using *lm* (package *stats*). To describe differences in developmental time (defined as the time required for 50% ≤ of individuals to reach adulthood), a linear mixed effect model was initially run (function *lme* from package *nlme*) where 'line' was a fixed effect and the 'time block' (representing the two feeding arenas upon which replicates were allowed to lay eggs) were considered as a random effect. As determined by the *anova* function from package *stats*, the random effect of time block was insignificant, hence data was finally analyzed with a linear model using *lm,* where every plate was treated as an independent biological replicate. Post hoc Dunnett test was subsequently carried out to perform multiple comparisons with the control line, using the *glht* function in the package *multcomp* (adjusted p-value <0.05) (Westfall et al., 2011).

When normality or homogeneity of variance was not met, a non-parametric Kruskal-Wallis test was performed (adjusted p-value <0.05). Pairwise comparisons, using Dunn's-test for multiple comparisons with one control, were done with *dunn.test.control* function from the *PMCMR* package (adjusted p-value <0.05) (Dunn, 1964).

To test for significant monotonic trends in the frequency of etoxazole-resistant homozygotes, a Mann–Kendall test was performed using package *Kendall* (Hipel and McLeod, 1994). Under the assumption of Hardy-Weinberg equilibrium, it is expected that allele and genotype, and therefore phenotype, frequencies will remain relatively stable in our experimental set-up. Data included in the analysis spanned period between the first month (expected to represent the stable frequency of genotypes at Hardy–Weinberg equilibrium) and the ninth month, when the experiment was terminated.

The analysis of life history raw data of the different lines was based on the lifetable R script (Maia et al., 2014). Specifically, line name, female ID, age and number of eggs laid per female at each oviposition date, proportion of females in the offspring, and ISS were used as input. The intrinsic rate of increase (*rm*) was calculated with the equation

$$\sum_{x=x_0}^{\Omega g} e^{-rm} lxmx = 1$$

where *lx* is the proportion of females surviving to age *x* and *mx* is the mean number of female progeny per adult female at age *x*. The net reproductive rate or mean number of daughters produced per female was calculated from

$$R0 = \sum_{x=x_0}^{\Omega g} lxmx$$

and the mean generation time from T = ln(R0)/rm. The finite rate of increase and doubling time were inferred from equations LM = e^{rm} and DT = ln2/rm, respectively. Variance for the FLT parameters was estimated with Jackknife resampling method (Quenouille, 1956). Since the Jackknife method is an asymptotic procedure sensitive to highly skewed distribution (Maia et al., 2000), prior the analysis, dataset's symmetry was measured with function *skewness* from package *moments* (Sheskin, 2011). Subsequently, mean Jackknife values and their standard errors (SE) were calculated for the five FLT parameters (Meyer et al., 1986). Mean jackknife values for lines carrying mutations were then compared to the control line using Dunnett test (adjusted p-value <0.05). Statistical analysis was conducted within the R framework [version 3.1.2 (RCoreTeam, 2014)].

5.3. RESULTS

5.3.1. Development time, sex ratio, and ISS

The three lines CHS1_R1-R3 exhibited a significantly longer median developmental time than their susceptible counterpart (CHS1_C). Both males and females of CHS1_C matured on average between 12 and 24 h earlier than those of CHS1_R1-R3 (TABLES 5.2 and S5.1, FIGURE 5.1). There was no significant difference in male and female emergence time between lines VGSC_R2, VGSC_R3, and their susceptible control (TABLES 5.2 and S5.1). Males of the lines cytb_R1, R2,R3 did not differ from the susceptible control, while females of the cytb_R2 and R3 lines emerged significantly later than those of the control strain Wasatch (TABLES 5.2 and S5.1). Males and females of lines GluCl1+3_R2 and GluCl1+3_R3 had a significantly longer total developmental time when compared with the susceptible reference line (GluCl1+3_C) (TABLES 5.2 and S5.1).

Values of ISS and the ratio of females in the offspring for line CHS1_R2, differed significantly from line CHS1_C (TABLES 5.2 and S5.1). VGSC_R2 had a significantly higher ISS and proportion of females in surviving progeny than VGSC_C1 (TABLES 5.2 and S5.1). The ISS and female ratio were significantly higher in cytb_R1 and R2 in comparison to Wasatch (TABLES 5.2 and S5.1). GluCl1+3_C showed significantly higher ISS values than the lines GluCl1+3_R1, R2, R3. Additionally, lines GluCl1+3_R1 and R3 were characterized by a significantly lower female ratio when compared to the control line. Line GluCl1+3_R1 exhibited an inverted sex ratio compared to the all other lines, with only 43% females in the progeny (TABLES 5.2 and S5.1).

5.3.2. Fecundity and adult longevity

Females of CHS1_R2 lived significantly longer than females of the susceptible control (CHS1_C) (FIGURE 5.2A, TABLES 5.3 and S5.1). Congenic lines VGSC_R2,3 and the susceptible control VGSC_C1 did not vary significantly in their life span (FIGURE 5.2B, TABLES 5.3 and S5.1). Similarly, there was no difference in longevity between the three cytb_R1,2,3 lines and susceptible Wasatch (FIGURE 5.2C, TABLES 5.3 and S5.1). Lastly, lines GluCl1+3_R1-3 and GluCl1+3_C did not differ significantly in their life span (FIGURE 5.2D, TABLES 5.3 and S5.1).

There was no significant difference between CHS1_C and CHS1_R1-3 in daily egg laying within the oviposition period per female. However, line CHS1_R2 showed a significantly higher total fecundity per female compared to CHS1_C (FIGURE 5.2E, TABLES 5.2 and S5.1). Lines VGSC_C1 and VGSC_R2,3 did not differ significantly in their daily and total fecundity (FIGURE 5.2F, TABLES 5.2 and S5.1). The cytb_R2 line exhibited a lower daily

TABLE 5.2 – Mean values ± SE of developmental time, immature stage survivorship (ISS), offspring sex ratio (FR), daily (FFD), and total (TF) fecundity in *Tetranychus urticae* near-isogenic lines and Wasatch.

Target-site	Line	N*	Developmental time ± SE		ISS ± SE	FR ± SE	FFD ± SE	TF ± SE
			Male	Female				
CHS1 (wt)	CHS1_C	906	**9.32 ± 0.12a**	**9.68 ± 0.08a**	0.59 ± 0.04a	0.65 ± 0.05a	6.28 ± 0.32a	91.03 ± 8.86a
CHS1 (I1017F)	CHS1_R1	710	**9.90 ± 0.06b**	**10.38 ± 0.08b**	0.63 ± 0.05a	0.67 ± 0.05a	5.52 ± 0.30a	105.41 ± 10.26a
	CHS1_R2	752	**9.96 ± 0.15b**	**10.39 ± 0.17b**	0.64 ± 0.05b	0.72 ± 0.05b	5.87 ± 0.30a	128.06 ± 10.42b
	CHS1_R3	1132	**9.93 ± 0.09b**	**10.36 ± 0.09b**	0.58 ± 0.04a	0.65 ± 0.04a	6.57 ± 0.58a	86.06 ± 7.27a
VGSC (wt)	VGSC_C	969	10.95 ± 0.23a	11.45 ± 0.20a	0.66 ± 0.03a	0.67 ± 0.01a	8.06 ± 0.21a	101.58 ± 6.33a
VGSC (L1024V)	VGSC_R2	1157	10.89 ± 0.16a	11.20 ± 0.18a	0.72 ± 0.02b	0.75 ± 0.03b	7.66 ± 0.20a	94.88 ± 5.69a
	VGSC_R3	1461	11.0 ± 0.06a	11.63 ± 0.07a	0.66 ± 0.03a	0.69 ± 0.03a	8.12 ± 0.30a	93.87 ± 6.76a
Cytb (wt)	Wasatch	1408	9.67 ± 0.38a	10.17 ± 0.38a	0.60 ± 0.04a	0.66 ± 0.04a	5.53 ± 0.28a	92.64 ± 7.85a
Cytb (P262T)	cytb_R1	1498	9.92 ± 0.46a	10.35 ± 0.50a	0.69 ± 0.04b	0.75 ± 0.03b	6.42 ± 0.28a	113.09 ± 8.04a
	cytb_R2	1078	10.17 ± 0.38a	10.73 ± 0.37b	0.63 ± 0.04b	0.76 ± 0.04b	4.42 ± 0.27b	71.46 ± 8.13a
	cytb_R3	1132	9.98 ± 0.18a	10.79 ± 0.23b	0.61 ± 0.04a	0.65 ± 0.04a	5.04 ± 0.19a	94.66 ± 6.45a
GluCl1 + 3(wt)	GluCl1+3_C	1124	9.78 ± 0.09a	10.16 ± 0.06a	**0.77 ± 0.03a**	0.77 ± 0.03a	**8.32 ± 0.30a**	123.09 ± 9.64a
GluCl1 +3 (G314D + G326E)	GluCl1+3_R1	1890	10.03 ± 0.06a	10.28 ± 0.06a	**0.35 ± 0.02b**	0.43 ± 0.03b	**7.22 ± 0.34b**	93.61 ± 9.12a
	GluCl1+3_R2	1244	10.29 ± 0.12b	10.48 ± 0.10b	**0.72 ± 0.03b**	0.76 ± 0.03a	**6.66 ± 0.36b**	105.81 ± 10.32a
	GluCl1+3_R3	1194	10.23 ± 0.06b	10.42 ± 0.06b	**0.66 ± 0.03b**	0.67 ± 0.03b	**7.33 ± 0.34b**	120.10 ± 10.47a

Development time (d): time required for 50% ≤ males and females to emerge, TF: eggs/female, FFD: eggs/female/day, within oviposition period, ISS: % females reaching adulthood, FR: % females in the progeny. Means followed by the letter 'a' within a column are not, while means followed by the letter 'b' are, significantly different from the control line (adjusted p-value ≤ 0.05). * Initial number of eggs. Comparisons where all mutation carrying lines significantly differ from the susceptible control are indicated in bold.

egg laying when compared to the Wasatch control. There was no significant difference between Wasatch and the cytb_R1-R3 in total fecundity per female (FIGURE 5.2G, TABLES 5.2 and S5.1). The control line GluCl1+3_C displayed the highest and significantly different daily fecundity compared to the three GluCl1+3_R1-3 lines, while values for the mean total fecundity did not differ significantly between control and the lines with mutation (FIGURE 5.2H, TABLES 5.2 and S5.1).

Of note, line cytb_R2 displayed an unusual egg laying pattern, lacking the characteristic oviposition peak, but displaying a prolonged plateau that extended over a substantial proportion of the oviposition period (FIGURE 5.2G). This may explain the observed low mean values for total and daily fecundity (TABLE 5.2). The plots of daily egg laying of lines CHS1_R3 and VGSC_R3 displayed an unusual shape with an additional oviposition peak around the 30th day after eclosion. In both experiments, the peak results from a prolonged oviposition period of one female mite (Figures 5.2E and 5.3F).

5.3.3. Pre-oviposition, oviposition, and post-oviposition periods

Lines CHS1_R1,R2,R3 did not differ significantly in the length of the pre-oviposition period compared to CHS1_C (TABLES 5.3 and S5.1). CHS1_C had significantly shorter oviposition periods compared to CHS1_R2. Lines CHS1_R1,R2,R3 and CHS1_C did not differ significantly in the duration of the post-

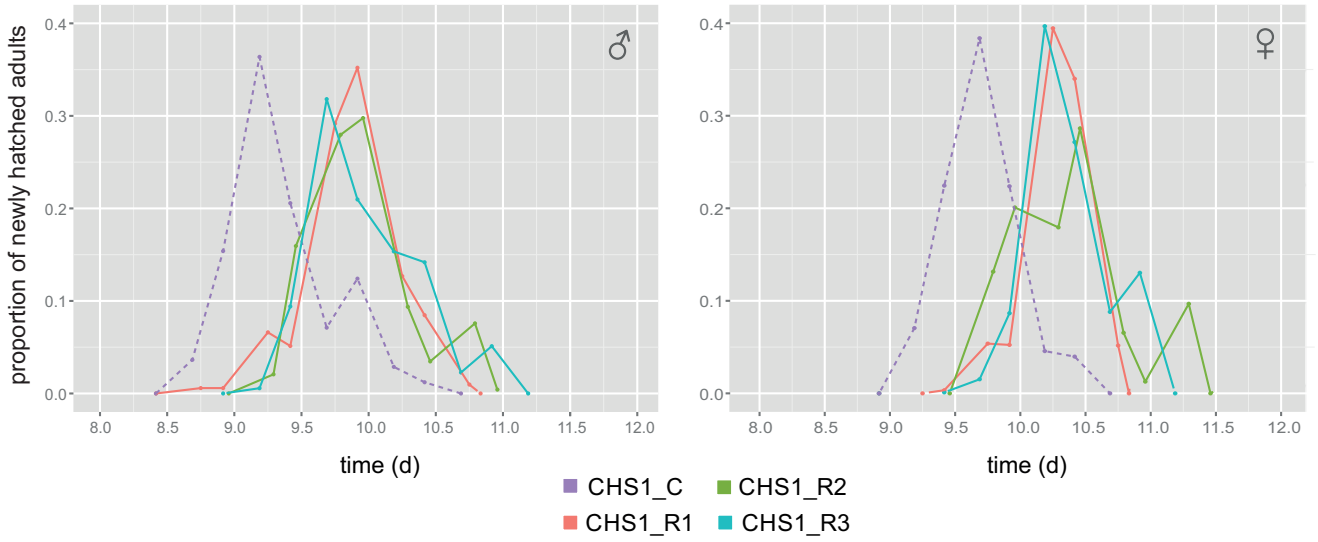

FIGURE 5.1 – Total development time of near-isogenic lines of *Tetranychus urticae* carrying I1017F in the *CHS1* gene and their susceptible control. The experiment consisted of mites grouped in two time blocks, differing in age by 4-5 h. The adult mites were scored in the intervals of 12 h. Total development time of males and females is presented in the left and right panel, respectively. Dashed purple line depicts adults of the susceptible EtoxR_C while solid lines red, green, and blue depict EtoxR_R1, EtoxR_R2, and EtoxR_R3, respectively.

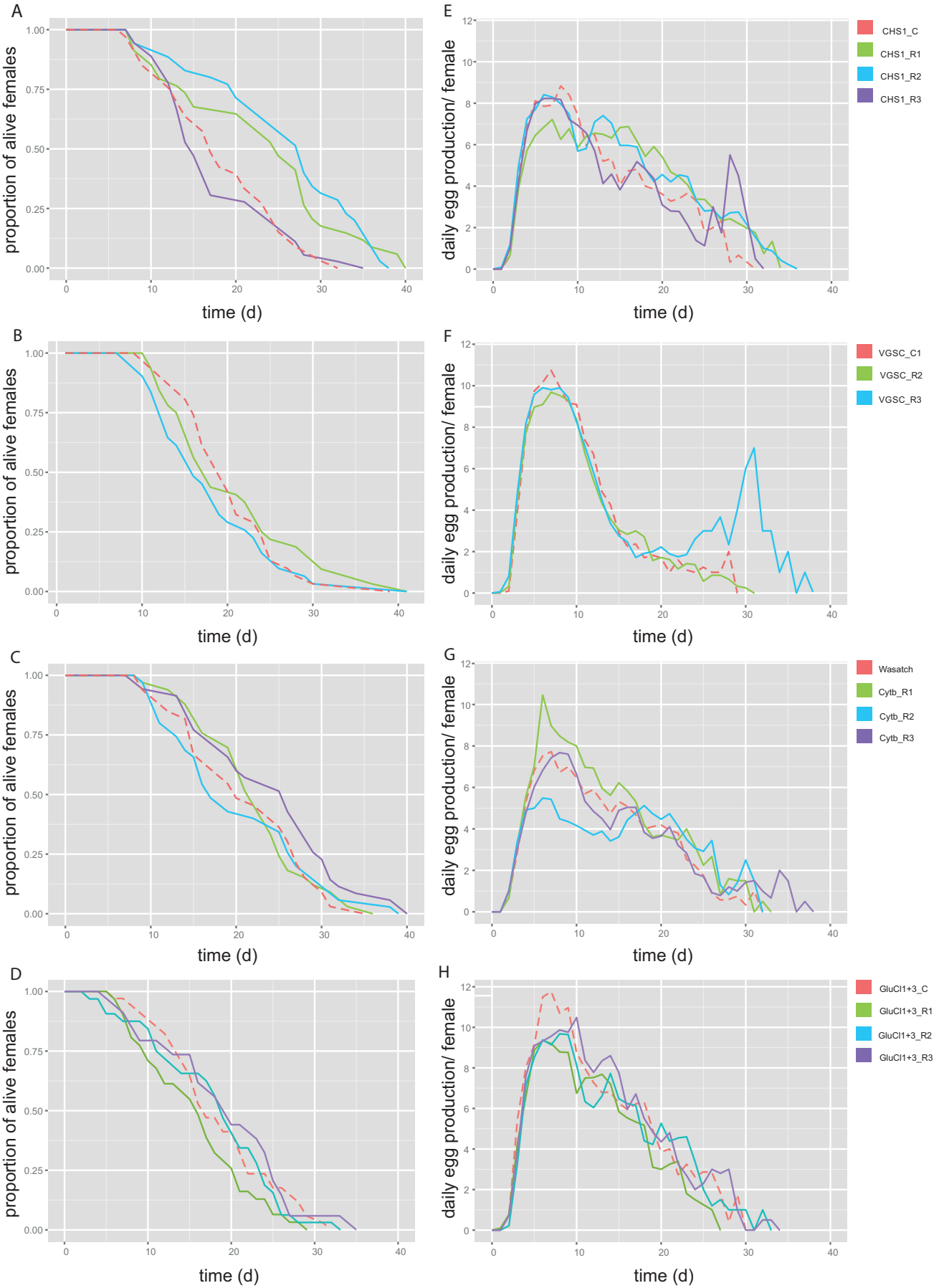

FIGURE 5.2 – Visualization of female adult longevity and daily egg laying per *Tetranychus urticae* female. Panel A, B, C, and D present adult longevity while panel E, F, G, and H depict the number of eggs/female/day for lines carrying I1017F in CHS1, lines carrying L1024V in VGSC, lines carrying P262T in cytb, and lines carrying G314D and G326E in GluCl1 and 3 and their susceptible control lines, respectively. Dashed lines depict data of the susceptible control, while solid lines of different colors represent three, or in case of L1024V mutation, two lines with target–site resistance mutation. TABLES 5.2 and 5.3 show the results of the statistical analysis on these data.

oviposition period (TABLES 5.3 and S5.1). Lines VGSC_R2, 3 and VGSC_C did not differ significantly in the length of pre-oviposition, oviposition or post-oviposition periods, neither did the lines cytb_R1,2,3 in comparison to Wasatch (TABLES 5.3 and S5.1). GluCl1+3_C had a significantly shorter pre-oviposition period than lines GluCl1+3_R1, R2, R3. The lines did not differ significantly in the length of the oviposition period but GluCl1+3_R2 had significantly shorter post-oviposition period than GluCl1+3_C (TABLES 5.3 and S5.1).

5.3.4. Fertility life table parameters

Mean Jackknife values of FLT parameters for lines with mutations in CHS1, VGSC, cytb, and GluCl channels and their respective control lines are summarized in TABLE 5.4. With an exception of the line VGSC_R2 (1.19), results of skewness test indicated that the data were fairly symmetrically distributed (rule of a thumb $-0.5 \leq x \geq 0.5$) or moderately skewed ($-1 \leq x \geq 1$), justifying the use of Jackknife resampling method (TABLE S5.2, FIGURES S5.1-S5.4).

Line CHS_R1 was found to have significantly smaller values of rm and LM and consequently significantly longer doubling time (DT) than CHS_C. Line CHS1_R2 was characterized with a higher net reproductive rate (R0) compared to CHS1_C. The control line had a significantly shorter generation time (T) when compared to CHS1_R1,R2,R3.

The VGSC_R2,3 lines did not differ significantly from the susceptible VGSC_C congenic line in any of the five FLT parameters. R0, DT, rm and LM, and T did not differ between control line Wasatch and cytb_R2 and R3. Line cytb_R1 showed the highest R0, rm, and LM and shortest DT (TABLE 5.4).

All FLT parameters were significantly different in GluCl1+3_R1-3 when compared to their susceptible congenic line (TABLE 5.4). The control line had significantly higher values of rm, LM, and R0 and consequently lower values for T and DT. As mentioned above, line GluCl1+3_R1 was characterized with an inverted sex ratio and consequently was repeatedly inferior to the remaining lines for all FLT parameters tested, with an exception of mean generation time (TABLE 5.4).

TABLE 5.3 – Mean values ± SE of female adult longevity. duration of pre-oviposition (Pre-OP), oviposition (OP) and post-oviposition periods (Post-OP) obtained for *Tetranychus urticae* near-isogenic lines and Wasatch.

Target-site	Line	N*	Longevity ± SE	Pre-OP ± SE	OP ± SE	Post-OP ± SE
CHS1 (wt)	CHS1_C	33	18.18 ± 1.22a	1.34 ± 0.05a	14.03 ± 1.19a	1.64 ± 0.43a
CHS1 (I1017F)	CHS1_R1	34	23.15 ± 1.68a	1.40 ± 0.05a	18.32 ± 1.41a	1.12 ± 0.30a
	CHS1_R2	34	25.66 ± 1.49b	1.20 ± 0.06a	21.44 ± 1.36b	2.15 ± 0.39a
	CHS1_R3	35	17.39 ± 1.17a	1.21 ± 0.06a	14.20 ± 1.07a	1.00 ± 0.35a
VGSC (wt)	VGSC_C	31	20.16 ± 1.07a	1.39 ± 0.05a	12.77 ± 0.87a	4.45 ± 0.78a
VGSC (L1024V)	VGSC_R2	32	20.50 ± 1.43a	1.36 ± 0.05a	12.84 ± 1.01a	4.81 ± 1.03a
	VGSC_R3	31	17.90 ± 1.31a	1.33 ± 0.07a	11.84 ± 1.07a	3.32 ± 0.63a
Cytb (wt)	Wasatch	33	21.12 ± 1.25a	1.59 ± 0.09a	16.45 ± 1.12a	1.82 ± 0.24a
Cytb (P262T)	cytb_R1	33	21.97 ± 1.1a	1.41 ± 0.09a	17.24 ± 0.97a	2.03 ± 0.43a
	cytb_R2	35	20.20 ± 1.43a	1.53 ± 0.09a	15.29 ± 1.34a	2.26 ± 0.45a
	cytb_R3	35	24.03 ± 1.41a	1.41 ± 0.07a	18.8 ± 1.19a	1.66 ± 0.41a
GluCl1+3 (wt)	GluCl1+3_C	34	19.53 ± 1.16a	**1.33 ± 0.05a**	14.74 ± 1.16a	2.26 ± 0.39a
GluCl1+3 (G314D+G326E)	GluCl1+3_R1	31	16.71 ± 1.14a	**1.66 ± 0.07b**	12.52 ± 1.10a	1.32 ± 0.32a
	GluCl1+3_R2	32	19.00 ± 1.32a	**1.54 ± 0.07b**	15.25 ± 1.36a	0.75 ± 0.22b
	GluCl1+3_R3	34	19.12 ± 1.35a	**1.56 ± 0.08b**	15.44 ± 1.29a	1.76 ± 0.38a

Time is expressed in days. Means followed by the letter 'a' and 'b' within a column are not significantly or significantly different from the control line, respectively (adjusted p-value <0.05). * Number of females. Comparisons where all mutation carrying lines significantly differ from the susceptible control are indicated in bold.

TABLE 5.4 – Jackknife estimates ± SE of five FLT parameters obtained for near-isogenic lines of *Tetranychus urticae* and Wasatch.

Target-site	Line	*N	R0 ± SE	T ± SE	DT ± SE	rm ± SE	LM ± SE
CHS1(wt)	CHS1_C	33	35.31 ± 3.44a	**17.96 ± 0.30a**	3.49 ± 0.06a	0.199 ± 0.003a	1.220 ± 0.004a
CHS1 (I1017F)	CHS1_R1	34	44.23 ± 4.31a	**20.15 ± 0.33b**	3.68 ± 0.07b	0.188 ± 0.004b	1.207 ± 0.004b
	CHS1_R2	34	58.83 ± 4.79b	**19.56 ± 0.30b**	3.32 ± 0.05a	0.208 ± 0.003a	1.232 ± 0.004a
	CHS1_R3	35	32.37 ± 2.74a	**18.28 ± 0.33b**	3.64 ± 0.06a	0.190 ± 0.003a	1.210 ± 0.004a
VGSC (wt)	VGSC_C	31	46.32 ± 3.03a	19.41 ± 0.33a	3.51 ± 0.05a	0.198 ± 0.003a	1.219 ± 0.003a
VGSC (L1024V)	VGSC_R2	32	51.33 ± 3.08a	19.35 ± 0.21a	3.403 ± 0.02a	0.204 ± 0.001a	1.226 ± 0.002a
	VGSC_R3	31	42.99 ± 3.10a	19.38 ± 0.33a	3.568 ± 0.04a	0.194 ± 0.002a	1.214 ± 0.003a
Cytb (wt)	Wasatch	33	36.93 ± 3.13a	19.52 ± 0.29a	3.74 ± 0.07a	0.185 ± 0.004a	1.203 ± 0.004a
Cytb (P262T)	cytb_R1	33	58.30 ± 4.15b	19.48 ± 0.25a	3.32 ± 0.05b	0.209 ± 0.003b	1.232 ± 0.004b
	cytb_R2	35	34.23 ± 3.89a	20.05 ± 0.4a	3.92 ± 0.09a	0.177 ± 0.004a	1.193 ± 0.005a
	cytb_R3	35	38.15 ± 2.54a	20.06 ± 0.27a	3.82 ± 0.07a	0.182 ± 0.003a	1.199 ± 0.004a
GluCl1+3 (wt)	GluCl1+3_C	34	**73.40 ± 5.75a**	**18.44 ± 0.22a**	**2.97 ± 0.03a**	**0.233 ± 0.003a**	**1.262 ± 0.003a**
GluCl1+3 (G314D+G326E)	GluCl1+3_R1	31	**13.99 ± 1.36b**	**19.18 ± 0.26b**	**5.03 ± 0.15b**	**0.138 ± 0.004b**	**1.148± 0.005b**
	GluCl1+3_R2	32	**57.61 ± 5.53b**	**19.53 ± 0.28b**	**3.33 ± 0.07b**	**0.208 ± 0.004b**	**1.231 ± 0.005b**
	GluCl1+3_R3	34	**52.78 ± 4.60b**	**19.65 ± 0.23b**	**3.43 ± 0.06b**	**0.202 ± 0.003b**	**1.224 ± 0.004b**

Means followed by the letters 'a' and 'b' within a column are not significantly and significantly different from the control line, respectively (adjusted p<0.05). * Number of females. Comparisons where all mutation carrying lines significantly differ from the susceptible control are indicated in bold.

5.3.5. Temporal dynamics of etoxazole resistance

Our experimental data revealed that, after approximately three generations (i.e., 1 month at 28°C), the percentage of mites that survived etoxazole exposure and thus were homozygous for the CHS1 resistance mutation, was 42, 18, and 7% for the R70, R50 and R30 populations, respectively. These values were below the frequencies expected under Hardy-Weinberg equilibrium (49, 25, and 9%, respectively). Over the next 8 months, the frequencies of resistant homozygous individuals dropped to 6.0, 4.1, and 0.5% for R70, R50, and R30, respectively. While the frequency of resistant females in the R30 population was low and relatively stable, the frequency of resistant females in line R70 appeared to largely fluctuate over time. The monotonic downward trend was significant only for the population R50 ($\tau = -0.67$, $p<0.05$) (FIGURE 5.3).

5.4. DISCUSSION

According to a long-standing hypothesis, mutations that underlie target-site resistance can carry a fitness disadvantage in an insecticide/acaricide-free environment (Crow, 1957), and arthropods with newly arisen resistant alleles are expected to be less competitive compared to susceptible conspecifics (ffrench-Constant, 2007; ffrench-Constant and Bass, 2017; Fisher, 1999). In the light of this theory, investigating biological weaknesses in resistant populations may be important in the context of Insecticide Resistance Management (IRM) as a factor mitigating resistance development (Crow, 1957; P. Georghiou and E. Taylor, 1977). The management tactics in the pesticide-sprayed areas could exploit selective advantage of susceptible individuals by creating unsprayed refugia where these can prevail, and/or use of pesticides of short residual activity, extending periods between treatments (Leeper et al., 1986). Where resistance is already present, use of pesticide with unrelated mode of action (MoA) which does not discriminate between susceptible and resistant individuals, could slow down the development of resistance or possibly even eliminate it from the field. In cases where pesticide resistance-related fitness cost is of a large magnitude, use of two unrelated MoA pesticides in rotation would allow time to restore susceptibility to one compound while the other compound is in use (Overmeer et al., 1975). On the other hand, target-site resistance mutations could have been present within pest populations as natural polymorphisms prior to the introduction of the insecticide into agriculture. These natural polymorphisms are not expected to be disadvantageous as they have been maintained in unsprayed pest populations (Crow, 1957; ffrench-Constant, 2007). However, in most cases, but with notable exceptions (Gould et al., 1997; Hartley et al., 2006), the origin and history of nucleotide polymorphisms associated with resistance is unknown, which makes it difficult to have an *a priori* expectation on potential fitness costs. In this study, we took advantage of a collection of near-isogenic lines to quantify any potential pleiotropic fitness effects of five key mutations associated with resistance in *T. urticae*. Surprisingly, only three target-site resistance mutations, I1017F in CHS1 and the co-occurring G314D and G326E mutations in GluCl1 and GluCl3, respectively, were shown to significantly and consistently alter certain fitness parameters in *T. urticae* (TABLE 5.2). The other two mutations, P262T in cytb and L1024V in VGSC, did not induce a consistent change in any fitness parameter that was analyzed in our study.

Chitin synthase, the rate limiting enzyme in the chitin biosynthesis pathway of arthropods, is targeted by a number of classes of insecticides/acaricides referred to as chitin synthesis inhibitors (IRAC 15, e.g., benzylourea; IRAC 16, buprofezin) and mite growth inhibitors (IRAC 10, clofentezine, hexythiazox, and etoxazole) (Demaeght et al., 2014; Douris et al., 2016; Van Leeuwen et al., 2012). The involvement of CHS1 in the mode of action of these compounds was first uncovered in a milestone study that used a population level bulked segregant analysis to map a case of monogenic, recessive etoxazole resistance (Van Leeuwen et al., 2012). In a follow-up study, high resistance to hexythiazox and clofentezine in *T. urticae* was shown to be also strongly associated with the I1017F substitution in CHS1 (Demaeght et al., 2014). Recently, Riga et al. (2017) provided unambiguous proof that this mutation is the main mechanism underlying the extremely high levels of resistance to mite growth inhibitors. In *Panonychus citri*, genetic crosses also revealed a recessive, monogenic inheritance of hexythiazox resistance, suggesting that this mutation occurs in multiple spider mite species (Yamamoto et al., 1995). Indeed, mutations at the position corresponding to I1017F of *T. urticae* CHS1 have been identified in the chitin synthesis inhibitor-resistant field strains of the lepidopteran diamondback moth, *Plutella xylostella* (I1042M/F) (Douris et al., 2016), thysanopteran western flower thrips *Frankliniella occidentalis* (I/ M) (Suzuki et al., 2017), and dipteran

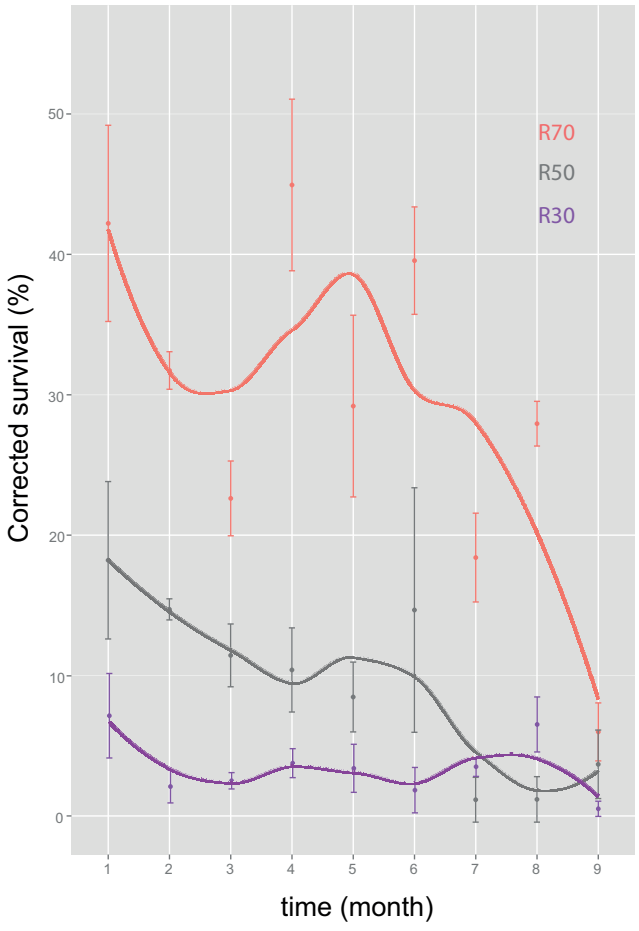

FIGURE 5.3 – Visualization of the temporal dynamics of etoxazole resistance in *Tetranychus urticae*. Nonparametric loess curves were fitted to the data using the *lowess* function in R. Red color depicts population R70, gray R50, and purple R30, i.e., populations with 70, 50, and 30% of resistant homozygous females in the starting population, respectively.

Culex pipiens (I1043M/L) (Grigoraki et al., 2017). Finally, CRISPR-Cas9-mediated genome-editing functionally validated that mutations at homologous positions of the *P. xylostella, C. pipiens,* and *T. urticae* chitin synthases confer high levels of etoxazole resistance in *Drosophila melanogaster* (Douris et al., 2016; Grigoraki et al., 2017).

As the I1017F mutation is located in a highly conserved region of CHS1*,* substitution could impair its enzymatic function. Within the Tetranychoidea superfamily, fitness costs of mite growth inhibitors resistance has been previously investigated in *T. urticae, P. citri,* and *Brevipalpus phoenicis* (Campos and Omoto, 2006; Herron et al., 1993; Stocco et al., 2016; Yamamoto et al., 1996). However, the experimental set-up of these studies was flawed as the examined strains were either not related or of an unknown genotype. Nonetheless, these studies suggest that resistance to mite growth inhibitors is associated with a significant fitness cost, because of the reproductive disadvantage of the resistant genotype (Stocco et al., 2016; Yamamoto et al., 1996), when competitive susceptible individuals are present (Campos and Omoto, 2006; Herron et al., 1993; Stocco et al., 2016; Yamamoto et al., 1996). In this study, the impact of I1017F on spider mite fitness was quantified by both analyzing single-generation life history traits, FLT parameters of individual near-isogenic lines, as well as by an inter-line competition experiment. Results indicate that the total development and mean generation time were significantly different between the strains carrying the I1017F mutation in *CHS1* gene in relation to the control strain (Figure 5.1, Tables 5.2-5.3). Interestingly, the expression of *T. urticae CHS1* is the highest in the obligate chrysalis stage wherein the mite molts to complete its development (Van Leeuwen et al., 2012). Mite development time could thus be affected by the I1017F mutation by inefficient molting and a longer chrysalis stage. In support of this theory, *P. xylostella* strains that carry the I1042M mutation had a significantly longer development time at 20 and 30°C, compared to strains lacking the mutation (Steinbach et al., 2017). In addition to a shorter development time, the I1042M mutation is also associated with a lower fecundity in the resistant than in the susceptible *P. xylostella* strains (Steinbach et al., 2017), although it is possible that other loci underlie this additional effect.

Although a delay in developmental time is predicted to heavily influence population growth (Snell, 1978), especially due to a later age of first reproduction (Caswell and Hastings, 1980; Cole, 1954), the longer development in the resistant mites did not induce a significant change in any lifetable parameter apart from generation time (Table 5.4). It should also be noted that CRISPR-Cas9 genome edited *D. melanogaster* flies that bear the *T. urticae* I1017F or the *P. xylostella* I1042M mutation do not exhibit a significant difference in time until eclosion, adult survival or average daily fecundity when isolated in an isogenic background, indicating that the I1017F/M mutations do not have pleiotropic effects in all organisms (Douris et al., 2016; Grigoraki et al., 2017).

Moreover, the I1017F resistance mutation was found relatively widespread and often present in a homozygous state in field and laboratory collections originating from a wide geographic range (Europe, Asia, and Africa) (Ilias et al., 2014; Kwon et al., 2014). This phenomenon appears to be partially supported by our analysis of the temporal dynamics of etoxazole resistance in the mixed population experiment (Figure 5.3). While in all three populations (R30, R50, and R70) the frequency of resistant homozygotes dropped below 10% in a period of 10 months, only population R50 showed a significantly downwards trend throughout the course of experiment. The frequency of the resistant individuals in population R70 was largely fluctuating, while population R30, as expected under Hardy-Weinberg equilibrium, reached a very low level of resistant individuals and remained relatively stable over time (Figure 5.3).

To conclude, the I1017F mutation in CHS1 of *T. urticae* affects the total development time of mites but its effect on overall fitness of the resistant population may be negligible, as supported by a high worldwide prevalence of the I1017F mutation (Ilias et al., 2014) and conservation of the resistance mechanism across arthropod taxa (Douris et al., 2016; Grigoraki et al., 2017; Steinbach et al., 2017).

The abamectin and milbemectin acaricides primarily target GluCl channels and have been used successfully against *T. urticae* populations for the last 30 years (Campos et al., 1995; Campos et al., 1996; Kwon et al., 2014; Stumpf and Nauen, 2002; Van Leeuwen et al., 2010). Abamectin resistance is known to arise through multiple mechanisms, involving target-site and biochemical/metabolic resistance, the latter likely mediated by cytochrome P450 monooxygenase and GST detoxification (Pavlidi et al., 2015; Riga et al., 2014). Two mutations, G314D in GluCl1 and G326E in GluCl3, have been reported to contribute to resistance (Dermauw et al., 2012; Kwon et al., 2010b) and act synergistically (Riga et al., 2017). Although *in vitro* functional validation of the G326E mutation in *Xenopus laevis* oocytes reveals the complete abolishment of abamectin binding (Mermans et al., 2017), near isogenic lines possessing both mutations have shown that target-site resistance is only of minor importance for the occasionally encountered high resistance levels (Ferreira et al., 2015; Ilias et al., 2014; Riga et al., 2017).

There are several studies investigating the potential fitness costs carried by abamectin/milbemectin resistance in *T. urticae*. However, as for studies on chitin synthesis inhibitors, caution is needed since investigated *T. urticae* strains were genetically unrelated or of unknown genetic basis of resistance. Nonetheless, previous approaches that followed the resistance dynamics over time indicate that under laboratory conditions a reversal to susceptibility in *T. urticae* can occur relatively rapid (Campos et al., 1995; Nicastro et al., 2010; Sato et al., 2005; Stumpf and Nauen, 2002).

In the current study, the near-isogenic GluCl1+3_R1-3 lines, that carry both G314D and G326E mutations, exhibited significantly lower ISS and longer pre-oviposition period as well as higher daily fecundity/female than the GluCl1+3_C control line (Table 5.2). Differences in single life history traits were reflected in FLT parameters. Here, the three resistant lines had significantly lower values of R0, rm, and LM and consequently higher T and DT compared to the susceptible control (Table 5.4). The lower value of rm is particularly informative, since the parameter takes into account the development time, immature stage survivorship, and timing and amount of births and deaths in the population (Birch, 1948; Ferrari and Georghiou, 1981).

Situated five amino acids away from G314D or G326E, the A309V (*T. urticae* numbering) mutation at the N-terminus of the third transmembrane helix (TM3) of the GluCl channel is strongly associated with abamectin resistance in *P. xylostella* (Wang and Wu, 2014). In parallel to our results, Wang and Wu

(2014) concluded that abamectin resistance carries significant fitness costs in *P. xylostella* after they had backcrossed the abamectin-resistant Roth-Abm strain carrying the A309V into the parental susceptible Roth strain. A subsequent study showed that the frequency of the resistance allele dropped from 94.7 to 9.6% after 20 generations in both populations where abamectin use was ceased, further confirming a pleiotropic effect of the A309V mutation (Wang et al., 2016). Although the mutated residues in *T. urticae* and *Plutella* are not located at the identical position, they are situated at the N-terminus of the TM3. The TM2, TM3, and TM2-TM3 linker region have been previously shown to be significant for the function of the ligand-gated chloride channels. For instance, residues in the TM2, TM3, and the linker region of GABA and glycine receptors are critical for the response to inhaled anesthetics (Mihic et al., 1997). Similarly, mutations in the conserved TM2–TM3 linker and the TM3 of glycine/glutamate-gated receptors have shown to decrease the efficacy of avermectin to gate the receptor (Kane et al., 2000; Lynagh et al., 2011; Wang and Wu, 2014). Additionally, associated with resistance, a mutation in GABA-gated ion channels, A302S in the M2, is responsible for the well-characterized dieldrin resistance (ffrench-Constant, 2013; ffrench-Constant et al., 1990; Zhang et al., 1994), with its fitness cost being most thoroughly and adequately investigated in *L. cuprina,* where the Rdl allele showed to have a lower representation in overwintering larvae under field conditions (McKenzie and Clarke, 1988).

Together, our results indicate a high fitness cost to populations carrying both G314D and G326E mutations in, respectively, GluCl1 and GluCl3. The phenomenon may explain a high efficiency of abamectin and the rare resistance reports, despite the acaricide has been used intensively for the last 3 decades (Van Leeuwen et al., 2010). Even though combination of two mutations does not confer a high resistance level to abamectin (Riga et al., 2017), it may act as a resistance-limiting factor. Mutations may be selected by high concentrations of abamectin as an additional mechanism to a putative P450/GST-mediated metabolic resistance, and prevent highly resistant populations from being established (Pavlidi et al., 2015; Riga et al., 2014). This hypothesis finds its support in the study by Ilias et al. (2014), where in a world-wide survey, the combination of the two mutations was present in only two field collections (i.e., 0.06% of all samples) both originating from roses grown in abamectin-treated greenhouses (Ilias et al., 2014). A recent electrophysiological study on *T. urticae* GluCl3 has shown that homomeric wild type and G326E GluCl3 channel does not differ in performance in the absence of acaricides when expressed in *X. laevis* oocytes (Mermans et al., 2017). Therefore our results also raise the question, how different combinations of target-site mutations affect channel performance and whether there is an *in vivo* prevalence to form hetero- over homomeric GluCl channels in *T. urticae*. As G326E occurs relatively frequently in world-wide isolates of *T. urticae (*21% of all samples*),* compared to the G314D mutation or the combination of both mutations (Ilias et al., 2014), hence the G326E mutation alone may not impose significant fitness costs on the mite.

Pyrethroid resistance in *T. urticae* has been reported globally with resistance levels exceeding 10,000 fold (Ay and Gürkan, 2005; Herron et al., 1993; Tsagkarakou et al., 2009; Van Leeuwen et al., 2005). Possibly due to differences in amino acid positions within and at the periphery of the VGSC binding pocket, the kdr and super-kdr mutations that cause DDT and pyrethroid insensitivity in insects, are so far not found in *T. urticae* (Van Leeuwen and Dermauw, 2016). Instead, the VGSC mutations F1538I in domain III L6 and L1024V in domain II L6 (*Musca domestica* numbering) are located at unique positions and have been found at relatively high frequency in *T. urticae* populations in many regions of the world (Ilias et al., 2014; Kwon et al., 2014). Here, we followed the single generation life history of L1024V mutation-carrying lines and their susceptible congenic line. Results indicated no fitness cost related to the presence of mutation. There was no consistent difference between VGSC_R2,3 and the control strain in any of the examined life history traits (Tables 5.2 and 5.3) nor the FLT parameters (Table 5.4).

The costs of kdr and/or super-kdr mutations in insects have been previously studied in near-isogenic lines. They did become manifest in altered life history parameters like oviposition in *Aedes aegypti* (Brito et al., 2013) and survival to adulthood in *Culex quinquefasciatus* (Berticat et al., 2008) but more strongly in behavioral characteristics such as indifference to temperature changes or temperature sensitivity in *M. domestica* (Foster et al., 2003), lowered ability of host seeking in *Anopheles gambiae* (Diop et al., 2015), and increased locomotor activity in *A. aegypti* (Brito et al., 2013). Therefore, even though our study presents a detailed analysis of mite life history parameters, we acknowledge that by focusing purely on biological characteristics, subtle pleiotropic effects on mite behavior may be overlooked.

The Complex III inhibitors including bifenazate have been introduced for the control of mite infestation relatively recently (Dekeyser, 2005; Grosscurt and Avella, 2005; Kinoshita et al., 1999). Nonetheless, high levels of resistance to bifenazate have been detected in the field (Van Leeuwen et al., 2008; Van Nieuwenhuyse et al., 2009). Resistance has been found to be maternally inherited and attributed to mutations in conserved regions of cd1 (S141F+G126S and I136T+G126S) and ef (P262T) helices of cytb (Van Leeuwen et al., 2008; Van Nieuwenhuyse et al., 2009). The residue P262 is known to bind head group of atovaquone in yeast cytb and is located next to the E263, which is thought to be a ligand for ubiquinol (Van Nieuwenhuyse et al., 2009; Wenz et al., 2007). Given the extent of sequence conservation and possibly important role of P262 in ubiquinol oxidation, the P262T substitution has been analyzed through examination of single generation life history parameters.

Here, there was no consistent difference seen between Wasatch and cytb_R1-R3 lines neither in the individual life history parameters (Tables 5.2 and 5.3) nor in the life table parameters (Table 5.4). Similar results have been previously found for the mutations in the cd1 helices of cytb, S141F + G126S, where in the absence of bifenazate selection (Van Leeuwen et al., 2008) no significant differences were found in life history traits or in intrinsic rate of increase (rm) between genetically related *T. urticae* resistant and susceptible strains. Nonetheless, considering data obtained from the wide-ranging survey of Ilias et al. (2014), where the P262T was present only in one out of 33 mite collections and S141F was not reported at all, it is surprising that mutations in cytb were not found to carry significant fitness cost.

In this study, we demonstrate consistent significant differences in certain fitness parameters for the near-isogenic lines that carry a target-site resistance mutation in CHS1 and two co-

occurring mutations in GluCl1 and GluCl3. The target-site mutations in cytb and VGSC did not significantly alter any fitness parameters that was analyzed. However, experiments performed in defined conditions may overlook the subtle pleiotropic effects on biology or behavior that only become visible under specific conditions, such as food shortage, high population size and density etc. (McKenzie, 1996). Hence, assumption of presence or absence of fitness cost has to be made with caution, because an individual's fitness is influenced by the sum of all stressors, which can act additively, antagonistically, and/or synergistically (Brady et al., 2017).

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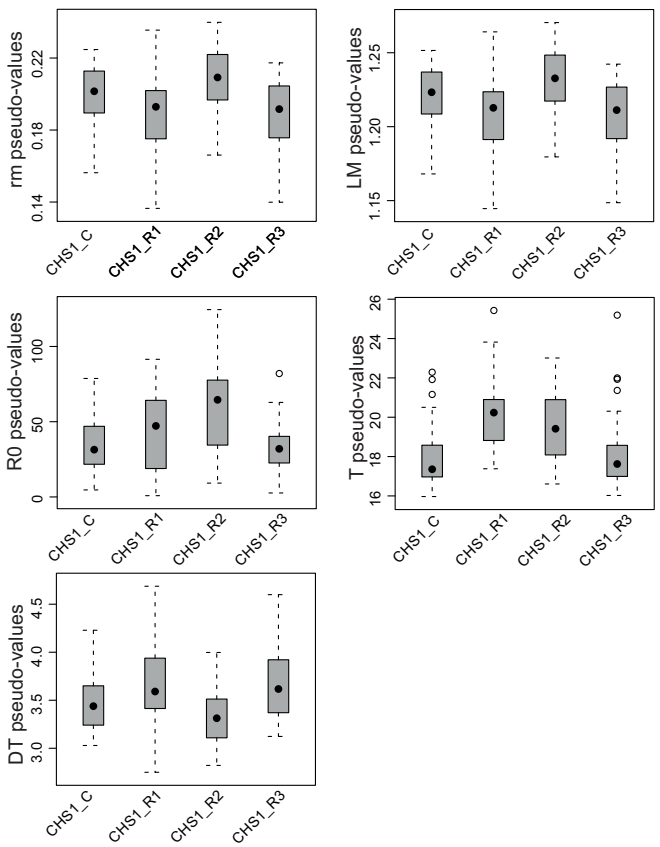

FIGURE S5.1 – The dispersion of Jackknife pseudo-values for R0, rm, DT, LM, and T estimated for the *Tetranychus urticae* lines EtoxR_R1, R2, R3 and their susceptible control EtoxR_C. The bottom and top edges of the box are located at the 25th and 75th percentiles of the sample. The dot is drawn at the median. The whiskers extend from the box as far as the data extend, to a distance at most 1.5 interquartile range. Outliers, observations located beyond the distance of 1.5 interquartile, are depicted with circles (Maia et al., 2014).

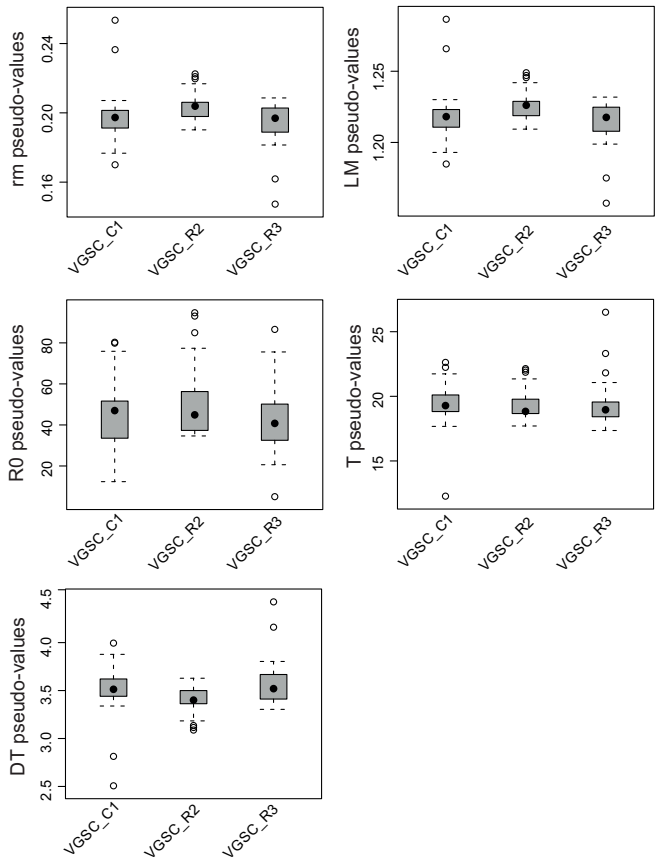

FIGURE S5.2 – The dispersion of Jackknife pseudo-values for R0, rm, DT, LM, and T estimated for the *Tetranychus urticae* lines VGSC_R2 and R3 and their susceptible control VGSC_C1. The bottom and top edges of the box are located at the 25th and 75th percentiles of the sample. The dot is drawn at the median. The whiskers extend from the box as far as the data extend, to a distance at most 1.5 interquartile range. Outliers – the observations located beyond the distance of 1.5 interquartile, are depicted with circles (Maia et al., 2014).

TABLE S5.1 – Results of the analysis of variance.

Parameter	Sex	CHS1_R1,2,3	Cytb_R1,2,3	VGSC_R2,3	GluCl1+3_R1, R2, R3
Developmental time	male	$F_3 = 7.64$, p<0.05	$Chisq_3 = 6.00$, p>0.05	$F_2 = 0.19$, p>0.05	$F_3 = 7.49$, p<0.05
	female	$F_3 = 9.99$, p<0.05	$Chisq_3 = 10.84$, p<0.05	*$Chisq_2 = 1.68$, p<0.05	$Chisq_3 = 14.37$, p<0.05
ISS		$Chisq_3 = 7.83$, p<0.05	$Chisq_3 = 51.24$, p<0.05	$Chisq_2 = 14.65$, p<0.05	$Chisq_3 = 15.8$, p<0.05
Offspring sex ratio		$Chisq_3 = 13.13$, p<0.05	$Chisq_3 = 71.479$, p<0.05	$Chisq_2 = 21.02$, p<0.05	$Chisq_3 = 10.09$, p<0.05
Daily fecundity		$Chisq_3 = 2.13$, p>0.05	$Chisq_3 = 26.87$, p<0.05	$Chisq_2 = 3.48$, p>0.05	$Chisq_3 = 18.89$, p<0.05
Total fecundity		$Chisq_3 = 10.35$, p<0.05	*$Chisq_3 = 13.12$, p<0.05	$Chisq_3 = 5.87$, p>0.05	$Chisq_3 = 5.87$, p>0.05
Longevity		$Chisq_3 = 19.29$, p<0.05	$Chisq_3 = 4.66$, p>0.05	$Chisq_2 = 3.23$, p>0.05	$F_3 = 0.99$, p>0.05
Pre-oviposition		*$Chisq_3 = 8.30$, p<0.05	$Chisq_3 = 1.59$, p>0.05	$Chisq_2 = 0.08$, p>0.05	$Chisq_3 = 12.12$, p<0.05
Oviposition		$Chisq_3 = 19.49$, p<0.05	$F_3 = 1.63$, p>0.05	$Chisq_2 = 1.86$, p>0.05	$Chisq_3 = 3.71$, p>0.05
Post-oviposition		$Chisq_3 = 5.55$, p>0.05	$Chisq_3 = 2.23$, p>0.05	$Chisq_2 = 1.23$, p>0.05	$Chisq_3 = 10.88$, p<0.05

*There is a significant difference within the mutation carrying lines but not between the susceptible control and the mutation-carrying lines (see TABLES 5.2 and 5.3).

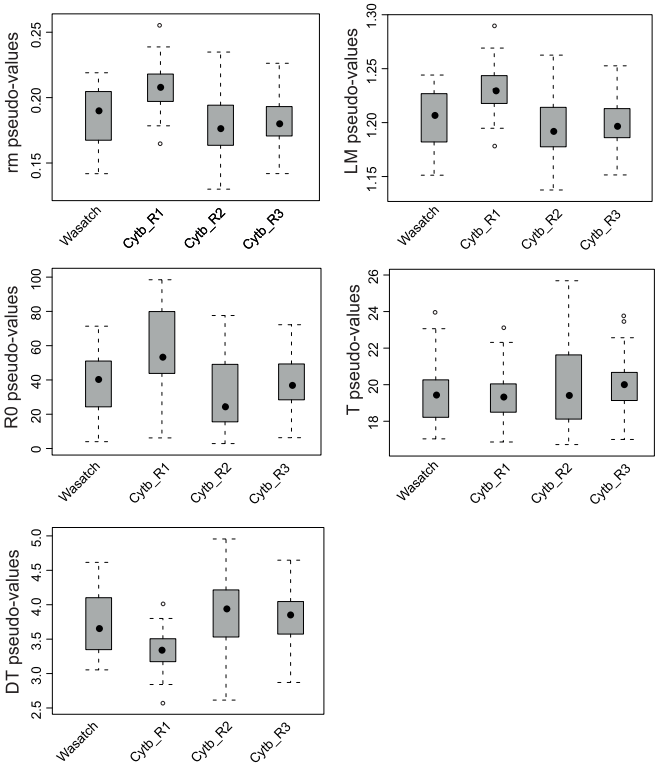

FIGURE S5.3 – The dispersion of Jackknife pseudo-values for R0, rm, DT, LM, and T estimated for the *Tetranychus urticae* lines cytb_R1 and R2 and R3 and susceptible control, strain wasatch. The bottom and top edges of the box are located at the 25th and 75th percentiles of the sample. The dot is drawn at the median. The whiskers extend from the box as far as the data extend, to a distance at most 1.5 interquartile range. Outliers – the observations located beyond the distance of 1.5 interquartile, are depicted with circles (Maia et al., 2014).

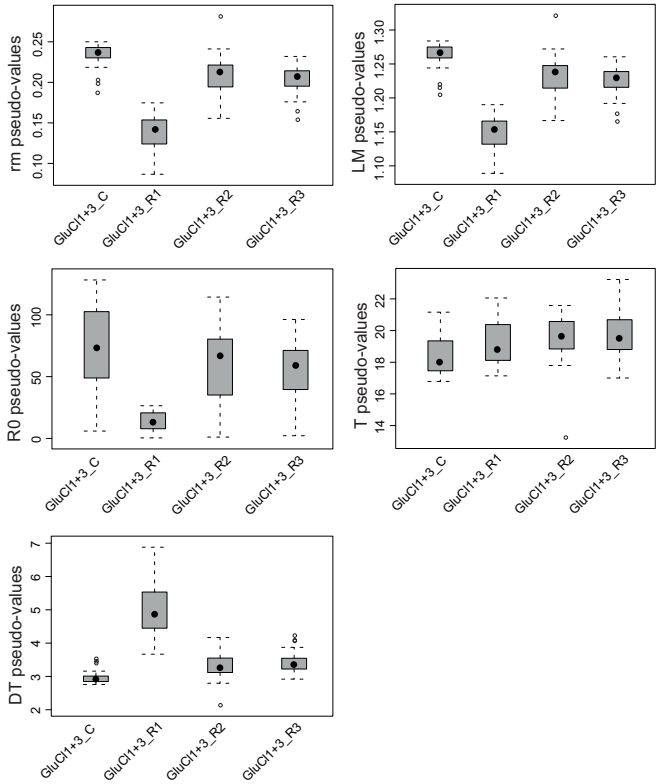

FIGURE S5.4 – The dispersion of Jackknife pseudo-values for R0, rm, DT, LM, and T estimated for the *Tetranychus urticae* lines GluCl1+3_R1, R2 and R3 and their susceptible control, GluCl1+3_C. The bottom and top edges of the box are located at the 25th and 75th percentiles of the sample. The dot is drawn at the median. The whiskers extend from the box as far as the data extend, to a distance at most 1.5 interquartile range. Outliers – the observations located beyond the distance of 1.5 interquartile, are depicted with circles (Maia et al., 2014).

TABLE S5.2 – Measure of skewness of the data distribution (total fecundity/ female within a line) for the *Tetranychus urticae* lines used in this study.

Line	Skewness
CHS1_C	0.44
CHS1_R1	-0.11
CHS1_R2	0.06
CHS1_R3	0.78
VGSC_C	0.25
VGSC_R2	1.19
VGSC_R3	0.55
Wasatch	-0.18
Cytb_R1	-0.14
Cytb_R2	0.47
Cytb_R3	-0.03
GluCl1+3_C	-0.08
GluCl1+3_R1	-0.01
GluCl1+3_R2	-0.32
GluCl1+3_R3	-0.48

6

General discussion

6.1. A TRANSCRIPTOMIC APPROACH TO STUDY THE MOLECULAR MECHANISMS UNDERLYING PESTICIDE RESISTANCE AND OTHER BIOLOGICAL TRAITS IN ARTHROPOD PESTS

Transcription is a vital step in the regulation of enzyme activity, and therefore essential for growth and development. The quantification of transcript levels across different conditions helps to deduce gene function. Measurements of transcription levels already began in the late 70's, with Sim et al. (1979) investigating mRNA levels in silk moth chorion by constructing a cDNA library. Throughout the 90's, Expressed Sequence Tag (EST) sequencing became a method of choice and allowed for the rapid and efficient profiling of tissue- and even cell-specific gene expression (Adams et al., 1993; Rudd, 2003).

The currently leading transcriptomic techniques are gene expression microarrays (GE microarrays) and RNA sequencing (RNAseq) and were developed in the mid-90's and the beginning of 21st century, respectively. GE microarrays estimate transcript abundance via the hybridization of cyanine-labelled mRNA to an array of complementary probes. Here, fluorescent intensity reflects the gene expression level. Gene expression microarrays are able to accurately quantify levels of gene expression on a genome-wide level but require a previously sequenced and annotated reference genome/transcriptome for the synthesis of the complementary probes, which limits discovery application of this approach (Hughes et al., 2001). Currently, an RNAseq approach is favored for most organisms and a multitude of different RNAseq methodologies have been developed. In 2005, 454 Life Sciences launched the first next-generation sequencer using pyrosequencing methodology which is often referred to as 454 RNA sequencing. In recent years however, more popular technologies have been established including those developed by Solexa and Illumina (Adiconis et al., 2013; Han et al., 2015; Nookaew et al., 2012). Here, single-end or paired-end reads are produced. Depending on the sequencing technology, RNAseq can produce reads of 30 to 10,000 bp in length but the typical read length is 100 nucleotides. Using mapping software packages such as Bowtie2 and STAR, reads are mapped to a reference annotated genome/transcriptome and mapped reads are subsequently counted per individual gene and statistically compared across treatments (Mortazavi et al., 2008). However, in contrast to GE microarrays, RNAseq does not necessarily depend on a previously sequenced and annotated genome/transcriptome. Indeed, a transcriptome can also be made *de novo* using only the RNAseq data at hand and assemblers such as CLC (http://www.clcbio.com/), SOAPdenovo (http://soap.genomics.org.cn/) and Trinity (http://trinityrnaseq.sourceforge. net/) (Honaas et al., 2016). The reliability of a *de novo* transcriptome assembly heavily depends on the sequence variability in the sample (Landman et al., 2014), the sequencing technology and the choice of assembler. If RNA is obtained from, e.g., a mixed field sample, the assembler will have difficulty to deal with the allelic variation, and the final transcriptome will contain a lot of broken, incomplete read sequences. Intuitively, assembly of an inbred line, will be more straightforward. Whereas single-end sequencing is more cost effective and gives a sufficient yield to quantify expression levels, paired-end sequencing generates higher quality contigs after assembly (Ozsolak and Milos, 2011). Moreover, recent advances in Illumina technology now also allow to preserve strand-specific information. Strand-specificity improves transcriptome assemblies by identifying the transcription of genes that occur at an overlapping genomic location but are located on opposite strands (Levin et al., 2010). Honaas et al. (2016) describes a comprehensive comparison of the quality of 99 assemblies of the *Arabidopsis thaliana* leaf transcriptome, produced by a set of six *de novo* assemblers, including CLC, Trinity, SOAP, Oases, ABySS and NextGENe. These authors argue that the quality of *de novo* transcriptome assemblies is best assessed using the four metrics that are outlined in TABLE 6.1. This table also lists *de novo* assemblies of mites that belong to the Acariformes clade and, if published, presents the respective values for the quality metrics. Unfortunately, in the majority of cases, the only available statistic is the total number of unigenes, which, as Honaas et al. (2016) show, by itself does not provide sufficient information to assess transcriptome quality. Our *Panonychus ulmi* transcriptome was assembled with CLC, which according to Honaas et al. (2016) is one of the best performing *de novo* assemblers and was based on strand-specific paired-end reads of 2 x 100 bp, where only female adult mites were used to isolate the RNA sequences (Gong et al., 2017).

6.1.1. Transcriptomic analysis and functional characterization of genes and their encoded enzymes that underlie insecticide resistance in arthropod pests

Previous studies have shown that a transcriptomic approach has great potential for identification of the genes that underpin insecticide and acaricide resistance. In these studies, a transcriptomic analysis is often followed by *in vitro* functional characterization of the enzymes coded by genes that are differentially expressed in the resistant vs. susceptible populations

TABLE 6.1 – Four metrics that assess the quality of *de novo* transcriptome assemblies and a list of examples of mite transcriptomes of the Acariformes lineage.

Species	Sequencing technology	Assembly	Mapped reads (%)	Conserved gene recovery	N50 (bp)	No. of unigenes	Reference
Panonychus ulmi	Illumina	CLC Genomics Workbench	91.7	-	2087	27,777	Bajda et al., 2015
Panonychus citri	Illumina	SOAPaligner/ soap2	-	-	-	64,149	Niu et al., 2012
Panonychus citri	Illumina	SOAPdenovo	-	-	-	32,217	Liu et al., 2011
Tetranychus evansi	454 pyrosequencing	MIRA	-	-	1461	31,263	Villarroel et al., 2016
Dermanyssus gallinae	454 pyrosequencing	Newbler gsAssembler	81.5	-	1144	19,813*	Schicht et al., 2016
Phytoseiulus persimilis	454 pyrosequencing	GS Assembler	-	-	-	12,556	Cabrera et al., 2011
Metaseiulus occidentalis	454 pyrosequencing	Newbler and PTA (Paracel Transcript Assembler) v.3.0.0 (Paracel, Pasadena, CA)	-	-	-	30,691	Hoy et al., 2013
Tetranychus urticae[1]	Illumina	Trinity	-	-	-	22,941	Bu et al., 2015
Neoseiulus barkeri	Illumina				2126	34,211	Cong et al., 2016

[1] Red morph of *T. urticae*, considered by authors as a separate species *Tetranychus cinnabarinus* (Bu et al., 2015)
*Large contigs metrics (contig size ≥ 500 bp)

(Gong et al., 2017; Grigoraki et al., 2015, 2016; Mitchell et al., 2012; Müller et al., 2008; Yang and Liu, 2011).

Surprisingly, despite the high economic importance of spider mite pests (SECTION 1.3.1), research on identification of the molecular mechanisms underlying spider mite adaptation to acaricides and insecticides has not kept pace with that of insects over the last 20 years. However, recently, with the release of the high-quality Sanger-sequenced genome of *Tetranychus urticae* in 2011, custom-made GE microarrays have now begun to unravel the complex transcriptomic changes that are associated with *T. urticae* resistance to several commonly used acaricides (Demaeght et al., 2013; Dermauw et al., 2013; Khalighi et al., 2016; Pavlidi et al., 2017; Riga et al., 2014). For example, in one of the first studies, transcriptomic profiling revealed that transcription of *CYP392E7* and *CYP392E10* is constitutively up-regulated in *T. urticae* populations resistant to spirodiclofen, and that *CYP392E10* expression is highly induced upon exposure to spirodiclofen (Demaeght et al., 2013). Spirodiclofen, spirotetramat and spiromesifen inhibit acetyl-coenzyme A carboxylase (ACCase) and belong to spirocyclic tetronic acid derivatives, one of the most recently introduced mite control agents (Bretschneider et al., 2007). Functional characterization showed that the CYP392E10 enzyme metabolizes spirodiclofen and spiromesifen but not the systemic insecticide spirotetramat (Demaeght et al., 2013). Additional tests indicated however, that CYP392E10 is not able to metabolize spirodiclofen-enol, a presumed active metabolite of spirodiclofen (Brueck et al., 2009; Demaeght et al., 2013). Microarray analysis also revealed that a single CCE (CCE04) was highly overexpressed in the two spirodiclofen-resistant, genetically distinct spider mite strains (Demaeght et al., 2013). More in-depth molecular studies showed presence of two tautomeric forms of this gene, one identical to the CCE of the spirodiclofen susceptible strain and one that was overexpressed in spirodiclofen-resistant strains, carrying multiple amino acid substitutions (Demaeght et al., 2013). It is now suspected that the 'susceptible' allele of CCE may hydrolyze spirodiclofen to spirodiclofen-enol, as is clearly the case for spirotetramat-enol (Brueck et al., 2009), while the 'resistant' allele is involved in spirodiclofen-resistance in *T. urticae*. This is a plausible theory, since the functionally expressed resistant allele is less inhibited by spirodiclofen than the susceptible one, although the nature of the inhibition is not clear (Demaeght, 2015). Hence, the low levels of inhibition in the resistant strain might indicate that spirodiclofen is less efficiently metabolized to the enol-form which potentially protects mites from rapid intoxication, allowing the CYP392E10 to detoxify spirodiclofen (Demaeght, 2015). The transcriptional analysis in CHAPTER 2 of this thesis suggests that spirodiclofen resistance may be mediated by a similar molecular mechanism in *P. ulmi* as outlined for *T. urticae,* since both P450 (contig_01016/PuCYP_18) and CCE (contig_00577/PuCCE7) are one of the most up-regulated genes in the spirodiclofen-resistant PSR-TK strain (TABLE 2.8). However, these candidate genes still await functional characterization and thus their relative importance has not been confirmed yet.

To identify arthropod adaptations to insecticides via the transcriptomic level, previous studies on insects have indicated three important aspects of the experimental design. First, tissue-specific analysis of those tissues that are most abundant in transcripts coding for detoxification enzymes or channels should be performed. For instance, midgut-specific transcriptome assemblies and differential transcription analysis in insects greatly enhanced our understanding of Bt toxin resistance (Pauchet et al., 2009; Xie et al., 2012). Similarly, tissue-specific transcriptome of Oriental Armyworm, *Mythimna separate,* revealed that majority of genes putatively involved in insecticide resistance, were expressed in gut and salivary glands (Liu et al., 2016). Analysis of the salivary gland transcriptome of *Frankliniella occidentalis* identified genes likely to be involved in detoxification and inhibition of plant defense responses (Stafford-Banks et al., 2014). We did not analyze the tissue-specificity of the transcription of the detoxification genes involved in *P. ulmi* resistance (whole bodies of female adult spider mites were used, see SECTION 2.2.2), as dissecting different tissues is only possible with laser-based surgical techniques. In addition, it is currently not very clear which mite tissues, next to the gut, would be prime targets, and the location of detoxification has not yet been investigated. For instance, in Jonckheere et al. (2016), the authors did not succeed to dissect salivary glands of *T. urticae* and instead, were forced to dissect the whole mite's proterosomas to investigate gene expression levels of salivary genes. However, recent advances in *in situ* hybridization (Jonckheere et al., 2016, 2016) and immunohistochemistry (Van Leeuwen lab, unpublished) using cryosec-

tioning protocols may provide more insight into the localization of detoxification in spider mite. These advances would allow to at least partially dissect the spatial transcript patterns of detoxification enzymes such as the P450s and CCEs in *T. urticae* and *P. ulmi*.

Second, identification of life stage-specific resistance combined with stage-specific transcriptome sequencing can contribute to understanding more detailed mechanism related to physiological status, development and age. For instance, the different life stages of the whitefly *Bemisia tabaci* exhibit variable levels of resistance to neonicotinoids, thiamethoxam and imidacloprid, whereas transcriptome profiling revealed correlated stage-specific gene expression for several detoxification genes (Nauen et al., 2008a; Jones et al., 2011; Yang et al., 2013). Since previous studies in *T. urticae* show a correlation between the transcription of the spirodiclofen-metabolizing P450 monooxygenase CYP392E10 and resistance levels in eggs compared to the other life stages, it would be interesting for future studies to identify similar correlation patterns using other candidate resistance genes that we revealed in *P. ulmi* (Table 2.8).

Third, investigation of constitutive vs induced expression of detoxification genes is yet another factor to consider when designing transcriptomic experiments. Constitutive expression of detoxification enzymes is thought to be energetically costly, hence they become activated only when their action is required (Terriere, 1984). P450s are known for their ability to become induced by various chemicals, including pesticides (Feyereisen, 2012), and induction of P450s by several xenobiotics was previously linked to insecticide tolerance in, e.g., larvae of *Aedes aegypti* (David et al., 2013; Poupardin et al., 2008) and a susceptible strain of *T. urticae* (Van Pottelberge et al., 2008). Also, *CYP392E10* is constitutively overexpressed in resistant *T. urticae* lines, but has been found to be also strongly induced by spirodiclofen in the susceptible (48-fold) strain. Moreover, again in the resistant strain exposure further increased transcript levels > 150-fold. It would certainly be interesting to find out if overexpressed PuP450_18 in the PSR-TK strain of *P. ulmi* can also be induced by spirodiclofen.

Although we did not yet functionally characterize the enzymes coded by the genes of interest in spirodiclofen resistance in *P. ulmi,* we did perform an isoelectric focusing (IEF) gel electrophoresis on *P. ulmi* protein extracts to reinforce the overexpression of esterases (Figure 6.1). More specifically, we isolated protein samples from PSR-TK and HS strains and stained esterase activity after separating esterase isoforms according to their isoelectric point. There are several reports in which isozymes show more intense staining in resistant strains, for instance in the apple moth, *Epiphyas postvittana,* and the tobacco budworm, *Heliothis virescens* (Armstrong and Suckling, 1988; Harold and Ottea, 2000). IEF revealed the presence of an esterase band of high intensity in the resistant PSR-TK compared to the susceptible HS strain. This band presumably represents contig_00577/PuCCE7 which is overexpressed in PSR-TK. Additionally, there are two high intensity activity zones in the HS strain that are only vaguely visible in the corresponding region of the PSR-TK strain. These bands might reflect the two esterases (contig_00445/PuCCE2 and contig_06333) that are upregulated in HS (Table S2.9). A more in-depth functional characterization of the *P. ulmi* genes of interest can be achieved by using several experimental avenues. For instance, ectopic expression in *D. melanogaster* can reveal an association between the introduction of the gene and resistance levels. For instance, transgenic expression of the *Aedes aegypti CYP9J28* gene confers some level of pyrethroid resistance in *Drosophila melanogaster* (Pavlidi et al., 2012) while tissue-specific expression of the *D. melanogaster CYP6G1* and *Tribolium castaneum CYP6BQ9* genes provides evidence of this gene's involvement in resistance to multiple pesticides (Chung et al., 2009; Zhu et al., 2010). Ectopic expression of *T. urticae* resistance genes has been achieved in Riga et al. (2015), where *Drosophila* with high levels of *T. urticae* CYP392A11 transcription were found to be 2.6 fold more resistant to fenpyroximate than wild type flies. The applicability of this approach for the verification of the metabolic properties of *P. ulmi* PuCYP_18 and PuCCE7 remains to be verified, as susceptibility of *D. melanogaster* to spirocyclic tetronic acid derivatives is not known at present (Brück et al., 2009; Cantoni et al., 2008; Kontsedalov et al., 2008; Nauen et al., 2002, 2008b; Wachendorff et al., 2000).

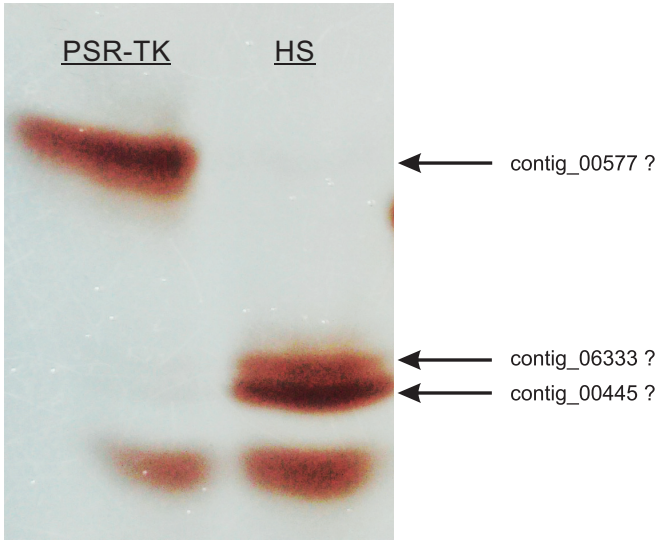

Figure 6.1 – IEF gel stained for esterase activity. The native esterase profile of *Panonychus ulmi* strains PSR-TK (resistant, left) and HS (susceptible, right) is depicted. An extra esterase band, visible in the resistant strain (potentially an esterase coded by contig_00577), is missing in the susceptible strain. In turn, the two bands, specific for the spirodiclofen-susceptible strain are not present in the PSR-TK.

6.1.2. Transcriptomics as a tool to investigate other evolutionary processes in arthropod pests

The xenobiotic metabolism of arthropod herbivores not only mediates resistance to synthetic insecticides, but in the first place allows these herbivores to cope with natural toxins that are produced as defense molecules by their host plant. Using a GE microarray platform, Dermauw et al. (2013) showed that transcription levels of acaricide resistance in *T. urticae* exhibit a strong positive correlation with transcription levels that are associated with host plant change. These results support the hypothesis that the evolution of gene families that allow *T. urticae* and other polyphagous herbivores to feed on a large number of plant species has predisposed these pests to rapidly evolve pesticide resistance (Gordon, 1961; Krieger et al., 1971). Therefore, an experimental set-up for a transcriptomic analysis that is designed to identify detoxification genes that underlie insecticide

resistance can also be applied to studies that focus on host plant adaptation and the detoxification of naturally occurring toxins. Previous transcriptomic studies have, for instance, characterized spider mite adaptation to tomato and grapevine (Díaz-Riquelme et al., 2016; Wybouw et al., 2015), the response of generalist and specialist caterpillars to *Arabidopsis* glucosinolates (Schweizer et al., 2017) and drosophilid adaptation to cactus (Hoang et al., 2015). *Panonychus ulmi* feeds almost exclusively on plants of the Rosaceae family (Migeon and Dorkeld, 2006-2017). These plants often defend themselves against herbivory by producing cyanogenic glucosides that release toxic cyanide upon herbivore attack. Wybouw et al. (2014) showed that upon adaptation of *T. urticae* to a cyanogenic bean cultivar, constitutive overexpression of a β-cyanoalanine synthase allows to detoxify cyanide. Mining the *P. ulmi* transcriptome, we annotated two contigs that are predicted to code for a β-cyanoalanine synthase enzyme. It would thus be very interesting for future studies to show that the presumable duplication of β-cyanoalanine synthase genes is related to the high levels of cyanide in Rosaceae. As *P. ulmi* shows a higher level of specialization to cyanogenic plants than *T. urticae*, it can be hypothesized that the *P. ulmi* β-cyanoalanine synthase enzymes have further specialized to efficiently detoxify cyanide.

6.1.3. Challenges and disadvantages of a transcriptomic approach to study evolutionary processes

Despite the advantages of RNAseq and *de novo* assembly of transcriptomes, the approach is not without its limitations. The length of the assembled read is a first critical factor that has an immediate effect on the quality and reliability of the transcriptome annotation effort. *De novo* assemblies are rich in short contigs of incomplete open reading frames (ORFs) that do not show homology to other genes in public databases at a given E-value. A second caveat of RNAseq studies is the identification of an appropriate depth of coverage. Genes that exhibit low transcription may not appear in the final transcriptome due to low coverage levels. Here, it is difficult to determine if such genes are truly absent from the genome or absence is a result of its undetectable gene-expression level (Maza et al., 2013; Tarazona et al., 2011). This is especially true for the non-normalized assembles, like the one of *P. ulmi*, where highly abundant transcripts may hamper de novo identification of low-expressed genes (Maza et al., 2013; Vogel and Wheat, 2011). Normalized transcriptomes do not allow for differential expression analysis but the variance in gene expression levels is reduced and normalized assembles provide a more even coverage across genes, increasing chance for discovery of lowly expressed genes (Maza et al., 2013; Vogel and Wheat, 2011). For instance, we initially failed to identify the β-cyanoalanine synthase gene from the *P. ulmi* CLC transcriptome assembly. As we knew from other genomic and transcriptomic sources that *P. ulmi* should have a homologue, we also investigated the alternative, Velvet/Oases assembly and identified the two β-cyanoalanine synthase contigs. A third obstacle is how to distinguish between allelic variants and true paralogues in *de novo* transcriptomic resources. A common assumption in transcriptome annotation pipelines is that paralogues are more divergent than alleles. For the annotation of *P. ulmi* and *Panonychus citri* transcriptomes (SECTION 2.2.4), we used a threshold of 94% nucleotide identity to call a contig an allelic variant. This arbitrary cut-off value was based on the nucleotide identity value obtained for two divergent ORFs that still mapped to the same region on the assembly (and were therefore very likely alleles). As a direct result of this inability to distinguish between allele and paralogue, identification of gene duplication as the molecular mechanism underlying the evolution of an adaptive trait is impossible (SECTION 1.2.2.1). As documented by Zimmer et al. (2017), gene duplication and its subsequent neofunctionalization is implicated in the evolution of imidacloprid resistance in the brown planthopper, *Nilaparvata lugens*. Here, gene duplication was found by mapping of Illumina HiSeq2500 sequenced DNA reads to the coding sequence of the investigated *CYP6ER1* and two reference genes, both of which are single copy genes in *N. lugens* (Zimmer et al., 2017). Finally, genes have recently been discovered in arthropod genomes that were horizontally acquired from fungi and bacteria (Wybouw et al., 2016, 2018). For example, the *T. urticae* genome harbors two vitamin B5 biosynthesis genes of bacterial origin that are predicted to help spider mites gain more independence from their often nutritionally poor host plants. In assemblies, horizontally transferred genes can only be reliably distinguished from contaminating bacterial reads based on genome continuity, when these genes have been shown to reside on large genomic regions that hold normal eukaryotic neighboring genes. As a result, a transcriptome assembly cannot reliably detect horizontally transferred genes and incorporate these in downstream analyses. This shortcoming is especially valid for spider mites, since their genomes have shown to be true hot spots for horizontally transferred genes (Wybouw et al., 2018). Hence, whole genome sequencing is the next logical step in *P. ulmi* molecular research and would greatly facilitate such techniques as genetic mapping. Below, I briefly discuss the *T. urticae* genetic mapping performed in CHAPTER 3 of this thesis and outline several cutting-edge methodologies that allow to map the molecular basis of pesticide resistance in more detail using a genomic assembly.

6.2. GENETIC MAPPING APPROACHES FOR STUDYING MONO- AND POLYGENIC RESISTANCE IN ARTHROPODS

The genetic architecture of insecticide resistance ranges from a monogenic trait, involving genes with large effect size, to a polygenic one with many genes involved (see also SECTION 1.2.1 for more information). In strains of tetranychid mites, classic genetic crosses reveal a polygenic basis for a range of acaricides, including ATP synthase inhibitors, cyhexatin (Mizutani et al., 1988) and propargite (Keena and Granett, 1990), uncoupler of oxidative phosphorylation via disruption of the proton gradient, chlorfenapyr (Van Leeuwen et al., 2004), inhibitor of acetyl CoA carboxylase, spirodiclofen (Van Pottelberge et al., 2009a), mitochondrial complex I electron transport inhibitor, tebufenpyrad (Van Pottelberge et al., 2009b) and glutamate-gated chloride channel (GluCl) allosteric modulator, abamectin (Clark et al., 1994). In CHAPTER 3 of this thesis, we explored the genetic architecture of pyridaben resistance in *T. urticae* using a traditional recombination mapping and QTL analysis. In that study, the resistance locus was mapped to a region on the 7th scaffold of the *T. urticae* genome assembly (FIGURE 3.4). Unfortunately, our microsatellite-based QTL mapping approach lacked the resolution to clearly decipher whether the *PSST* locus alone is responsible for pyridaben resistance (target-site resistance) or that neighboring P450 monooxygenases also play a role (metabolic resistance). The often complex genetic architecture of pesticide resistance

in mite and insect pests calls for statistically more powerful methodologies that work at higher resolutions. Below, I discuss such newly developed methodologies and outline their relevance for the study of polygenic resistance in arthropods.

Genome-wide association studies (GWASs) examine the genomic sequences of individuals within certain phenotypically divergent populations and are able to identify with a high resolution genetic polymorphisms that are associated with the phenotype of interest (Hirschhorn and Daly, 2005; Korte and Farlow, 2013). A range of GWA methodologies have been developed; in humans, the most common approach uses the case-control set-up (Clarke et al., 2011) where two large groups of individuals are compared against each other. The *Drosophila* Genetic Reference Panel (DGRP) (Mackay et al., 2012), a large collection of inbred *D. melanogaster* lines with sequenced genomes and transcriptomes was exposed to different concentrations of azinphos-methyl (Battlay et al., 2016). Based on LC_{50} values of the lines and associated genotypes, Battlay et al. (2016) identified four novel candidate genes associated with azinphos-methyl resistance and confirmed the role of *CYP6G1* in cross-resistance patterns in *D. melanogaster*. Weetman et al. (2010) genotyped 1500 resistance-phenotyped wild mosquitoes from Ghana and Cameroon using a 1536-SNP array enriched for candidate insecticide resistance gene SNPs, and showed that despite low linkage disequilibrium in wild pyrethroid-resistant populations of *Ae. gambiae,* association mapping can still identify the genetic basis of resistance.

Bulk segregant analysis (BSA) is yet another mapping approach where pools of progeny are examined that have been generated by a controlled cross between phenotypically and genotypically divergent parents (see SECTION 1.2.2.2 for more information). The BSA mapping method was originally developed for the identification of disease-resistance-linked genetic markers in plants (Michelmore et al., 1991) but has been successfully employed to map the genetic basis of several traits in various organisms, including arthropod species like *T. urticae* and the red flour beetle, *T. castaneum* (Demaeght et al., 2014; Jagadeesan et al., 2013; Van Leeuwen et al., 2012b). However, for optimal BSA mapping accuracy and resolution, the experimental set-up of the controlled cross, the number of screened progeny, and the specific mapping analysis all are important factors which I outline below in light of spider mite biology.

Since spider mite males are haploid, a cross between a resistant male and susceptible females introduces only one single resistant genotype in the cross and among the offspring. Adopting this single male set-up, Demaeght et al. (2014) were able to increase the BSA resolution in mapping the mite growth inhibitors (IRAC 10) resistance loci compared to a previous study, where several resistant males were crossed with susceptible females (Van Leeuwen et al., 2012a). Female spider mites can mate with their sons, a fact that can be used to create isogenic and inbred lines. Setting up a controlled cross using inbred lines that are homozygous on a genome-wide scale greatly enhances the mapping resolution and has, for example, been used to explore the albinism locus in spider mites (Bryon et al., 2017). An enormous amount of progeny from the controlled cross must be screened in order to maintain the diversity of recombination break-points. Indeed, based on simulation, Ehrenreich et al. (2010) showed that BSA can detect many small-effect loci with high resolution when mapping is performed on the progeny of crossed populations of a size that exceeds 10^5 individuals. Since *T. urticae* and other spider mite species exhibit high reproductive rates, this can easily be achieved. Investigation of polygenic traits in tetranychid mites could be additionally facilitated by creating a large number of replicates from the initial bulked population, hence in each replicate recombination under acaricide selection pressure, could occur independently. That would allow application of a permutation-based test that uses information from replicates to verify, that peaks are due to selection rather than genetic drift.

6.3. Pleiotropy

Pleiotropy describes the phenomenon where a single genotype has more than one distinctive phenotypic manifestation. However, depending on the research context, the term pleiotropy may refer to very distinct 'one-to-many' scenarios. Paaby and Rockman (2013) proposed three distinct definitions of pleiotropy that are not necessary mutually exclusive but rather represent different research interests. Molecular-gene pleiotropy defines the number of molecular functions that a gene has. In developmental pleiotropy, the unit of interest is a mutation rather than a certain gene. Here, pleiotropy underlies for example, phenotypic manifestations of genetic syndromes and evolutionary/developmental importance of cis-regulatory vs protein-coding variants (evo-devo theory) (Albin, 1993; Brunner and Van Driel, 2004; Carroll, 2008; Goodman and Coughlin, 2000). Finally, selectional pleiotropy refers to the number of fitness components that are affected by a certain gene and its encoded enzyme (Fisher 1999; Orr, 2000; Otto, 2004). The section below builds on the results in CHAPTER 5 of this thesis and further discusses the selectional pleiotropic effects of pesticide target-site genes in arthropods and how to reliably quantify them.

As predicted by classical theory based on Fisher's geometrical model of adaptation, *de novo* mutations that underlie pesticide resistance may carry a fitness disadvantage in a pesticide-free environment (Fisher 1999). Therefore, arthropods with newly arisen resistant alleles are expected to be less competitive compared to susceptible conspecifics. Thus, *de novo* mutations are likely to disappear in populations that are no longer exposed to pesticides, a process that can be used in agricultural crop protection strategies to counteract the development and spread of pesticide resistance (Crow, 1957).

6.3.1. Study of pleiotropy in arthropods – obstacles of the experimental set-up

It is not straightforward to unambiguously asses if a certain genomic variant is indeed responsible for a resistance phenotype and/or if it carries a pleiotropic fitness cost. Over the years, a vast amount of research has been performed on the potential fitness costs of a variety of resistance mechanisms to chemical compounds of different MoA (for a review, I refer to Kliot and Ghanim, 2012). Unfortunately, the reliability and quality of these studies varies considerably due to their experimental set-ups and methodologies. In a recent review, ffrench-Constant and Bass (2017) outline that studies of the pleiotropic fitness costs of pesticide resistance must be designed using a number of basic criteria. First, the principal resistance mechanisms must have evolved in the field and not under laboratory conditions. In laboratory-selected strains, prolonged and repetitive pesticide application may lead to the evolution of resistant genotypes which may not be representative for the genotypes found in the field

(McKenzie, 1994). For instance, despite the fact that different P450s can cause DDT resistance in the laboratory-selected strains of *D. melanogaster*, field-collected strains always show over-expression of the same P450 gene, *CYP6G1* (Daborn et al., 2001; Daborn et al., 2007). In CHAPTERS 4 and 5 of this thesis, all investigated target-site resistance mutations were originally identified in field or greenhouse populations with a history of acaricide application. Hence in-depth examination of those genotypes has a clear benefits for the Insecticide resistance management (IRM) of acaricide target-site resistance in the field.

Second, ffrench-Constant and Bass (2017) stated that the genotypes of susceptible and resistant strains should be known and compared within an identical genetic background. Indeed, by ensuring an identical genomic background, potential different fitness measures can be directly linked to the causal resistant allele. In this thesis, we have met this strict criterion by generating isogenic lines by marker-assisted back-crossing of the resistant allele into a susceptible back-ground over the course of 6 to 9 generations. Although this represents a unique genetic resource, a weakness of back-crossing may, however, surface when a number of resistance loci are located close to one another. Hospital (2001) estimated that without background selection, the expected length of an intact donor segment on one side of the locus of interest is 10 cM even after 10 generations of back-crossing (under the assumption that the locus of interest is located 100 cM from the chromosome end). Hence, this second criterion is not easily met due to the biology of the arthropod pest under study. *Panonychus* mites are characterized by the same reproductive strategy and similar reproductive characteristics as *T. urticae*, but cannot be cultivated on fast growing plants like *Phaseolus vulgaris*, which are used in our lab as hosts for *T. urticae*. Nonetheless, for an on-going study (the inbreeding of the *P. ulmi* HS strain), I have shown that individual *P. ulmi* mites can be manipulated and that controlled crosses can be performed on detached plum leafs, resting on a wet cotton pad.

Third, ffrench-Constant and Bass (2017) favor experiments where resistant genotypes directly compete with their susceptible conspecifics to measure selectional pleiotropy. In my view however, experiments that quantify single-generation life history parameters may be equally, if not more, informative. Although direct competition studies are the most efficient and straightforward way to assess potential fitness costs, they do not single out the specific fitness component that put a resistant strain at a disadvantage. To study in detail if and how certain genomic variants hinder fitness, one should gather as many data on various life history traits as possible and examine their impact on the population's intrinsic rate of increase and /or other life table parameters, and consequently on the population buildup over time. On the other hand, I acknowledge that with the experimental set-up used in CHAPTER 5, we might have overlooked some specific affected fitness components. In several cases, the pleiotropic effect of resistance to insecticides that affect the insect nervous system, such as organophosphates, DDT and pyrethroids, has been associated with changes in the behavior of insects, which ultimately lowered mating efficiency, the ability to find a blood meal and survival under certain conditions such as predator attack, hence influencing the overall success of the population (Brito et al., 2013; Diop et al., 2015; Foster et al., 2000, 2003). Based on life history traits and calculated life table parameters, we have observed no fitness cost associated with the L1024V mutation in the voltage-gated sodium channel, conferring pyrethroid resistance (TABLES 5.2, 5.3 and 5.4). A logical next step would be therefore to investigate the mutation-carrying lines for, e.g., mobility, ability to find a mate, feeding efficiency or vulnerability to predators.

Finally, ffrench-Constant and Bass (2017) argue that experiments should be performed under field conditions, including seasonal temperature and humidity alterations, spatial and temporal plant host switches, predation and competition for food resources (Roush and Daly, 1990). To measure the pleiotropic fitness costs of target-site mutations, we have maintained the experimental isogenic lines under laboratory settings. Although we are aware of the short-comings of this approach, we feel that migrant *T. urticae* genotypes carrying other resistance mechanisms would severely bias our results if we would have placed our isogenic lines in the field. Previous studies have indicated that temperature and host plant have the biggest influence on population growth in spider mite species (TABLE 1.2) (Badii et al., 2003; Kaur and Zalom, 2017; Praslicka and Huszar, 2004; Sohrabi and Shishehbor, 2008; White and Liburd, 2005). We therefore believe that it would be most interesting for future studies to repeat and expand our laboratory experiments by testing our near-isogenic lines under varying temperature regimes, while mites infest different host plants (FIGURE 1.2).

6.4. CRISPR – A FUNCTIONAL GENETICS AT HAND

A key purpose of genetics is to identify which genes are responsible for certain phenotypes. Forward genetics is a hypothesis-driven approach, where the casual gene is mapped based on its phenotypic manifestation. Advent of genome sequencing and genome-wide technologies revolutionized forward genetics, allowing robustness and a statistically powerful analysis of gene expression patterns, genetic polymorphism and gene regulation, significantly improving our ability to associate a certain phenotype or disease state with a particular genotype (McCarthy et al., 2008; Zhu et al., 2016). Nonetheless, when gene function is unknown, an in-depth analysis must be performed to investigate possible pleiotropic effects. If this is the case, a candidate-driven reverse genetics approach like genome editing is the way to go.

The last 20 years have witnessed the development of genome editing tools that allow selective mutagenesis of precise genomic regions. These technologies are based on DNA binding factors that recognize certain sequential patterns within DNA. Upon recognition of a target-site, these factors act through introducing a double stranded break (DSB) in a DNA molecule. Depending on the purpose of the experiment, DSB can be then repaired by non-homologous end joining (NHEJ) or homologous recombination (HR) (Bibikova et al., 2002). NHEJ is preferred by the living cell, but is an error-prone strategy, usually yielding deletion or insertion of a few bases at the cutting site (Bibikova et al., 2002). HR occurs only in the presence of a template, which in excess has to be provided to the cell. In genome editing procedures, HR is typically used to introduce a defined alteration to the target sequence (Beumer et al., 2006, 2008; Bibikova et al., 2003). Zinc finger nucleases (ZFNs) are artificially engineered endonucleases consisting of a zinc finger DNA-binding domain and a DNA-cleavage domain. Zink finger technology has been used to manipulate genomes of many plants and animals including *Arabidopsis* (Zhang et al., 2010), soybean (Curtin et al., 2011), *Drosophila* (Bibikova et al., 2003), frogs (Young et al., 2011) and

cattle (Yu et al., 2011). The major drawback of this procedure is binding specificity, because binding preference of each Zink finger within the multimeric ZFN complex depends on the context of surrounding zinc fingers and DNA (Bulyk et al., 2001; Del Rio and Setzer, 1993), which complicates the use of ZNF on a broader scale. Yet another class of DNA-binding proteins used for genome editing is called transcription activator like effectors (TALEs). TALEs are engineered by fusing a TAL effector DNA-binding domain to a DNA cleavage domain. A TALE DNA-binding domain consists of highly repetitive stretches of 33 amino acids, which bind to a DNA molecule in a highly predictive manner upon recognition by two amino acids, the so-called repeat variable diresidue (RVD; (Boch et al., 2009; Moscou and Bogdanove, 2009). TALEs can be designed to provide exquisite binding specificity. Unfortunately, the large size of the resulting polypeptides may be problematic for manipulation.

As described in section 1.2.2.2., clustered regularly interspaced short palindromic repeats (CRISPR-Cas9) are a form of acquired immune response in bacteria but have been co-opted as a RNA-mediated nuclease for use in targeted genome engineering (Cong et al., 2013; Deltcheva et al., 2011; Garneau et al., 2010; Gratz et al., 2013). For the last 5 years CRISPR-Cas9 system has been revolutionizing the field, since it is easier to design and handle, more efficient and less cytotoxic than other genome editing approaches. Fusion of the crRNA and tracrRNA into a ~100 nt synthetic single guide or chimeric RNA (sgRNA or chiRNA) simplified the system, which now consists of only two components, the nucleotide-binding domain sgRNA/chiRNA and the Cas9 endonuclease, while a template for homologous recombination is optional, depending on the experimental purpose (Cong et al., 2013; Jinek et al., 2012; Mali et al., 2013). Specificity of binding in the CRISPR-Cas9 system relies on recognition of a 20 nt sequence at the 5' end of the sgRNA. The oligonucleotide can be designed to match any DNA fragment. The only limitation to this design is imposed by a protospacer adjacent motif (PAM) consisting of an NGG triad, which must follow the sequence recognized by the guide RNA, in order for efficient cleavage to occur. Considering the NGG motif occurs approximately every 8 nucleotides within a genome, it usually does not present significant hindrance to the experimental design.

Due to its status as model organism and the ample availability of molecular tools, *Drosophila* was used for CRISPR-Cas9 manipulations before any other insect, and a multitude of web resources facilitating successful design of CRISPR-Cas study have become available for this fruit fly (reviewed in Bassett and Liu, 2014). A number of groups have used the CRISPR-Cas9 system to edit the *Drosophila* genome using different approaches to supply the flies with Cas9 and sgRNA molecules, which yielded different rates of successful modifications (Bassett et al., 2013; Gratz et al., 2013; Kondo and Ueda, 2013; Ren et al., 2013; Yu et al., 2013). An approach that gave one of the most promising results was originally proposed in (Port et al., 2014; Ren et al., 2013), and adopted in chapter 3 of this thesis, to introduce point mutations into the 20kDa subunit of the PSST homologue of *D. melanogaster*. The procedure presents a solution to avoid potentially problematic somatic editing by restricting Cas9 expression to the germline cells, under the control of nanos (Port et al., 2014; Ren et al., 2013) or vasa promoters (Sebo et al., 2014). The donor templates for HR and sgRNAs are delivered in the form of plasmids, where in vivo reverse transcription of sgRNAs is driven by the U6 promoter. Another method, that has shown to give > 90% of flies that have at least one mutated offspring, was presented by Kondo and Ueda (2013). Here, two transgenic lines are produced, one expressing Cas9 in the germline under the *nanos* promoter, and a second with ubiquitous expression of the sgRNA, again driven by the U6 promoter. Crossing those two lines results in highly efficient mutagenesis and does not involve embryo injection, which makes it more reproducible. The downside of this method, however, is the time-consuming generation of the transgenic flies and elimination of the Cas9 protein, once the mutant is produced.

So far, Cas9-mediated DSB has been used most frequently to introduce loss-of-function mutations. In this case, NHEJ typically introduces a mutation which frequently leads to a coding frame-shift and premature termination of translation. Screening of loss-of-function mutants has already been used in a high-throughput approach for the genome-wide knockout analysis in mammalian cells (Koike-Yusa et al., 2014; Shalem et al., 2014; Wang et al., 2014). In arthropods, frameshift mutations were successfully introduced in *D. melanogaster* and several other species including silk worm, *Bombyx mori*, the water flea *Daphnia magna*, the mosquito *Culex quinquefasciatus* and *Plutella xylostella* (Huang et al., 2016; Itokawa et al., 2016; Nakanishi et al., 2014; Wang et al., 2013). Other applications of CRISPR-Cas9 involve a HDR-mediated, precise knock-in of a whole gene (Gilles et al., 2015; Ma et al., 2014; Yu et al., 2014) as well as precise single-nucleotide substitutions (Douris et al., 2016; Grigoraki et al., 2017; Somers et al., 2015; Zimmer et al., 2016). One of the most innovative approaches using Cas9 technology is engineering evolutionary stable gene drives for the control of invasive insect species and insect vector-borne diseases (Esvelt et al., 2014; Gabrieli et al., 2014; Li and Scott, 2016), where the artificially introduced gene spreads through the target population much faster than in case of natural genetic inheritance [for an overview of the procedure see Hammond et al. (2016); Sinkins and Gould (2006)]. Screening opportunities using Cas9 extend beyond coding genes. Catalytically inactive Cas9 (dCas9) has been shown to modulate levels of gene transcription, without introducing irreversible mutations to the genome. This approach has not yet been applied in arthropods but satisfactory and moderate levels of repression have been shown in *Escherichia coli* and mammalian cells (Bikard et al., 2013; Larson et al., 2013). A custom-designed sgRNA library can be used for the unbiased identification of regulatory sequences by covering non-coding genomic regions with sgRNAs (Cheng et al., 2013) while the epistatic interaction between pairs of genes can be facilitated by the delivery of multiple sgRNAs (Kampmann et al., 2013).

6.4.1. CRISPR for insecticide resistance research

Up to date, nine studies have used Cas9 for a functional validation of genes involved in resistance (Table 6.2). Six studies exploited *D. melanogaster* as an experimental subject for its ease of manipulation and availability of molecular tools (Bajda et al., 2017; Douris et al., 2016, 2017; Grigoraki et al., 2017; Somers et al., 2015; Zimmer et al., 2016). Two studies were performed on *Helicoverpa armigera* (Wang et al., 2016a, 2017), one on *C. quinquefasciatusa* (Itokawa et al., 2016). The majority of alterations relied on a single nucleotide substitution, that alters the target-site of an insecticide (Table 6.2). The two frame-shift mutations in-

TABLE 6.2 – Overview of studies that use CRISPR-Cas9 to investigate pesticide resistance related targets.

Species	Target of modification	Expected modification	Result	Reference
Drosophila melanogaster	a6 subunit of the nicotinic acetylcholine receptor	Single nucleotide alteration (P146S)	Decrease in sensitivity to spinosad	Somers et al., 2015
Drosophila melanogaster	a6 subunit of the nicotinic acetylcholine receptor	Single nucleotide alteration (G275E)	Decrease in sensitivity to spinosad	Zimmer et al., 2016
Helicoverpa armigera	Cadherin	Frame shift mutation	Decrease in sensitivity to Cry1Ac	Wang et al., 2016
Culex quinquefasciatus	CYP9M10	Frame shift mutation	Decrease in resistance to pyrethroid	Itokawa et al., 2016
Drosophila melanogaster	Chitin synthase	Single nucleotide alteration (I1056M/F/L)	Cross-resistance to etoxazole, benzoylureas and buprofezin	Douris et al., 2017; Grigoraki et al., 2017
Drosophila melanogaster	PSST subunit of respiratory Complex I	Single nucleotide alteration (H95R)	inconclusive	Bajda et al., 2017
Drosophila melanogaster	Ryanodine receptors	Single nucleotide alteration (G4946V)	High RRs to flubendiamide and chloranthraniliprole and low RR, to cyantraniliprole	Douris et al., 2017
Helicoverpa armigera	HaABCA2	Frame shift mutation	High RR to Cry2Aa and Cry2Ab	Wang et al., 2017

duced in *H. armigera* showed that, disruption of cadherin causes resistance to the BT toxin Cry1Ac while knockout of HaABCA2 leads to high levels of resistance to both Cry2Aa and Cry2Ab but not to Cry1Ac (TABLE 6.2). Knockout of *CYP9M10* in *C. quinquefasciatus* showed that the P450 product of that gene plays an important role in pyrethroid metabolism (TABLE 6.2).

Despite its well-established use in *Drosophila*, interpretation of CRISPR-Cas9 results may be sometimes difficult. In CHAPTER 3 of this thesis, I attempted to validate the function of a H92R mutation in the PSST subunit of Respiratory complex I found in METI-I resistant *T. urticae*. When this mutation was introduced at the homologous position in *D. melanogaster,* it could not be brought to homozygosity in any of the independently generated lines, and hemizygous males were not found, rendering the results of the approach inconclusive. As discussed in SECTION 3.4, any off-target effect of our CRISPR-Cas9 approach is highly unlikely, given the specificity of the used sgRNAs. In addition, outcrossing and allowing recombination of the X-chromosome failed to generate hemizygous males carrying the mutation, and so the phenotype could not be uncoupled from the mutation. Based on that evidence, we assumed that the H92R mutation is lethal. Further deliberations, however, brought to conclude that the cause of lethality cannot be unambiguously determined based on the available data. *Drosophila* flies may gain deleterious mutations in a natural way and if a lethal locus is located very close to the *Drosophila's* 20kDa homologue of PSST, recombination on the X-chromosome may not be able to disentangle two loci, or segregation may occur at a very low frequency. The only reliable strategy to eliminate the effect of closely linked lethal loci would be to perform a complementation tests with flies that contain the 20kDa duplication on a autosomal chromosome (in wild type flies, the 20kDa subunit is encoded on the sex chromosome). By using the autosomal duplication, one can be sure that a functional copy of 20kDa will always be present in the progeny. In case the 'lethality' is associated with X-linked H92R, crossing of heterozygous females carrying the mutated allele with hemizygous males carrying the wild type gene at the autosome would result in complementation and presence of a viable male progeny carrying the H92R allele. In turn, wild-type-only male progeny would be a clear indication that the lethality is not associated with 20kDa, and lies somewhere else on the X chromosome, in a region outside of the duplicated one, since it does not complement it.

Where in model organisms issues as the one described above can be resolved thanks to the availability of a broad range of molecular tools, in non-model species CRISPR design and potential problem solving is much more difficult or even not possible. This is especially troubling in case of agriculturally important pest species such as *T. urticae*. Recently, Bryon et al. (2017) aimed at disrupting phytoene desaturase using CRISPR-Cas9 to investigate its role in carotenoid accumulation and diapause in *T. urticae*. Out of 20,000 F1 male progeny, 15 males were identified that either lacked all pigmentation or were partially albino. Further experiments suggested, however, that the screen recovered genotypes unrelated to the CRISPR editing, but were most likely rare alleles that were segregating at very low frequencies in the original population of *T. urticae* (Bryon et al., 2017). One may speculate about the reasons of a failed approach. A flaw in the approach was the strategy for delivering the CRISPR-Cas9 components to the *T. urticae* cells, where microinjections were performed not on eggs, but on gravid females. Efficient delivery of genome-editing proteins into the target cells is challenged by their proteolytic instability and poor membrane permeability (Fu et al., 2014). Wang et al. (2016b), proposed that the success of delivery of the anionic Cas9:sgRNA complex can be greatly enhanced when combined with bio-reducible lipid nanoparticles, acting as delivery vehicles.

6.5. REFERENCES

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Summary

'Genetics and fitness costs of acaricide resistance in spider mites'

The Tetranychidae family (Arthropoda: Chelicerata: Acariformes) holds over 1,250 spider mite species, and includes some of the most damaging agricultural pests. In this thesis, I focused on the two-spotted spider mite, *Tetranychus urticae,* and the European red mite, *Panonychus ulmi,* since the management of populations of both spider mite species via chemical compounds is becoming increasingly challenging due to their exceptional ability to adapt to any acaricide class that have been used for their control.

In CHAPTER 1, I outlined how agricultural intensification, crop domestication, and artificial selection of elite cultivars have increased vulnerability of modern agro-ecosystems to arthropod-related production loss, making them dependent on continuous human intervention. The most accessible and efficient way of crop protection against arthropod-induced damage is still achieved through application of insecticides or acaricides. Regardless the abundance of different chemical classes and various modes of action (MoAs), arthropod species typically evolve resistance to the applied pesticides. Phytophagous mites and insects adapt to natural and synthetic toxins using a multitude of strategies. These strategies can be classified by the nature of the physiological changes that lead to higher resistance levels or, as recently proposed, by the underlying molecular genetic mechanisms. Following the physiological based classification, these strategies are categorized as either toxicodynamic or toxicokinetic. Toxicokinetic modifications affect the enzymatic modification, penetration through different cell layers, sequestration, and excretion of a pesticide. Toxicodynamic modifications on the other hand, typically involve alterations of the pesticide target-site. For managing resistance and estimating evolutionary dynamics of resistance development, the molecular genetic classification might be more relevant than understanding the physiological implications. Hence, with the advances in molecular biology, insecticide resistance management strategies rely on molecular studies for identification of specific mutations and mechanisms governing pesticide resistance. Identification of these mechanisms allows for more efficient pest control, for instance by avoiding rotational use of the same MoA pesticides. In CHAPTER 1, I also highlight the importance of research on fitness cost associated with pesticide-resistant genotypes and its application as a potential Integrated Pest Management (IPM) strategy. I also introduce *T. urticae* and *P. ulmi* and describe their biological, behavioral, and demographic characteristics in light of their adaptive advantages that led to the successful colonization of so many annual and perennial plant species.

In CHAPTER 2, we produced the first big genomic data, a strand-specific RNA-seq based transcriptome resource for *P. ulmi*, providing means to initiate molecular research on this devastating mite species. RNA sequences were derived from spirodiclofen-susceptible and -resistant strains of *P. ulmi*, since spirodiclofen, although relatively recently introduced, is a widely used acaricide and its detoxification mechanism in *P. ulmi* is not known.

From a *de novo* assembly of the *P. ulmi* transcriptome, we manually annotated detoxification enzyme families, target-sites of commonly used acaricides, and horizontally transferred genes implicated in plant-mite interactions and pesticide resistance. In a comparative analysis that incorporated sequences available for *Panonychus citri, T. urticae*, and various insect species, we identified radiations for detoxification gene families following the divergence of *Panonychus* and *Tetranychus* genera. Finally, we used the replicated RNA-seq data from the spirodiclofen susceptible and resistant strains to describe gene expression changes associated with resistance. A cytochrome P450 monooxygenase, as well as multiple carboxylcholinesterases, were differentially expressed between the susceptible and resistant strains, and provide a molecular entry point for understanding resistance to spirodiclofen. We showed that the *de novo* transcriptome provides a valuable resource to accelerate molecular research on non-model arthropod species, including investigation of pesticide detoxification mechanism and target-site resistance.

In CHAPTER 3, we investigated strains of *T. urticae* for presence of target-site resistance to Mitochondrial Electron Transport Inhibitors I (METI-Is). METIs act on respiratory complex I (NADH: ubiquinone oxidoreductase), a large multi-subunit membrane protein with a central role in oxidative phosphorylation. After their initial introduction in the early 90s, METIs gained immense popularity amongst growers, for they act fast, have good knock-down activity, and provide sufficient residual control. Nonetheless, resistance to these compounds was reported relatively quickly after their initial launch. Resistance mechanisms are known to be complex and hydroxylation of the tertiary butyl group is thought to be a common mechanism of detoxification, while there are no reports identifying target-site resistance. Here we exploited the available whole genome sequence of *T. urticae* to identify, annotate, and sequence the relevant complex I subunits that are thought to form the binding site of METI-Is in both susceptible and characterized METI-I resistant strains. A single point mutation, H92R, in the *T. urticae* homologue of the *Bos taurus* PSST subunit was identified and its potential role in METI-I resistance was further studied by modeling, selection experiments, and genetic mapping. Using marker-assisted introgression, we introduced the mutation into a *T. urticae* line with susceptible genetic background and studied its effect on the resistant phenotype. Last, we used a complementary approach to evaluate the effect of a resistance mutation in *Drosophila* as a model. We used the CRISPR-Cas9 genome editing tool to introduce the mutation conferring resistance to METI-I in the *Drosophila* PSST homologue. Entire experimental evidence gathered in CHAPTER 3 including the discovery of H92R in METI-I resistant strains, supplemented with evidence coming from genetic crosses and the location of the mutation being relevant for inhibitor binding, supports a causal link between H92R and METI-I resistance in *T. urticae*. In addition, our study contributes to a general understanding of the respiratory complex I structure and the mechanism of inhibitor binding.

The relative contribution of target-site resistance mutations to the resistance phenotype has remained poorly understood in

most arthropod pests. Therefore, in CHAPTER 4, we used the marker-assisted backcrossing approach from CHAPTER 3 to create 30 congenic lines of *T. urticae*. These lines share a common pesticide-susceptible genomic background, except for 9 loci that carry mutations (alone, or in combination in a few cases) that are associated with resistance to avermectins, pyrethroids, mite growth inhibitors, and mitochondrial complex III (QoI) inhibitors. Toxicity tests revealed that mutations in the voltage-gated sodium channel, chitin synthase 1, and cytochrome b confer high levels of resistance and, when fixed in a population, these mutations alone can result in field failure of acaricide treatment. In contrast, the glutamate-gated chloride channels mutations do not lead to the high resistance levels that are often reported in abamectin-resistant strains of *T. urticae*. Overall, this study functionally validates reported target-site resistance mutations in *T. urticae*, by uncoupling them from additional mechanisms, allowing to finally investigate the strength of the conferred phenotype in vivo.

Consequently, in CHAPTER 5, we took advantage of the near-isogenic lines created in CHAPTER 4 to investigate possible fitness costs that are associated with the well-characterized acaricide target-site resistance mutations. Theory predicts that although resistance mutations may exhibit advantageous effect when acaricide selection pressure is present, they might convey negative pleiotropic effects on other aspects of fitness. Resistance due to *de novo* mutations is thus likely to disappear in the absence of the insecticide/acaricide treatment if such fitness costs are in place, a process that would counteract the spread of resistance in agricultural crops. We analyzed *T. urticae* lines carrying P262T in the mitochondrial cytochrome b, the combined G314D and G326E substitutions in the glutamate-gated chloride channels, L1024V in the voltage gated sodium channel, and I1017F in chitin synthase 1 in comparison to their susceptible conspecifics. Five fertility lifetable parameters and six single-generation life history traits were quantified and compared across a total of 15 mite lines. In addition, we monitored the temporal resistance level dynamics of populations with different starting frequencies of the chitin synthase-resistant allele to further support our findings. Three target-site resistance mutations, I1017F and the co-occurring G314D and G326E mutations, were shown to significantly and consistently alter certain fitness parameters in *T. urticae*. The other two mutations, P262T and L1024V did not result in any consistent change in the studied fitness parameters. Our findings are important considering the global spread of *T. urticae* pesticide resistance, and within an IPM context.

Last, in CHAPTER 6 I discussed my findings in a larger perspective, deliberating possible methodological improvements and future research directions. I briefly introduced transcriptome technology, discussed its advantages and disadvantages and showed how a transcriptome is a valuable resource for future toxicological studies on arthropods. Further, in the light of CHAPTERS 3 and 4, I discussed how polygenic traits can now be mapped using statistically powerful, bulk segregant analysis (BSA). I analyzed the caveats and complexities of the experimental set-up of pesticide-resistance-related fitness cost studies in the light of research on Tetranychidae mites. I then moved on to discuss the achievements of functional genetics in the field of resistance and mode of action research, in the age of the CRISPR-Cas9 reverse genetic tool. Last, I further discussed the possible methodological improvements that could have been applied to the CRISPR approach used in the CHAPTER 3 of this thesis.

Samenvatting

'Genetica en fitnesskosten van acaricide-resistentie bij spintmijten'

Spintmijten uit de familie van de Tetranychidae (Arthropoda: Chelicerata: Acariformes) behoren tot de belangrijkste plagen van gewassen wereldwijd. De bonenspintmijt *Tetranychus urticae* en de Europese rode spintmijt *Panonychus ulmi* beschikken over een groot adaptief vermogen waardoor populaties van beide plaagsoorten snel resistentie ontwikkelen tegen allerlei chemische bestrijdingsmiddelen (acariciden). In dit proefschrift worden de moleculaire mechanismen die ten grondslag liggen aan de acarideresistentie in *T. urticae* en *P. ulmi* onderzocht.

In HOOFDSTUK 1 wordt in grote lijnen de stand van zaken beschreven met betrekking tot plaagbestrijding in de land- en tuinbouw, met een nadruk op de evolutionaire ontwikkeling van verschillende detoxificatieroutes in plaagsoorten binnen de groep van de geleedpotigen. Ik bespreek ook de gevolgen van resistentieontwikkeling voor andere levensgeschiedeniskenmerken van plaagsoorten en beschrijf hoe deze pleiotropische kosten van belang zijn voor chemische bestrijdingsstrategieën.

Gebruikmakend van *RNA-seq* technologie wordt in HOOFDSTUK 2 een eerste genoombrede sequentiedatabank gegenereerd voor de spintmijt *P. ulmi*. De bekende genfamilies van detoxificatie enzymen werden geannoteerd en hun evolutionaire geschiedenis werd fylogenetisch onderzocht. Deze analyses toonden onder andere aan dat de cytochroom P450 genfamilie een substantiële expansie heeft ondergaan in spintmijten binnen het geslacht *Panonychus*. Het *P. ulmi* transcriptoom diende vervolgens als referentie voor het ontcijferen van de genetische basis van spirodiclofenresistentie in deze olifage plaagsoort. Genoombrede transcriptieniveaus werden bepaald in een spirodiclofen-gevoelige en een spirodiclofen-resistente *P. ulmi* populatie. Statistische vergelijking van deze transcriptieniveaus wees één bepaalde cytochroom P450 paraloog aan als kandidaatgen voor spirodiclofenresistentie.

In HOOFDSTUK 3 richt ik mij op *target-site* resistentie in de spintmijt *T. urticae* tegen *Mitochondrial Electron Transport Inhibitors I* (*METI-I*s), een belangrijke groep van acariciden die wereldwijd al wordt gebruikt sinds 1990. Veldresistentie tegen *METI*-Is heeft zich echter relatief snel ontwikkeld en vormt tegenwoordig een significant probleem in de gewasbescherming. Met behulp van de ontcijferde sequentie van het genoom van *T. urticae* was ik in staat om de sequentie van het ademhalingscomplex I, de *target-site* van *METI-I*s, te analyseren in verscheidene resistente en gevoelige spintmijtpopulaties. Er kon een strikte associatie tussen de puntmutatie H92R in het PSST gen en METI-I resistentie worden geïdentificeerd. In een reeks experimenten werd de potentiële rol van H92R als moleculair mechanisme voor *METI-I* resistentie nader onderzocht en ondersteund via modellering, experimentele evolutie, en genetische kartering. Vervolgens heb ik een terugkruisingsmethode voor *T. urticae* ontwikkeld, die mij in staat stelde H92R in een bestaande, gevoelige genoomachtergrond te introduceren. Dit experiment toonde aan dat de mutatie in *T. urticae* alleen verantwoordelijk gesteld kan worden voor intermediaire niveaus van *METI-I* resistentie. Na introductie via het CRISPR-Cas9 genmodificatie systeem bleek de H92R mutatie lethaal te zijn in de fruitvlieg *Drosophila melanogaster*. Deze studie genereerde aldus via een multi-disciplinaire aanpak bewijs voor een causaal verband tussen H92R en *METI-I* resistentie in spintmijten.

De relatieve bijdrage van *target-site* resistentie aan de hoge resistentie niveaus die in populaties van geleedpotige plaagsoorten frequent worden waargenomen is in grote lijnen nog onbekend. Om dit recht te zetten heb ik in HOOFDSTUK 4 de terugkruisingstechniek uit HOOFDSTUK 3 benut om het specifiek effect van een selectie van bekende *target-site* mutaties op acarideresistentie in *T. urticae* te karakteriseren. Meer specifiek heb ik de bijdrage aan resistentie tegen avermectinen, pyrethroïden en inhibitoren van groei en van de mitochondriale ademhaling gemeten. Toxiciteitsproeven toonden aan dat de puntmutaties in de natriumkanaal, chitine synthase en mitochondriale cytochroom b genen hoge resistentieniveaus induceren. De puntmutaties in de glutamaat-afhankelijke chloride kanaalgenen lieten een ander beeld zien; hier werden na terugkruisen slechts middelmatige resistentieniveaus gemeten. Hiermee levert deze studie een functionele validatie van negen vaak gerapporteerde target-site resistentiemutaties in spintmijten.

Evolutionaire theorie voorspelt dat *de novo* mutaties die een positief effect hebben op het resistentieniveau een negatief pleiotropisch effect kunnen hebben op andere fitness componenten van de plaagsoort. In HOOFDSTUK 5 wordt deze hypothese getoetst door de impact van eerder gekarakteriseerde *target-site* mutaties op meerdere fitnesscomponenten van *T. urticae* te onderzoeken. In deze experimenten is gebruik gemaakt van de teruggekruiste lijnen die in HOOFDSTUK 4 werden gegenereerd. Vijf fecunditeitsparameters en zes levensgeschiedeniskenmerken werden bepaald en statistisch vergeleken tussen de verschillende gevoelige en resistente mijtenlijnen. Drie resistentie puntmutaties (I1017F, en de combinatie van G314D en G326E) hadden een significant negatief effect op bepaalde fitnessparameters. Dit hoofdstuk sluit af met een discussie over de manier waarop deze resultaten kunnen bijdragen aan een betere gewasbescherming.

In HOOFDSTUK 6, ten slotte, bediscussieer ik mijn bevindingen in de HOOFSTUKKEN 2 tot 5 in een breder kader. Ik geef een korte inleiding in de nieuwe transcriptoomtechnologieën en technieken in het genetisch karteren van kenmerken en argumenteer hoe deze kunnen bijdragen aan het onderzoek naar resistentiemechanismen. Ik bespreek ook de nieuwste ontwikkelingen in de functionele genetica, met een nadruk op de toepassing van CRISPR-Cas9 technologie in de fruitvlieg *D. melanogaster*.

Authors contributions

Chapter 2 – Transcriptome profiling of a spirodiclofen susceptible and resistant strain of the European red mite Panonychus ulmi using strand-specific RNA-seq
S. Bajda, W. Dermauw, R. Greenhalgh, R. Nauen, L. Tirry, R.M. Clark & T. Van Leeuwen

SB, WD, RMC and TVL designed research. SB and WD performed experiments. WD, SB and RG analyzed data. SB, WD and TVL wrote the manuscript, with input from RMC, RG, RN and LT.

Chapter 3 – A mutation in the PSST homologue of complex I (NADH:ubiquinone oxidoreductase) from Tetranychus urticae is associated with resistance to METI acaricides
S. Bajda, W. Dermauw, R. Panteleri, N. Sugimoto, V. Douris, L. Tirry, M. Osakabe, J. Vontas & T. Van Leeuwen

SB, TVL, WD, VD and MO designed research. SB, RP and NS performed experiments. SB, WD and VD analyzed data. SB, TVL wrote manuscript with input from WD, VD, LT, MO and JV.

Chapter 4 – The relative contribution of target-site mutations in complex acaricide resistant phenotypes as assessed by marker assisted backcrossing in Tetranychus urticae
M. Riga, S. Bajda, C. Themistokleous, S. Papadaki, M. Palzewicz, W. Dermauw, J. Vontas & T. Van Leeuwen

TVL, MR, SB designed research. MR and SB performed the experiments with contribution of SP, CT and MP. MR and SB analyzed data. SB, MR and TVL wrote the manuscript, with input from WD and JV.

Chapter 5 – Key point mutations that underlie target-site resistance in the two-spotted spider mite Tetranychus urticae reveal only limited fitness costs
S. Bajda, M. Riga, N. Wybouw, S. Papadaki, E. Ouranou, S. Masoumeh Fotoukkiaii, J. Vontas & T. Van Leeuwen

TVL, SB and MR designed research. SB and MR performed experiments with contribution of NW, SP, EO and S.M.F. SB, NW and MR analyzed data.

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Biography

Sabina Bajda-Wybouw was born on 2nd of June 1986 in Brzesko, Poland. After completing her secondary school degree in 2005 (Kazimierz Wielki upper secondary school in Bochnia, Poland), Sabina moved to Krakow to pursue higher education at the University of Agriculture and enrolled in a Biotechnology bachelor's degree program. Since 2008, Sabina continued her bachelor's studies at the Edinburgh Napier University, Scotland, where in 2010 she was awarded a bachelor's in science, BSc (Hons) degree in Microbiology and Biotechnology. In 2011 Sabina received her Master's degree in Biomedical Science and from 2012 began working as a research assistant at the Advanced Pest Solutions Ltd. Dundee, Scotland. Later in 2012, Sabina started her PhD-project in the group of Prof. Dr. Ir. Thomas Van Leeuwen and Prof. Dr. Ir. Luc Tirry (Department of Crop Protection) at the faculty of Bioscience-Engineering in Gent, Belgium. In 2014, Sabina relocated to Amsterdam and completed her PhD research at the Institute for Biodiversity and Ecosystem Dynamics (IBED), University of Amsterdam, The Netherlands. From October 2017, Sabina has been working as a researcher in the group of Thomas Van Leeuwen, at the faculty of Bioscience-Engineering in Gent, Belgium.

Publications

Publications in peer-reviewed journals (*Shared first authorship)

Bajda, S*., Riga, M*., Wybouw, N., Papadaki, S., Ouranou E., Fotoukkiaii, S.M., Vontas, J., Van Leeuwen, T. (2018). *Evolutionary Applications*. Fitness cost of key point mutations that underlie acaricide target-site resistance in the two-spotted spider mite *Tetranychus urticae*. Accepted for publication. (IF: 5.671)

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Sabina
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